Supplementary Material

Reagents. Antibodies used in the present studies include polyclonal antibodies to insulin receptor α (IR-α) and β (IR-β), phospho-AKT (pAKT, Thr308), phospho-AKT (pAKT, Ser473), AKT, Phospho-GSK3β (pGSK3β, Ser9), GSK3β, phospho-TSC2 (Thr1462), TSC2, mTOR, phospho-S6K (pS6K, Thr389), S6K, phospho-4EBP1 (p4EBP, Thr37/46) and 4EBP1, NLRP3, TXNIP, cGAS, STING, phospho-TBK1 (pTBK1, Ser172), TBK1, Bax, Bcl-2, Cleaved Caspase3 (C-cas3) and Cytochrome c (Cyt c) all of which were purchased from Cell Signaling Technology (Danvers, MA). Anti-HADHB antibody was from Abcam. Mouse anti-GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Bicinchoninic acid (BCA) protein Assay Kit was from Pierce (Rockford, IL, USA).

Animal care. These *Myl1*$^{tm1(cre)Sjb}$ mice selectively express the cre recombinase in skeletal muscle, which is driven by the myosin light polypeptide 1 (*Myl1*) promoter. The skeletal muscle-specific insulin receptor knockout mice (MIRKO) were generated by crossing insulin receptor *loxP* mice (IRlox) (Jackson Laboratory; stock No. 006955) with skeletal muscle-specific *Myl1* Cre mice (Jackson Laboratory; stock No. 024713). Genotyping was performed by PCR using genomic DNA isolated from the tip of the tail of 3- to 4-week-old mice. Insulin receptor (IR) was deleted selectively in skeletal muscle tissue, but not in the liver, iWAT, or heart. Six-week-old male MIRKO and IRlox control mice were fed with a normal chow diet (ND) or a high-fat diet (HFD; 60% Kcal from fat, Research Diets) for 24 weeks. All animals were maintained in an environmentally controlled facility with a diurnal light cycle and free access to water and a standard rodent chow. All experiments involving animals were performed in compliance with approved institutional animal care and use protocols according to NIH guidelines (NIH publication

no.86-23 [1985]).

Body Composition Analyses. Mice body weight was measured once every two weeks. MIRKO and IRlox control mice were fed with ND or HFD for 24 weeks. Measurements of fat, lean, and water masses was determined using EchoMRI^{TM}.

Insulin and Glucose Tolerance Test. In brief, insulin tolerance test (ITT) was performed on 6-hrs fasting mice. Mice were subjected to insulin (1.0 U/kg body weight; Novolin, Novo Nordisk) by intraperitoneal injection (i.p.). Oral glucose tolerance test (OGTT) was performed on mice fasted overnight. Mice were gavaged with glucose (2.5 g/kg body weight) and blood glucose was determined at 0, 30, 60, and 120 mins with a One Touch Ultra 2 glucometer (Lifescan, Milpitas, California). Then, to determine whether glucose tolerance exists, the area under the curve (AUC) was calculated. Each trapezoidal area is calculated by multiplying the base time (0.5 hour) by the average of two starting and ending height.

Insulin, IGF-1, Total Ketone Bodies (TKB), and Triglyceride Analyses. Insulin in serum was analyzed by Mouse Insulin ELISA kit (Mercodia, 10-1247-01) according to the manufacturer's instructions. Insulin-like growth factor (IGF-1) was analyzed by Mouse IGF-1 ELISA Kit (Thermo Fisher Scientific, Catalog # EMIGF1). Total ketone bodies (TKB) in serum was analyzed by measuring 3-hydroxybutyric acid (BOH) and acetoacetic acid (AcAc) using Ketone body assay kit (Sigma, No. MAK134) according to the manufacturer's instructions. Triglyceride in serum was analyzed using Triglyceride quantification colorimetric/fluorometric kit (Sigma, No. MAK266) according to the manufacturer's instructions.

