AMPKα2 regulates expression of estrogen related receptor-alpha, a metabolic transcription factor related to heart failure development

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Running title: **AMPKα2 regulates estrogen related receptor-alpha**

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Supplementary Methods

Western Blots and Quantitative Real-time PCR

Whole cell lysate from heart tissue or cardiomyocytes was resolved on SDS-PAGE gel and transferred to PVDF membrane (Amersham Biosciences). Primary antibodies against AMPKα1, AMPKα2, COX-3, and PGC1α are from Santa Cruz Biotech; antibodies against phos-AMPK^{Thr172}, phos-ACC^{Ser79}, and cytochrome C are from Cell Signaling; antibody against CPT-1b is from Alpha Diagnostics; antibody against MCAD is from Cayman Chemical; ERRα antibody is from Millipore Bioscience; antibodies against α-sarcomeric actin and UCP3 are from Sigma. HRP conjugated secondary antibodies were from BioRad Laboratories and Sigma, and were detected by enhanced chemiluminescence (Amersham Biosciences).

For RT and quantitative real-time PCR, total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The real-time PCR reaction was performed using the 7900HT Fast Real-Time PCR System from Applied Biosystems. Please see supplementary **Table S2** for primers used for qPCR amplification. Results were normalized to GAPDH levels.

Cell Culture, Transfection, Reporter Gene Assay and ChIP assay

Neonatal cardiac myocytes were isolated as previously described [1]. Cells were seeded in DMEM with 10% FBS and incubated at 37°C with 5% CO_2 overnight, which allows the cells to attach and spread. The next day, cells were incubated in DMEM (Sigma) with no serum and 0.1mM BrdU for another day before being used for transfection and other studies. Reporter genes were transfected with Lipofectamine 2000 (Invitrogen) as described by the manufacturer. For specific silencing of AMPKα2 gene expression, non-target control and AMPKα2 specific siRNA (Ambion) were transfected into neonatal cardiac myocytes using DharmaFECT 3 reagent (Dharmacon). Forty-eight hours after transfection, cells were treated with AICAR or infected with adenovirus for 24 hours. Then, cells were harvested for reporter assay, real-time PCR, Western blot analysis or ChIP assay. ERRα promoter was amplified by PCR using primer pair GGA 5'-GGT ACC ACG ATG GAG GAA AGC GTC AA-3' and 5'-GGA AGA TCT CTG GCT GCT TGT AGG ACA CAA A-3' , confirmed by sequencing, then subcloned into pGL3B vector (Promega). Deletions of ERRα promoter was generated using the existing restriction sites as indicated in Figure 7C. LacZ reporter driven by CMV promoter was used as an internal control for transfection, and relative luciferase activity was determined by normalizing luciferase activity to the corresponding LacZ activity. ChIP assay was performed as previously described [1]. Primers 5'-CTC CGC TTC CTC CAG CTG AC-3' and 5'- GTT AGG CCC CAC CCC TTA TG-3' were used to amplify ERRα promoter region with potential Sp1 binding sites.

Electron microscopy

Heart was first perfused with Millonig's buffer and then with 2.5% Glutaraldehyde solution. LV papillary muscles were then isolated and postfixed in 1% Osmium Tetroxide in distilled water and embedded in polyEmbed 812.

TUNEL staining and MTT assay

Rat neonatal cardiac myocytes were seeded in 96-well plate and transfected with AMPKα2 specific siRNA for 48 hours and then infected with GFP or ERRα adenovirus for an additional 24hours. Then cells were treated with 100μM H_2O_2 for 2 hours. After the treatment, some of the cells were stained with In Situ Cell Death Detection kit (Roche) for apoptotic cells. Others were

incubated in fresh media with 0.5 mg/ml 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) for an additional 4 hours and then the converted dye was dissolved in acidic isopropanol (0.04M HCl in absolute isopropanol). The absorbance was measured at 570 nm.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Data from two groups was compared with unpaired t-test. Tissue samples were obtained from 5-6 mice from each strain. In vitro studies were performed at least three times. For studies of AICAR treated neonatal cardiac myocytes, two-way analysis of variance (ANOVA) was used to test each variable for differences among the treatment groups. Statistical significance was defined as $p < 0.05$.

