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## Peroxisome Proliferator-activated Receptor $\delta$ Regulates Mitofusin 2 Expression in the Heart

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### Abstract

Mitofusin 2 (Mfn2) has been proposed as an important mitochondrial protein in maintaining mitochondrial network and bioenergetics. Mfn2 is highly expressed in the heart, but is downregulated in response to hypertrophic stimuli. However, little is known about how Mfn2's expression is regulated in cardiomyocytes. Here, we have investigated how Mfn2 expression in the heart responds to fasting condition and determined if Mfn2 is one of those PPAR $\delta$ -selective target genes that are involved in myocardial energy metabolism. Fasting for 48 hours in mice led to a robust increase of Mfn2 expression in the heart. On the other hand, cardiomyocyte-restricted PPAR $\delta$  deficiency in mice led to substantially diminished cardiac expression of Mfn2 transcript and protein compared to that of controls. Fasting induced cardiac expression of Mfn2 was blunted in cardiomyocyte-restricted PPAR $\delta$  deficient hearts. Moreover, PPAR $\delta$ -selective ligand treatment in cultured cardiomyocytes induced elevated Mfn2 expression. A functional PPRE consensus sequence located at –837 to –817bp upstream of the mouse Mfn2 promoter was identified and confirmed by Electrophoretic Mobility Shift Assays and Luciferase Promoter Reporter Assays. We conclude that Mfn2 is a PPAR $\delta$ -selective target, which may play an important role in regulating myocardial energy homeostasis.

### Introduction

Mitochondria are essential organelles of eukaryotic cells with their capability in bioenergy generation. The heart, as a continuously working pump, is the most energy demanding organ in the body. Under normal conditions, it has been estimated that a cardiomyocyte contains more than 1,000 mitochondria [1]. Mitochondria in cardiomyocytes appear in a uniform fashion and form a network along the entire length of the myofibrillar apparatus. Mitochondria in a cardiomyocyte play a critical role in cardiac cellular energy metabolism, calcium homeostasis, biogenesis, and regulation of cell death. Mitochondrial dysfunction has been identified in majority of cardiac pathologies and the implication is complex without fully understood.

An emerging group of proteins plays crucial roles in regulating the mitochondrial network and dynamics. Mitofusins (Mfn) 1 and 2 are two of these proteins located on the outer membrane

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of mitochondria and involved in the dynamic formation of the mitochondrial network [2,3]. They are the homologs of Fzo, a GTPase first discovered in *D. Melanogaster* [4] [5]. Knockout mouse studies unraveled that both Mfns are required for proper development ([6]). However, studies on biochemical and morphological evidences indicate that these two proteins are not functionally redundant [6,7]. Mfn2 is highly expressed in heart, skeletal muscle and brain [8] [6] [9]. Mfn2 participates in mitochondrial fusion and contributes to the maintenance and operation of the mitochondrial network [8] [6]. Mutations in Mfn2 were reported in patients with Charco-Marie-Tooth neuropathy type 2A [10] [11]. In *Drosophila*, Mfn2 deficiency leads to aberrant sperm, “fuzzy onion”-like mitochondria [5].

It has been shown that repression of Mfn2 reduces glucose oxidation, mitochondrial membrane potential, and cell respiration in L6E9 myotubes [8]. The importance of Mfn2 in the heart is recently evident by a study showing that upregulation of Mfn2 is involved in H<sub>2</sub>O<sub>2</sub>-induced apoptosis in cultured rat neonatal cardiomyocytes [12]. On the other hand, it was shown that diminished Mfn2 may be involved in an early activation of apoptosis in cardiomyocytes with increased mitochondrial fragmentation and decreased mitochondrial volume [13]. Another recent report demonstrated that Mfn2 expression was downregulated in hypertrophied cardiomyocytes induced by angiotensin II and phenylephrine and in hypertrophied hearts from multiple animal models [14]. Therefore, Mfn2 expression in the heart may have important implications in cardiac pathophysiology, especially, in myocardial energetic states. It has been recently demonstrated that Mfn2 can be induced directly by the cooperative actions of PPAR- $\gamma$  coactivator-1  $\alpha$  (PGC-1 $\alpha$ ) and estrogen related receptor (ERR $\alpha$ ) [15] [16] [17]. However, it is not clear if other important metabolic determinants such as PPARs also govern Mfn2 expression.

In the present study, we demonstrate that fasting in mice induces cardiac expression of Mfn2 and cardiac expression of Mfn2 is determined by PPAR $\delta$  signaling.

## Methods

### 1. Culture of rat neonatal cardiomyocytes

Rat neonatal cardiomyocytes were isolated by using a cardiomyocyte isolation system (Worthington, Lakewood, NJ) with minor modifications as described previously [18] [19]. Briefly, left ventricles of 1-day-old Sprague–Dawley rats were cut into small pieces and incubated with trypsin at 4°C overnight, following by a 45-minute collagenase digestion at 37°C. The resulted cells were pre-plated for 1 hour at 37°C in DMEM/F12, 15 mM HEPES, 5% FBS, 100 U/ml penicillin (Roche) and 100 mg/ml streptomycin (Roche, Indianapolis, IN) to allow adherence of noncardiomyocytes. Cardiomyocytes in the supernatant were re-suspended and plated at a field density of  $1 \times 10^5$  cells/cm<sup>2</sup> on 25-cm<sup>2</sup> culture flask in culture medium [MEM with 5% bovine calf serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin]. Cardiomyocytes were then incubated for 24 hours at 37°C with 0.5% FBS DMEM-F12 medium before 23 hour treatments of PPAR $\delta$ -selective ligand GW0742 (5  $\mu$ M, Cayman Chemical, Ann Arbor, Michigan) and PPAR $\alpha$ -selective ligand Wy14643 (10  $\mu$ M, Cayman Chemical, Ann Arbor, Michigan) or DMSO. For adenoviral mediated overexpression of Mfn2 in cultured cardiomyocytes, Ad-Mfn2 (100 MOI) and Ad-LacZ (100 MOI) as control were added with 0.5% FBS DMEM-F12 medium. Prior to each experiment, the cardiomyocytes were rinsed three times with 1 ml of warm (37°C) phosphate-buffered saline (PBS).