Histological Analyses. Three mice from each group were anesthetized with isoflurane. Heart samples were isolated and fixed with 4% paraformaldehyde for 48 hours. Fixed hearts were dehydrated and embedded in paraffin, and 5 μm sections were cut with a Leica RM-2162 (Leica Microsystems, Bensheim, Germany). Hematoxylin and Eosin (H&E) and Masson's trichrome staining were performed according to the manufacturer's instructions. The collagen volume fraction (CVF) was analyzed by Image J. Wheat germ agglutinin (WGA, Thermo Fish Scientific) staining was performed by using Alexa Fluor 488 conjugate dye.

Echocardiography Assay. Echocardiographic analysis was performed after 24 weeks of HFD feeding by using a Vevo 2100 Imaging System and an MS550D transducer (Visual Sonics, Toronto, ON, Canada). M-mode short axis images of the left ventricle were analyzed to measure the following parameters, including left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular internal diameter end-diastole and end-systole (LVIDd and LVIDs, mm), interventricular septal end-diastole and end-systole (IVSd and IVSs, mm), left ventricular posterior wall end-diastole and end-systole (LVPWd and LVPWs, mm).

ROS production, Lipid Peroxidation and Citrate Synthase Assay. The ROS production from isolated mitochondria from left ventricular homogenates was indirectly detected by quantitatively measuring hydrogen peroxide (H_2O_2) as previously described. Lipid peroxidation production in heart tissues was analyzed by measuring changes in malondialdehyde (MDA) using a TBARS kit (Cayman Chemical Company, Cat No.10009055) according to the manufacturer's instruction. Citrate Synthase (CS) activity in heart tissue homogenate was measured by analyzing

the production of SH-CoA by monitoring the absorbance of Citrate Synthase Developing Reagent using a Citrate Synthase Activity Assay kit (Cayman Chemical Company, No. 701040).

TUNEL staining and transmission electron microscopy (TEM) Assay. TUNEL staining was carried out by using an ApopTag® Fluorescein in Situ Apoptosis Detection Kit (S7110, Sigma) according to the manufacturer's instructions**.** TUNEL positive cell percentage was analyzed using ImageJ.

The mitochondrial ultrastructure in mouse cardiomyocytes was evaluated using transmission electron microscopy (TEM). Heart samples from the same site of the left ventricle were fixed in 5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 0.05% $CaCl_2$ for 24 hours. After washing in 0.1 M sodium cacodylate buffer, tissues were post fixed overnight in 1% OsO4 and 0.1 M cacodylate buffer, dehydrated, and embedded in EMbed-812 resin. The sections were stained with 2% uranyl acetate, followed by 0.4% lead citrate, and viewed with a JEOL JEM-2200FS 200KV electron microscope (Electron Microscopy Sciences in University of Texas Health Science Center at San Antonio). Lipid droplet diameter and sarcomere length was analyzed by ImageJ.

Mitochondrial DNA Copy Number Assay. Mitochondrial DNA (mtDNA) copy number was determined using a multiplexed real time PCR (RT-PCR) assay as previously described. Briefly, total DNA was extracted from the left ventricle using DNeasy Blood & Tissue Kit (QIAGEN, No. 69504) according to manufacturer's instruction. RT-PCR analysis was carried out using SYBR Green Master Mix (Thermo Fisher, USA). Mitochondrial-specific (ND1) and

Cyclophilin A control were determined in triplicated of each sample.

Quantitative RT-PCR analysis. In brief, total RNA of a subset of heart tissues was extracted using TRIzol following the manufacturer's instructions (Thermo Fisher, USA). 2 μg of total RNA was used for cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen). RT-qPCR analysis was carried out using SYBR Green Master Mix (Thermo Fisher, USA). The sequence of the primers used to detect these genes are listed in Supplemental Table 1.