Reference:

1. Hu X, Xu X, Huang Y, Fassett J, Flagg TP, Zhang Y, Nichols CG, Bache RJ, Chen Y. Disruption of Sarcolemmal ATP-Sensitive Potassium Channel Activity Impairs the Cardiac Response to Systolic Overload. *Circ Res.* 2008;103:1009-1017.

Table S1. General information of the human subjects.

Subject	Diagnosis	Age	Gender	LV ejection fraction	LVEDD (mm)
1	Donor	53	Female	N/A	N/A
2	Donor	65	Male	N/A	N/A
3	Donor	42	Female	N/A	N/A
4	Donor	48	Female	N/A	N/A
5	Donor	51	Male	N/A	N/A
6	DCM, mild CAD	62	Female	19%	83
7	DCM	54	Female	15%	83
8	DCM, AVR	64	Male	18%	73
9	DCM	30	Male	15%	75
10	DCM, SLE	34	Female	30%	60

DCM: Dilated cardiomyopathy; CAD: coronary artery disease; AVR = aortic valve replacement; SLE: systemic lupus erythematosus. N/A: not available.

Table S2. Primers used in real-time PCR

Gene name	sense	antisense
VLCAD	5'-ATGCAGTCGGCTCGGATGACCC-3'	5'-TGAGAAATTGTGCCTGTTCTTC-3'
VLCAD (rat)	5'-TCTTGTGCTTCATCAGCTTCG-3'	5'-TGAGTCGCAGTGGTGAACTGG-3'
MCAD	5'-CTAACCCAGATCCTAAAGTACCCG-3'	5'-GGTGTCGGCTTCCA AATGA-3'
CPT1b	5'-GAGTTCTCGATGGCTTTCCG-3'	5'-GACAGGACACTGTGTGGGTGAG-3'
CPT1b(rat)	5'-GCAGTTCCAGAGAATCCTCG-3'	5'-GCCCACTCTACCCTTCCTCCTG-3'
COX-1	5'-TGAAACCCCCAGCCATAAC-3'	5'-CCAGCGGGATCAAAGAAAG-3'
COX-3	5'-CATCGTCTCGGAAGTATTTTT-3'	5'-ATTAGTAGGGCTTGATTTATGTGG-3'

ERRα	5’-TGGAGCGGGAGGAGTACGTC-3’	5’-CAGCCTCAGCATCTTCAATGTG-3’

Primers were designed according to mouse cDNA sequences if not specifically labeled.

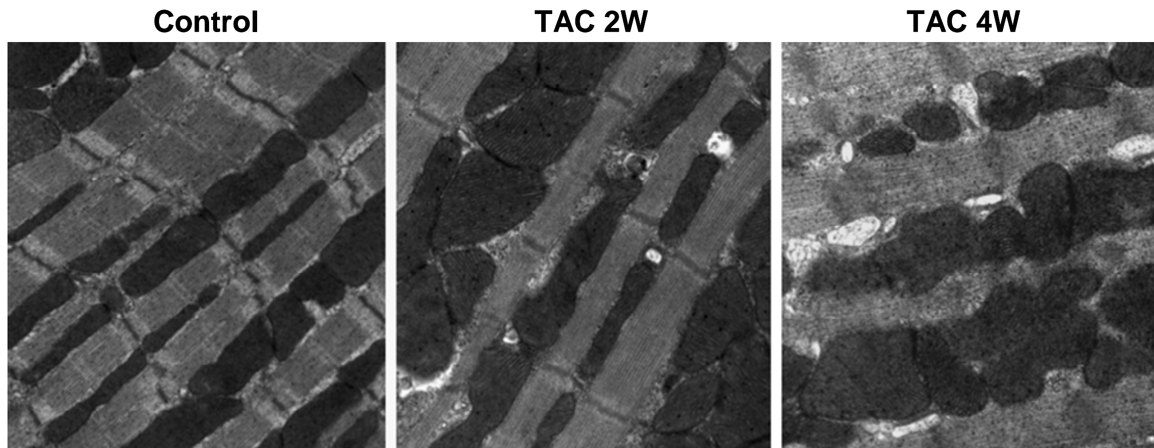

Figure S1. Myocardial mitochondria morphology in mice after TAC. Wild type mice were subjected to TAC for 2 or 4 weeks, which caused gradual mitochondrial damage.