### 2. Animal studies

Breeding of cardiomyocyte-restricted PPAR- $\delta$  knockout mice: Homozygous cardiomyocyte-restricted PPAR $\delta$  knockout mice (CR-PPAR $\delta^{\delta^{-/-}}$ ) [20] were generated by crossing the

transgenic Cre ( $\alpha$ -MyHC-Cre) mice (C57/BL6) [21] with the PPAR $\delta^{\text{flox/flox}}$  mice (C57/BL6) [22]. In this mouse line, PPAR $\delta$  was largely eliminated in ventricular cardiomyocytes shortly after birth.

Tamoxifen-inducible cardiomyocyte-restricted PPAR $\delta$  knockout line (TMPD): Homozygous TMPD mice (C57/B6) were generated by crossing a transgenic line with  $\alpha$ -MyHC driven Mer-Cre-Mer (MCM) overexpression ([23]) and the PPAR $\delta$  (PPAR $\delta^{\text{flox/flox}}$ ) line. Cardiomyocyte-restricted PPAR $\delta$  knockout was induced in 2-month old mice by 5 days of Tamoxifen treatment (2mg/200ul in sunflower oil, intraperitoneal injection). After tamoxifen treatment ending for 5 to 10 days, PPAR $\delta$  was largely eliminated in cardiomyocytes of mouse hearts from this line. PPAR $\alpha$  knockout line (C57/B6) was obtained from commercial source (Jackson Lab, Bar Harbor, Maine). Animals received food and water on an ad libitum basis, and lighting was maintained on a 12-hour cycle. All experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

### 3. Transcript analyses

Total RNA samples were extracted using a RNA extraction kit (Qiagen, Valencia, CA). Real Time-PCR analyses (Roche LightCycler PCR system, Carlsbad, CA) were carried out to determine transcript levels of target genes. Real-Time PCR results from each gene/primer pair were normalized to  $\beta$ -actin, and compared across conditions. The following primers were used: Mfn2: up-AAGTCCGGGAAGCTGAAAGT, low-TCTCGGTTATGGAACCAACC; PGC-1 $\alpha$ : up- CTCCTCCCACAACCTCCTCATA, low-GGGCCGTTTAGTCTTCCTTTCTC; ERR $\alpha$ : AGCAAGCCCCGATGGA, Low-GAGAAGCCTGGGATGCTCTT; PPAR $\delta$ : up-ATGGAACAGCCACAGGAGGA, low-ATCACAGCCCATCTGCAGCT and  $\beta$ -actin: up-AGATTACTGCTCTGGCTCCTA, low-CAAAGAAAGGGTGTAACG.

### 4. Protein analysis

Cytosolic protein samples were extracted using a nuclear protein extraction kit (Thermo Scientific, Rockford, IL). Protein samples (40 $\mu$ g) from each experiment were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Immunoblotting was performed using the enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL). A polyclonal anti-Mfn2 antibody (Sigma-Aldrich, St. Louis, MO) and a polyclonal anti-ATP synthase  $\beta$ -subunit (Abcam, Cambridge, MA) were used for the Western blot analyses. A Kodak Image Station 4000R (Kodak, Rochester, NY) was used for quantitative measurement of protein signals. Sample loadings were normalized by immunoblotting with an anti-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO).

### 5. Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Nuclear protein was extracted from left ventricles of  $\alpha$ -MyHC-Cre and CR-PPAR $\delta^{-/-}$  mice using a nuclear protein extraction kit (Thermo Scientific, Rockford, IL). Protein concentrations were determined with a Bio-Rad protein concentration assay kit (Bio-Rad Laboratories). Nuclear extracts were stored at  $-80^{\circ}\text{C}$  until used. EMSA was performed using LightShift $^{\circledR}$  Chemiluminescent EMSA Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Double-stranded (ds) PPRE oligonucleotide (5'-TTT CAT TTA GCC CAG TTT AT-3'), which corresponds to nucleotide  $-837$  to  $-817$  of the mouse Mfn2 gene promoter, was synthesized and its 3'-end was labeled with Biotin (Invitrogen). Nuclear extracts (10 $\mu$ g protein) were incubated with 1  $\mu$ g Poly (dI•dC), and the binding buffer for 20 minutes at room temperature. Bound DNA complexes were separated on a polyacrylamide gel

electrophoresis and transferred to a nylon membrane (Thermo Scientific, Rockford, IL). The nylon membranes were cross-linked and chemiluminescent detection was performed.

## 6. Luciferase transfection assays

Mouse Mfn2 promoter fragments with or without the predicted PPRE element were subcloned into a Luciferase reporter vector (pGL3-Basic). An embryonic rat heart cell line (H<sub>9</sub>C<sub>2</sub> cells) was seeded into 24-well plates. Vectors containing mouse Mfn2 promoter fragments with or without the putative PPRE consensus sequence were respectively transfected when the cells grew to a confluence of 50–80% after overnight culture. The above experimental groups were also co-transfected with pGL3-Basic vectors containing PPAR $\gamma$  co-activated  $\alpha$  (PGC-1 $\alpha$ ) and pRL-CMV (for normalization). In addition, the above cells were infected 5 hours later with adenoviral vectors containing mouse wild type PPAR $\delta$  (Ad-PPAR $\delta$ , 100 MOI). As we shown previously, the above infection lead to a 2 fold overexpression of PPAR $\delta$  protein [18] [19]. Finally, the above cultured H<sub>9</sub>C<sub>2</sub> cells were treated with GW0742 (5  $\mu$ M) or DMSO 23 hours before Luciferase assay. The relative Luciferase activity were tested and compared in different vectors to see whether the PPRE elements are still functionally active.

## 7. Statistics analyses

All data were analyzed by one factor or mixed, two-factor analysis of Variance (ANOVA) followed by post-hoc analysis with the Student–Newman–Keuls test and/or Bonferroni test to detect differences between experimental groups using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

## Results

### 1. Fasting induces a marked increase of Mfn2 expression in the heart

Given the importance of Mfn2 in maintaining mitochondrial architect and bioenergetics and it is highly expressed in the heart, it becomes intriguing if cardiac expression of Mfn2 is response to energy starvation conditions such as fasting. Mfn2 transcript levels in the heart were assessed by Real Time PCR measurement on samples from mice subjected to a 48 hour fasting. The results revealed that cardiac Mfn2 transcript expression was greatly increased in fasting mice compared with that of fed mice (Figure 1). In consistent with previous report [24], cardiac expression of PGC-1 $\alpha$  was also substantially elevated in fasting mice compared with that of fed mice (Figure 1B). These results indicates that Mfn2 may be involved in signaling pathways that are coactivated by PGC-1 $\alpha$  and potentially plays an important role in myocardial energy metabolism in starvation conditions.