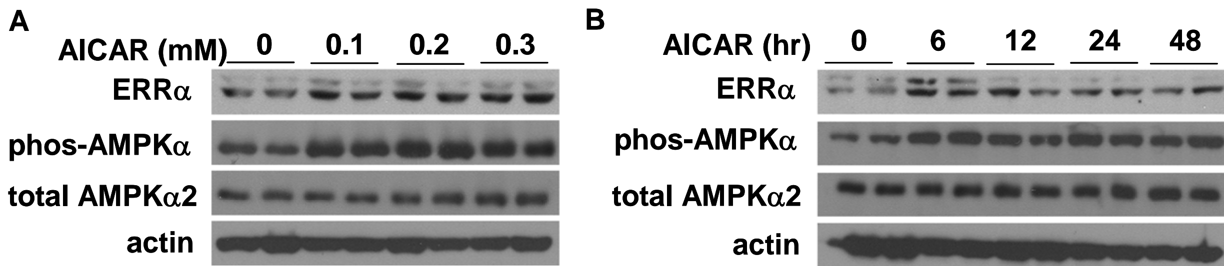

Figure S2. ERRα expression in response to different dose and time course of AICAR treatment. Rat neonatal cardiac myocytes were treated with AICAR at indicated doses and time course. The protein levels of ERRα was determined by Western blot.

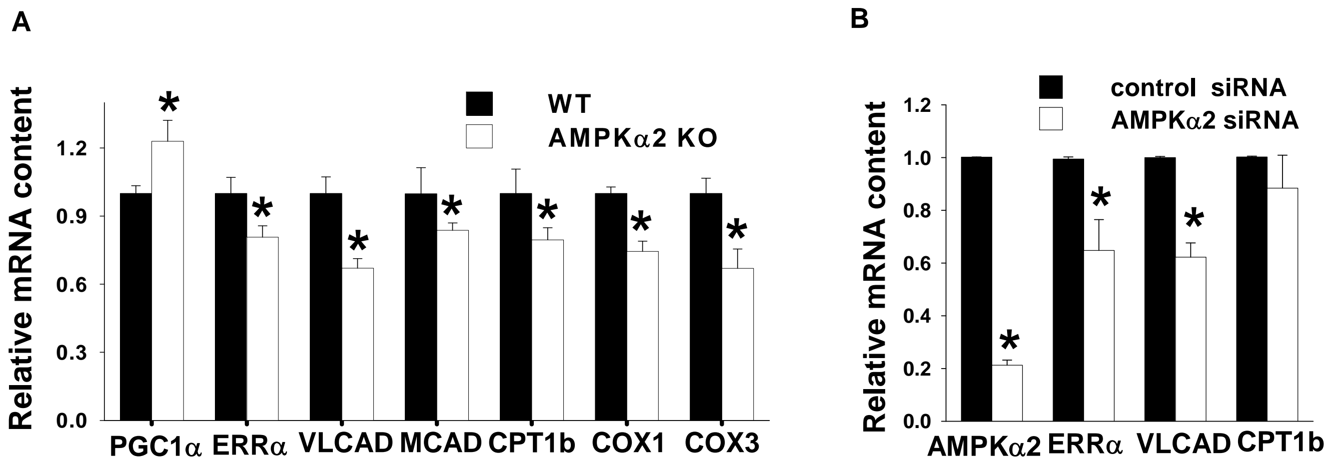

Figure S3. AMPKα2 regulates ERRα expression at the transcriptional level. The mRNA levels of ERRα and energy metabolism related genes were determined by real-time quantitative PCR. In AMPKα2 deficient mice (A), or rat neonatal cardiac myocytes treated with siRNA for AMPKα2 (B), the mRNA levels of ERRα, VLCAD and CPT1b were significantly decreased. * $p<0.05$ as compared with wild type controls (A) or as compared with control siRNA transfected cells (B).

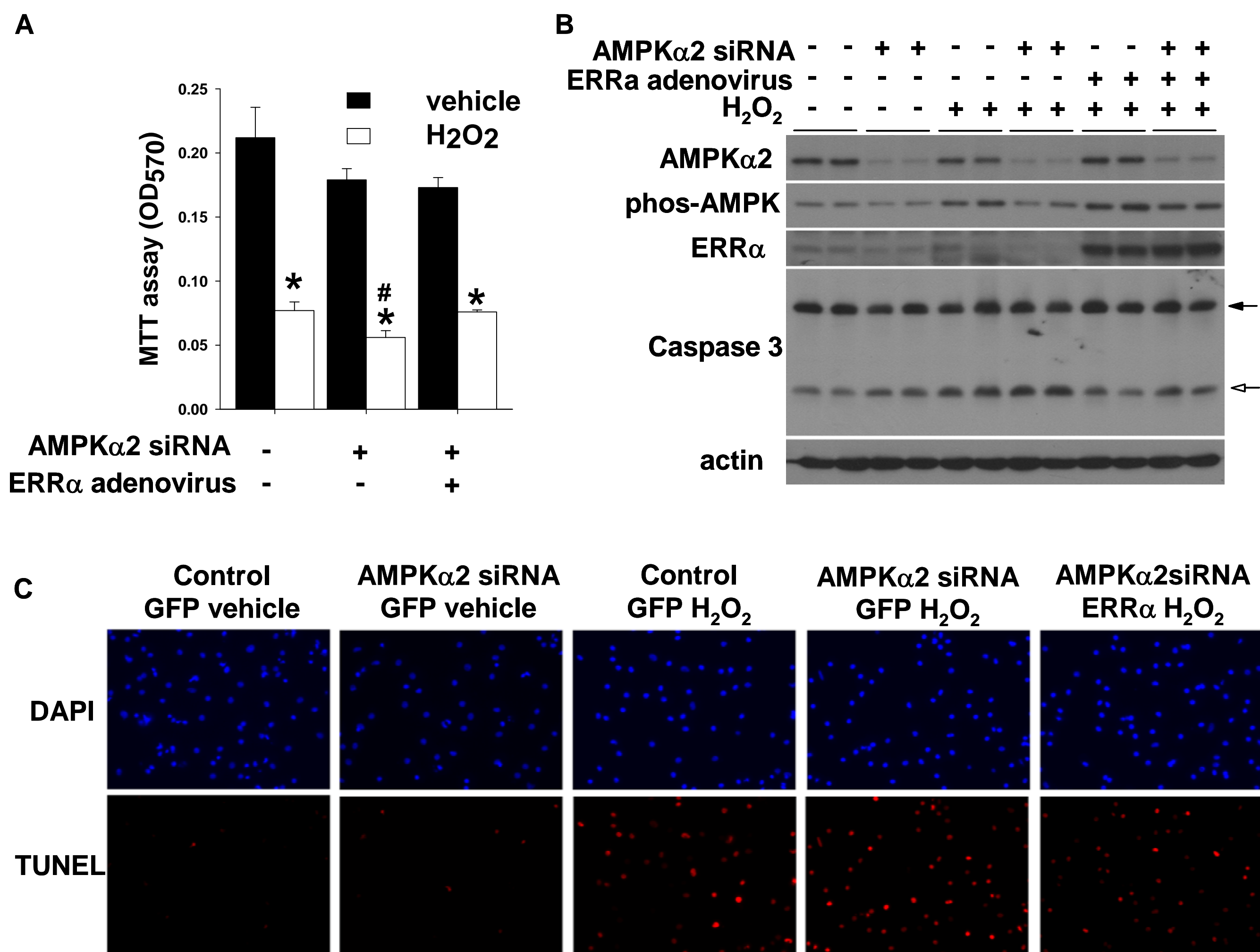

Figure S4. Effects of AMPK and ERRα overexpression on oxidative stress damage in rat neonatal cardiac myocytes. Rat neonatal cardiac myocytes were treated with H_2O_2 or vehicle. Inhibition of AMPKα2 expression by specific siRNA decreased cell viability (A), increased the level of cleaved caspase 3 (B) and the number of TUNEL positive cells (C). The enhanced oxidative stress damage caused by AMPKα2 repression could be partially rescued by ERRα overexpression.