### 2. Diminished Mfn2 expression in PPAR $\delta$ deficient hearts

Our previous study demonstrated that myocardial energetic defect and myocardial FAO depression were the main features of mice with cardiomyocyte-restricted PPAR $\delta$  deficiency (CR-PPAR $\delta$ <sup>-/-</sup>) [20] [25]. To investigate cardiac Mfn2 expression in CR-PPAR $\delta$ <sup>-/-</sup> hearts, expression levels of Mfn2 genes were evaluated by Real Time PCR measurement on RNA samples from CR-PPAR $\delta$ <sup>-/-</sup> and control mice before overt phenotypic changes. Mfn2 transcript levels in CR-PPAR $\delta$ <sup>-/-</sup> hearts were decreased by 50% relative to those of control hearts (Figure 2A.). Western blots also revealed that Mfn2 protein levels in CR-PPAR $\delta$ <sup>-/-</sup> hearts were decreased by about 30% compared with those of control hearts (Figure 2B). Western blot analyses also revealed that the abundance of another mitochondrial protein, ATP synthase  $\beta$  subunit, were not downregulated as mfn2 did in CR-PPAR $\delta$ <sup>-/-</sup> hearts (Figure 2C). These results indicate that downregulation of Mfn2 in CR-PPAR $\delta$ <sup>-/-</sup> hearts is not part of a general downregulation of mitochondrial proteins.

We further investigate whether *Mfn2* expression is also downregulated in *PPARα* knockout heart. Real Time PCR measurement revealed that *Mfn2* transcript levels in 2-month-old *PPARα* knockout hearts were not altered relative to those of control hearts (Figure 2D). To determine if the downregulation of *Mfn2* expression in CR-*PPARδ*<sup>-/-</sup> hearts is due to potential downregulation of *PGC-1α* and *ERRα* with *PPARδ* deficiency, transcript expression of *PGC-1α* and *ERRα* was assessed. Real Time PCR measurement revealed that the expression of both *PGC-1α* and *ERRα* in CR-*PPARδ*<sup>-/-</sup> hearts was not changed compared to controls (Figure 2E and F). Therefore, it is evident that the downregulation of *Mfn2* in CR-*PPARδ*<sup>-/-</sup> hearts should be the consequence of *PPARδ* deficiency.

In CR-*PPARδ*<sup>-/-</sup> mice, *PPARδ* is knocked out from ventricular cardiomyocytes shortly after birth. To exclude the possibility that the observed change of *Mfn2* expression in CR-*PPARδ*<sup>-/-</sup> heart is due to secondary responses to long-term knockout of *PPARδ* in cardiomyocytes, cardiac expression of *Mfn2* transcript was also assessed by Real Time PCR measurement on RNA samples from mice with short-term (5-10 days) cardiomyocyte-restricted *PPARδ* knockout. Cardiomyocyte-restricted *PPARδ* knockout was induced in 2-month old mice by Tamoxifen treatment. These mice (TMPD) were compared with age- and gender-matched MCM mice with the same Tamoxifen treatment, which designated as TMCM mice. At 10 days after Tamoxifen treatment, *PPARδ* was largely eliminated on RNA samples extracted from left ventricles of the TMPD mice compared with samples from control mice (TMCM) (Figure 3A). With this short-term *PPARδ* deficiency in the young adult mice, transcript levels of *Mfn2* in TMPD hearts were decreased by about 50% compared with those of the TMCM mice (Figure 3B). While fasting-induced transcript expression of *Mfn2* in the heart from TMCM and TMPD groups was consistent, it was blunted in TMPD mice compared with TMCM mice (Figure 3B). It is noted that the expression of *PPARδ* in the heart was not elevated by the same fasting condition (Figure 3C). Therefore, it is clear that *PPARδ* is essential for the heart to maintain normal *Mfn2* transcript levels in basal and at least partially in fasting conditions.

### 3. Activation of *PPARδ* induces elevated *Mfn2* expression in cardiomyocytes

To investigate if activation of *PPARδ* directly increases *Mfn2* expression in cardiomyocytes, cultured neonatal cardiomyocytes were treated respectively with a *PPARδ*-selective ligand (GW0742, 5 μM) and with a *PPARα*-selective ligand (Wy14624, 10 μM), for 23 hours. Real Time PCR measurement revealed that GW0742 treatment increased *Mfn2* expression by about 35% compared with vehicle treatment in neonatal cardiomyocytes (Figure 4A). On the other hand, Wy14643 treatment did not alter *Mfn2* expression in cultured neonatal cardiomyocytes (Figure 4B). These results demonstrated that *PPARδ* activation selectively directs *Mfn2* expression in cardiomyocytes and *Mfn2* may be a direct *PPARδ*-selected gene in the heart.

### 4. Identify a functional PPRE in *Mfn2* promoter

To further confirm that *PPARδ* activation directly upregulates *Mfn2* expression in cardiomyocytes, we assessed the theoretical promoter regions of mouse *Mfn2* gene using bioinformatics tools. A putative *PPAR* response element (PPRE) consensus sequence located at -837 to -817 bp upstream of the mouse *Mfn2* promoter was identified. Using a LightShift chemiluminescent EMSA kit (Bio-Rad Laboratories), we confirmed that the probe derived from a fragment of this putative PPRE was strongly bound in nuclear protein extracted from control ( $\alpha$ -MyHC-Cre) but not CR-*PPARδ*<sup>-/-</sup> mice in this in vitro condition (Figure 5). Similarly, no direct bound of the probe to nuclear protein extract from TMPD hearts either (data not shown). The functionality of the putative PPRE element on the *Mfn2* promoter was further investigated using the Luciferase reporter assay in cultured H<sub>9</sub>C<sub>2</sub> myocytes with *PGC-1α* expression and with adenoviral mediated *PPARδ* overexpression. Two fragments of the mouse *Mfn2* promoter with (*Mfn2*-918) or without (*Mfn2*-712) the putative PPRE

consensus sequence (Figure 6A), respectively, were cloned into the pGL3-Basic Luciferase reporter vector. Each of these vectors was transfected into H<sub>9</sub>C<sub>2</sub> myocytes with treatment of GW0742 (5 μM) or DMSO. Luciferase assay revealed that treatment with GW0742, but not DMSO, induced an over 2 fold increase in Luciferase activity in those cells transfected with the Mfn2 promoter fragment containing the putative PPRE. Conversely, GW0742 treatment did not increase Luciferase activity in cells transfected with another promoter fragment without the PPRE sequence (Figure 6B). These data provide strong evidence to support that Mfn2 as a PPARδ target gene.

## Discussion

In this study, we investigated a role of PPARδ in transcriptional regulation of Mfn2 expression in the heart, which may be in turn involved in maintaining myocardial energy homeostasis. The current results demonstrated that cardiac Mfn2 expression was elevated in mice subjected to fasting and downregulated in mice with PPARδ deficiency. We provide further evidence to demonstrate that PPARδ directly regulates Mfn2 expression via binding to the PPRE site in its promoter. The current results further support that Mfn2 is part of PPARδ signaling in regulating myocardial energy homeostasis.

Mfn-2 is ubiquitously expressed, especially at a high level in the heart [9]. Recent evidence demonstrates that Mfn-2 may be involved in regulating cell fate of cardiomyocyte under oxidative stress [12]. However, it remains unclear how Mfn-2 expression is regulated. Its deficiency in cardiomyocytes or in hearts undergoing pathological hypertrophy has also been revealed [14]. It is not clear yet if the downregulation of Mfn2 in hearts with pathological hypertrophy contributes to the development of cardiac hypertrophy or it is one of the hypertrophic responses in response to bioenergetic deficiency. The downregulation of Mfn2 expression in the cardiomyocyte-restricted PPARδ knockout hearts before the development of cardiac hypertrophy suggests that Mfn2 deficiency may contribute to the progression of cardiac hypertrophy and heart failure in these mice [20]. It is not likely the downregulation of Mfn2 is a consequence of cardiac hypertrophy. The downregulation of Mfn2 transcript in adult hearts with induced PPARδ knockout further support this notion with minimal confounding compensatory effects. One of the main phenotypic changes in the PPARδ deficient hearts is mitochondrial abnormalities such as matrix loss and enlarged, fragmented or even dramatic depletion in the severe cases, accompanied with a large quantity of lipid droplets [20]. An essential role of Mfn2 in maintaining mitochondrial network has been well established in many other cell types [8]. While it is difficult to establish a direct cause-effect relationship between the observed downregulation of Mfn2 and mitochondrial abnormalities in the CR-PPARδ<sup>-/-</sup> heart, it is possible that Mfn2 deficiency in the CR-PPARδ<sup>-/-</sup> hearts should at least partially contribute to the fragmentation of mitochondria network in the cardiomyocyte-restricted PPARδ knockout heart as reported previously [20].

Whereas deficiency of Mfn2 may contribute to cardiac pathological development, it has been reported recently that overexpression of Mfn2 exacerbates H<sub>2</sub>O<sub>2</sub> induced cardiomyocyte apoptosis [12]. It is conceivable that the appropriate expression of Mfn2 may be required in the heart. The finding that cardiac expression of Mfn2 is remarkably increased in response to fasting condition is intriguing. While the implication of fasting-induced cardiac Mfn2 overexpression on cardiac mitochondrial architectural integrity and energy metabolism may be complicated, it at least suggests that cardiac Mfn2 expression levels are directly correlated to the energy status of the heart. Therefore, understanding how the Mfn2 expression is regulated in the heart should provide insights into the role of Mfn2 in cardiac pathophysiology. Our results revealed that cardiac expression of PGC-1α was induced by fasting and it is consistent with previous finding [24]. It is well documented that PGC-1α is an important coactivator for a series of nuclear receptors, including PPARδ and ERRα [26,27,28,29]. ERRα, another nuclear

receptor has a similar effect as PPARs in regulating FAO gene expression, has been shown to directly target Mfn2 transcription expression in the presence of PGC-1 $\alpha$  [15,17]. It is likely that ERR $\alpha$  should have similar effect on controlling Mfn2 expression in the heart. Since cardiac expression of PPAR $\delta$  is not induced by fasting, upregulation of cardiac Mfn2 expression may be directed by fasting-induced PGC-1 $\alpha$  elevation, thus activating selected transcription factor signals including PPAR $\delta$  and ERR $\alpha$ . The upregulation of Mfn2 in the heart during starvation might help optimizing mitochondrial architecture and even function for the elevating energy demand.

The current study supports a major regulating role of PPAR $\delta$  in Mfn2 expression in the heart. We demonstrated that PPAR $\delta$  binds a novel PPRE sequence within the promoter of Mfn2 and this PPRE functions as a positive response element for PPAR $\delta$ -specific agonist. Subsequent analyses of the rat and human Mfn2 promoters also revealed sequences that have significant homology to the PPRE consensus sequence of the mouse one. Therefore, it is unlikely that the observed altered Mfn2 expression in response to PPAR $\delta$  deficiency or activation were the consequences of indirect effects from ERR $\alpha$  or other unknown regulators of Mfn2 expression. Instead, it appears that PPAR $\delta$  and ERR $\alpha$ , the two functionally similar nuclear receptors, exert their independent regulating function on Mfn2 transcription. PPAR $\alpha$  appears not to be involved in Mfn2 expression in cardiomyocytes but the underpinning mechanism is unclear. Further study on how Mfn2 expression is regulated by PPAR $\delta$  and ERR $\alpha$  in coordinate with their common transcription coactivator PGC-1 $\alpha$  is warranted. This further suggests that Mfn2 may be one of the mitochondrial proteins that play important roles in maintaining myocardial energy homeostasis.

Since Mfn1 may possess similar effects on mitochondrial fusion and function, future studies should be informative to see if Mfn1 is also regulated by PPAR $\delta$ . However, previous studies have shown that the two similar proteins are not redundant [6] [7]. It has been revealed that Mfn1 and 2 have a specific functional difference in how they regulate mitochondrial fusion [30]. Mfn2 is most abundantly expressed in cardiac and skeletal muscle. Therefore, the present study only focuses on PPAR $\delta$ 's role on the expression of Mfn2.

In summary, the present study demonstrates that Mfn2 expression in the heart is specifically regulated by PPAR $\delta$ . Cardiomyocyte-restricted knockout of PPAR $\delta$  in mice leads to Mfn2 deficiency and contributes to the development of myocardial bioenergetic defects. The discovery of the regulation effect of PPAR $\delta$  on Mfn2 expression should provide mechanistic insights into gene regulation of mitochondrial dynamic and energy metabolism in the heart and new therapeutic potentials for heart disease.

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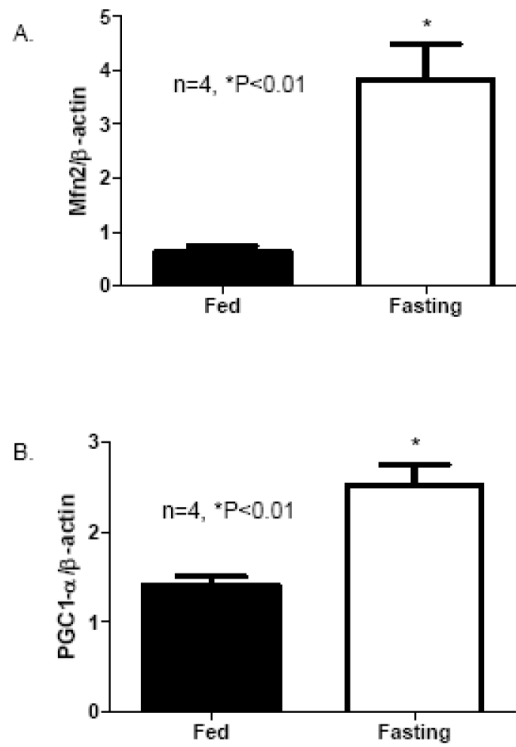
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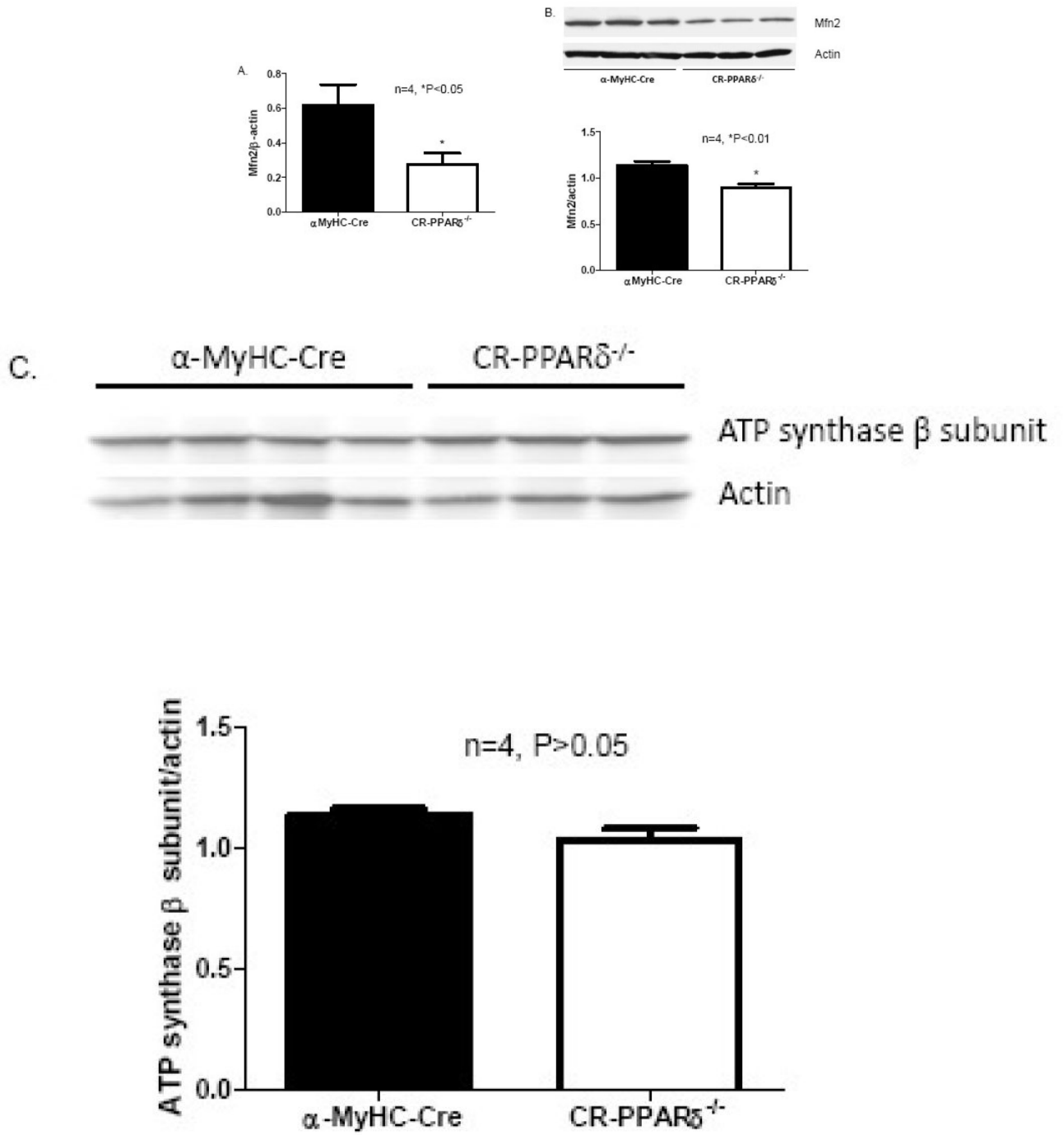


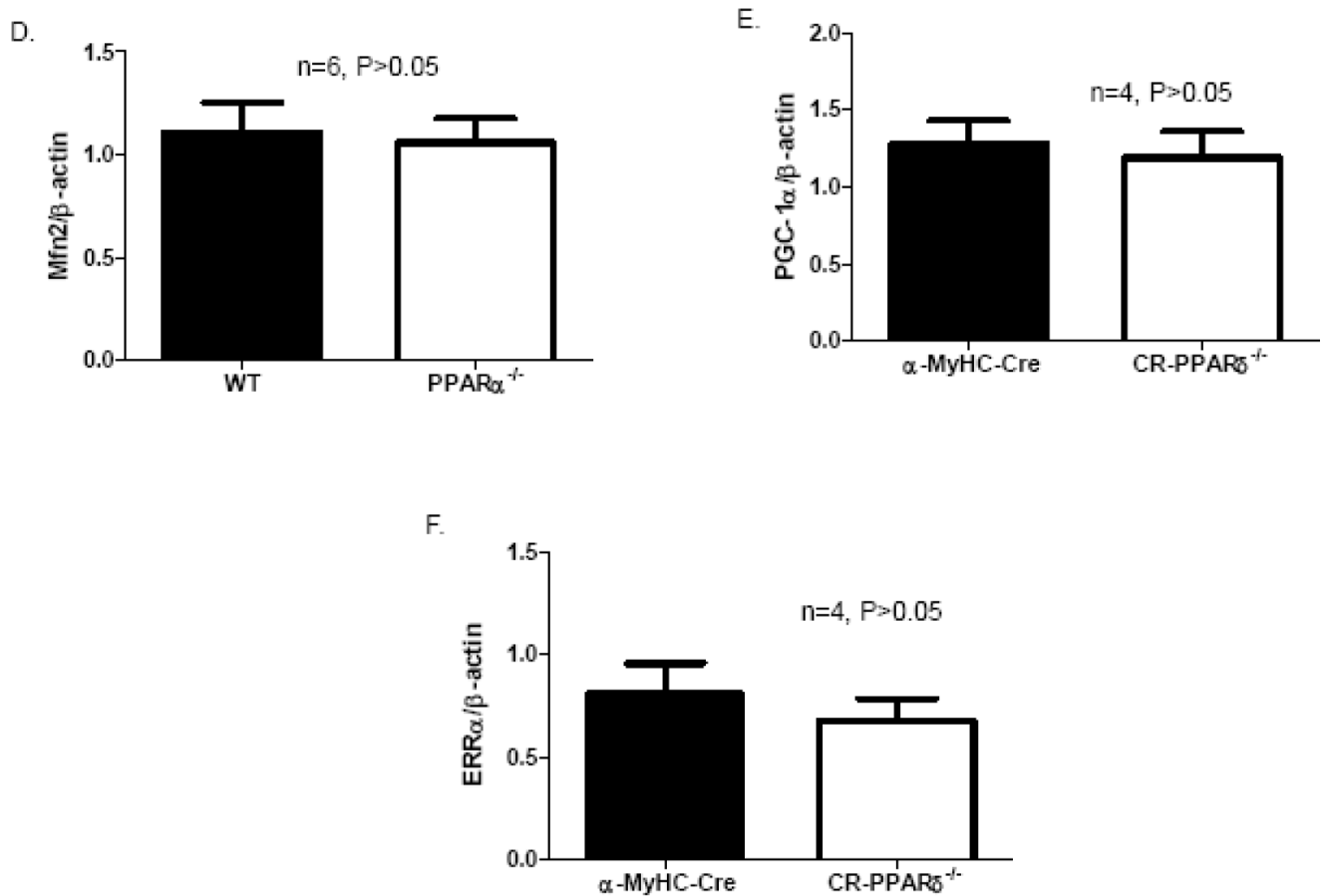
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**Figure 1.**

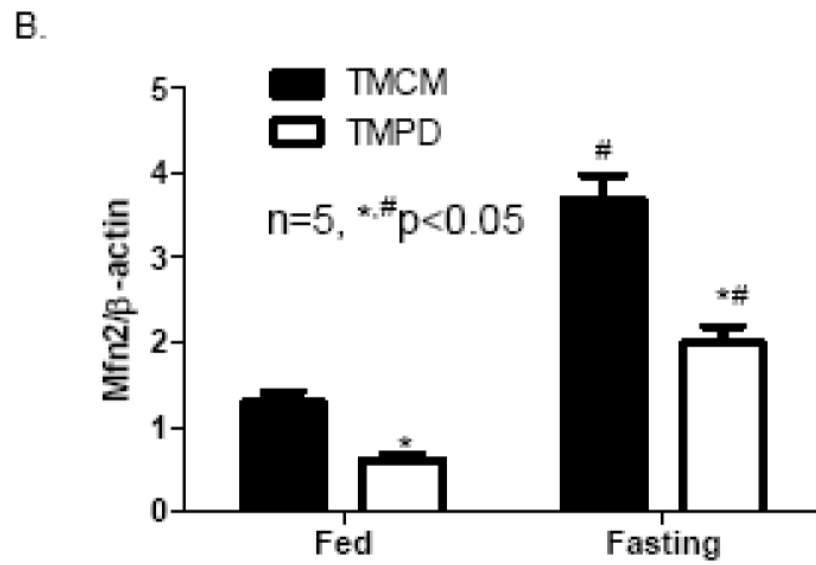
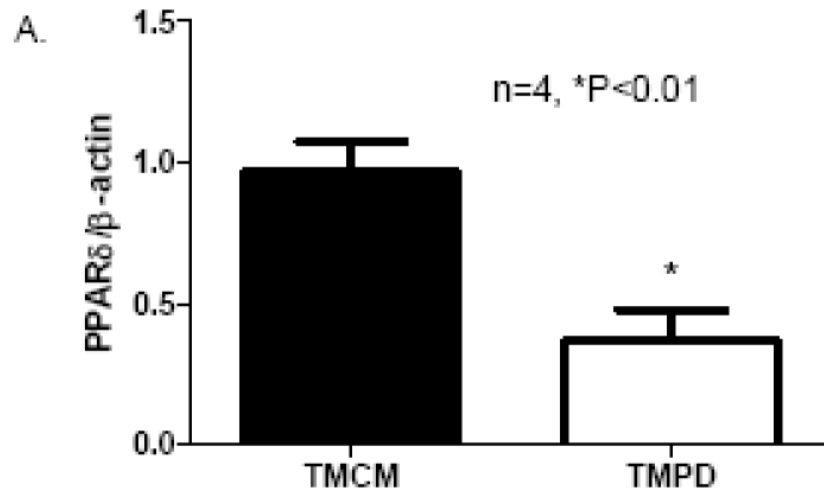
Cardiac Mfn2 and PGC-1 $\alpha$  transcripts in response to 48 hour fasting in mice: Hearts were collected from mice with and without fasting for 48 hour. A) Real Time PCR measurement Mfn2 transcripts normalized to  $\beta$ -actin transcript was conducted on samples extracted from the left ventricles of mice after 48 hour fasting. Data are expressed as mean $\pm$ SEM, n=4, \*P<0.01. B) Real Time PCR measurement PGC-1 $\alpha$  transcripts normalized to  $\beta$ -actin transcript was conducted on samples extracted from the left ventricles of mice after 48 hour fasting. Data are expressed as mean $\pm$ SEM, n=4, \*P<0.01.

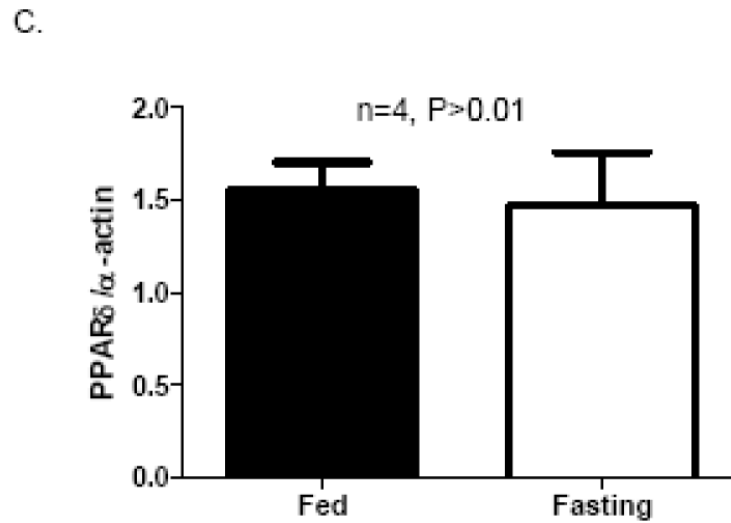




**Figure 2.**

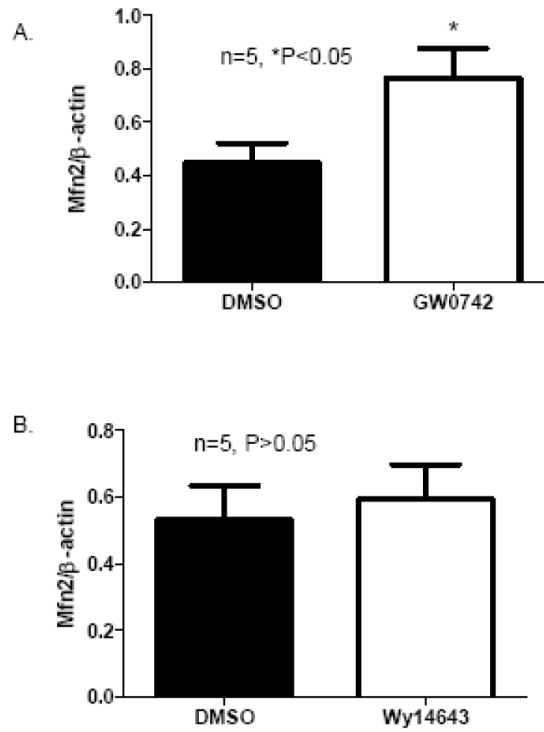
Mfn2 expression in long-term PPAR deficient hearts: A). Real Time PCR measurement of Mfn2 transcript normalized to  $\beta$ -actin transcript in samples from 2-month-old CR-PPAR $\delta^{-/-}$  and  $\alpha$ -MyHC-Cre hearts. Data are expressed as mean $\pm$ SEM, n=4, \*P<0.05. B) Western blot analyses of Mfn2 protein abundance in CR-PPAR $\delta^{-/-}$  and  $\alpha$ -MyHC-Cre hearts. The relative amount of Mfn2 was normalized to actin protein. Data are expressed as mean $\pm$ SEM, n=4, \*P<0.01. C) Western blot analyses of ATP synthase  $\beta$  subunit in CR-PPAR $\delta^{-/-}$  and  $\alpha$ -MyHC-Cre hearts. The relative amount of ATP synthase  $\beta$  subunit was normalized to actin protein. Data are expressed as mean $\pm$ SEM, n=4, P>0.05. D) Real Time PCR measurement of Mfn2 transcript normalized to  $\beta$ -actin transcript in cardiac samples from 2-month-old CR-PPAR $\alpha^{-/-}$  and wild type mice. Data are expressed as mean $\pm$ SEM, n=6, P>0.05. E) Real Time PCR measurement of PGC-1 $\alpha$  transcript normalized to  $\beta$ -actin in samples from 2-month-old CR-PPAR $\delta^{-/-}$  and  $\alpha$ -MyHC-Cre hearts. Data are expressed as mean $\pm$ SEM, n=4, P>0.05. F) Real Time PCR measurement of PGC-1 $\alpha$  transcript normalized to  $\beta$ -actin transcript in samples from 2-month-old CR-PPAR $\delta^{-/-}$  and  $\alpha$ -MyHC-Cre hearts. Data are expressed as mean $\pm$ SEM, n=4, P>0.05.





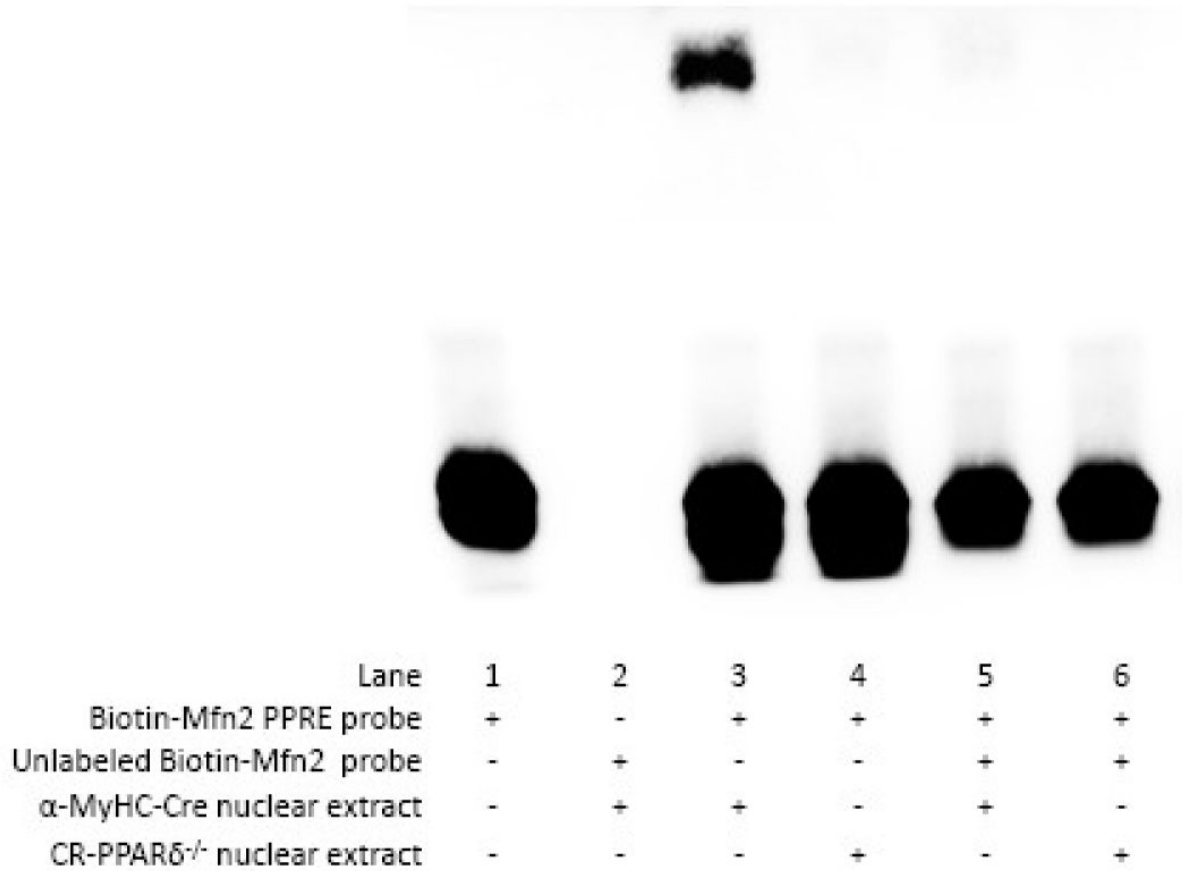
**Figure 3.**

Mfn2 expression in short-term PPAR $\delta$  deficient hearts: A) Real Time PCR measurement of PPAR $\delta$  transcript normalized to  $\beta$ -actin transcript in samples from TMPD and TMCM hearts. Data are expressed as mean $\pm$ SEM, n=4, \*P<0.01. B) Real Time PCR measurement of Mfn2 transcript normalized to  $\beta$ -actin transcript in samples left ventricles from TMPD and TMCM mice in Fed and fasting conditions, respectively. Data are expressed as mean $\pm$ SEM, n=5, \*P<0.05 (TMCM vs TMPD), # P<0.05 (Fasting vs Fed). C) Real Time PCR measurement of PPAR $\delta$  transcript normalized to  $\beta$ -actin transcript in left ventricles from mice in Fed and fasting condition. Data are expressed as mean $\pm$ SEM, n=4, P>0.05.



**Figure 4.**

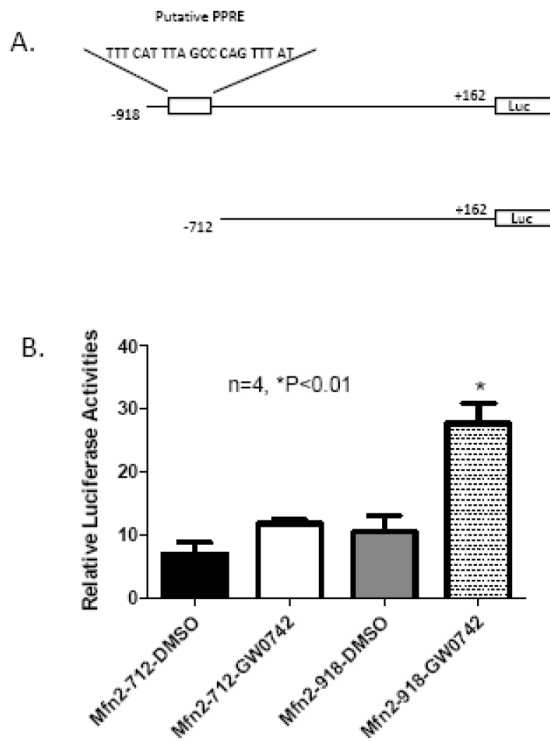
PPAR $\delta$  activation induced Mfn2 expression in cultured rat neonatal cardiomyocytes: A) Real Time PCR measurement of Mfn2 transcript normalized to  $\beta$ -actin transcript in samples from cultured cardiomyocytes treated with a PPAR $\delta$ -selective ligand, GW0742 (5  $\mu$ M in DMSO) or with DMSO. Data are expressed as mean $\pm$ SEM, n=5, \*P<0.05. B) Real Time PCR measurement of Mfn2 transcript normalized to  $\beta$ -actin transcript in samples from cultured cardiomyocytes treated with a PPAR $\alpha$ -selective ligand, WY14643 (10  $\mu$ M in DMSO) or with DMSO. Data are expressed as mean $\pm$ SEM, n=5, P>0.05.



**Figure 5.**

Electrophoretic-mobility shift assays (EMSA): EMSA was performed using LightShift® Chemiluminescent EMSA Kit (Pierce) to assess the direct binding of the Biotin-labeled oligonucleotides corresponding to the putative PPRE sequence (-837 to -817) in the mouse *Mfn2* promoter to endogenous PPAR $\delta$  in nuclear extracts. Nuclear extracts with or without PPAR $\delta$  were obtained from the  $\alpha$ -MyHC-Cre or the CR-PPAR $\delta^{-/-}$  hearts, respectively. Experiments were performed three times with similar results.





**Figure 6.**

Promoter reporter assays of PPAR regulation of Mfn2 transcriptional activities: A) A schematic diagram of the Mfn2 promoter fragments cloned into the pGL3-Basic luciferase reporter vector. Numbers in the names of plasmids represent the deletion points of the Mfn2 promoter fragments. Mfn2 -918, but not Mfn2-712, contains the putative PPRE sequence. Numbers are also given with reference to the translation start site of Luciferase (Luc). B). Luciferase assays: Cultured H<sub>9</sub>C<sub>2</sub> cells were transfected the above reporter constructs, PGC-1 $\alpha$  and Ad-PPAR $\delta$  followed by treatment with GW0742 (5  $\mu$ M) or vehicle (DMSO) alone. The results of Luciferase assays are the mean of 4 independent experiments and expressed as mean $\pm$ SEM, n=4, \*P<0.01.