RNA-sequencing library preparation and next generation sequencing. Approximately 500ng total RNA was used for RNA-seq library preparation by following the Illumina TruSeq stranded mRNA sample preparation guide. The samples were first purified for the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were then copied into first strand cDNA using reverse transcriptase and random primers, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. Strand specificity was achieved by replacing dTTP with dUTP in the Second Strand Marking Mix (SMM). These cDNA fragments then went through an end repair process, the addition of a single 'A' base, and then ligation of the adapters. The products were purified and enriched with PCR to create the final RNA-seq library. After RNA-seq libraries were subjected to a quantification process, they were pooled for cBot amplification and subsequent 50bp single read sequencing run with Illumina HiSeq 3000 platform. After the sequencing run, demultiplexing was performed using Bcl2fastq2 script (provided by Illumina, Inc) to generate the fastq file for each sample. The average of 37 M reads were obtained for this set of samples.

The raw reads were aligned to the reference Mus musculus genome (UCSC mm9) with TopHat2 (PMID: 19289445, 23618408). Genes were annotated and quantified (read count and RPKM (reads per Kilobase per million aligned reads) at each gene) by HTSeq (PMID: 25260700) with NCBI RefSeq. For differential gene expression analysis, DESeq [PMID: 20979621] was used to first normalize gene expression with the size factor method followed with comparisons between groups (knockout vs wild-type) to identify significantly differentially expressed genes (DEGs) by 1) adjusted p-value (for multiple tests using Benjamini-Hochberg method) < 0.05, 2) at least a 2-fold change, and 3) average RPKM > 1 in at least one group. Functional analyses of these DEGs were performed by R/Bioconductor (https://www. bioconductor.org) *topGO* package for function over-representation and *fgsea* package for gene set enrichment analysis. Genes were further annotated using biomarRt package with GO SLIM (Gene Ontology subset that provides overall sense of the key biological functions that are essential to the organism).

Supplemental Figures and Legends

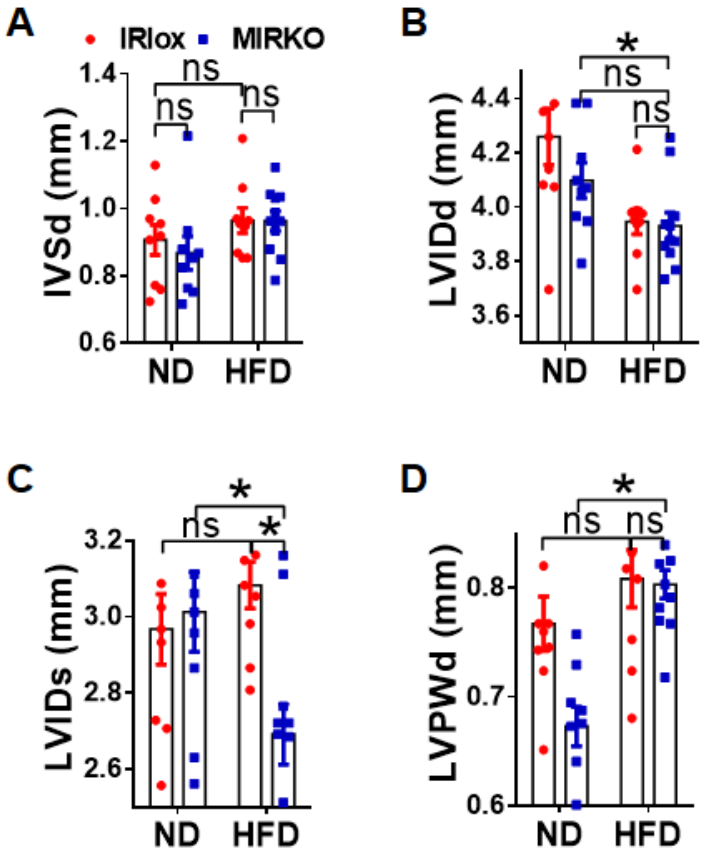

Figure S1. MIRKO mitigates DIO-induced LV dysfunction and adverse remodeling. *A-D*: Summary data of echocardiographic parameters, including interventricular septal end-diastole (IVSd) (*A*), LV internal diameter end-diastole and end-systole (LVIDd and LVIDs) (*B* and *C*), and LV posterior wall end-diastole (LVPWd) (*D*). n=10 per group. Data are expressed as means ± SEM; ns, nonsignificant, **P*<0.05.

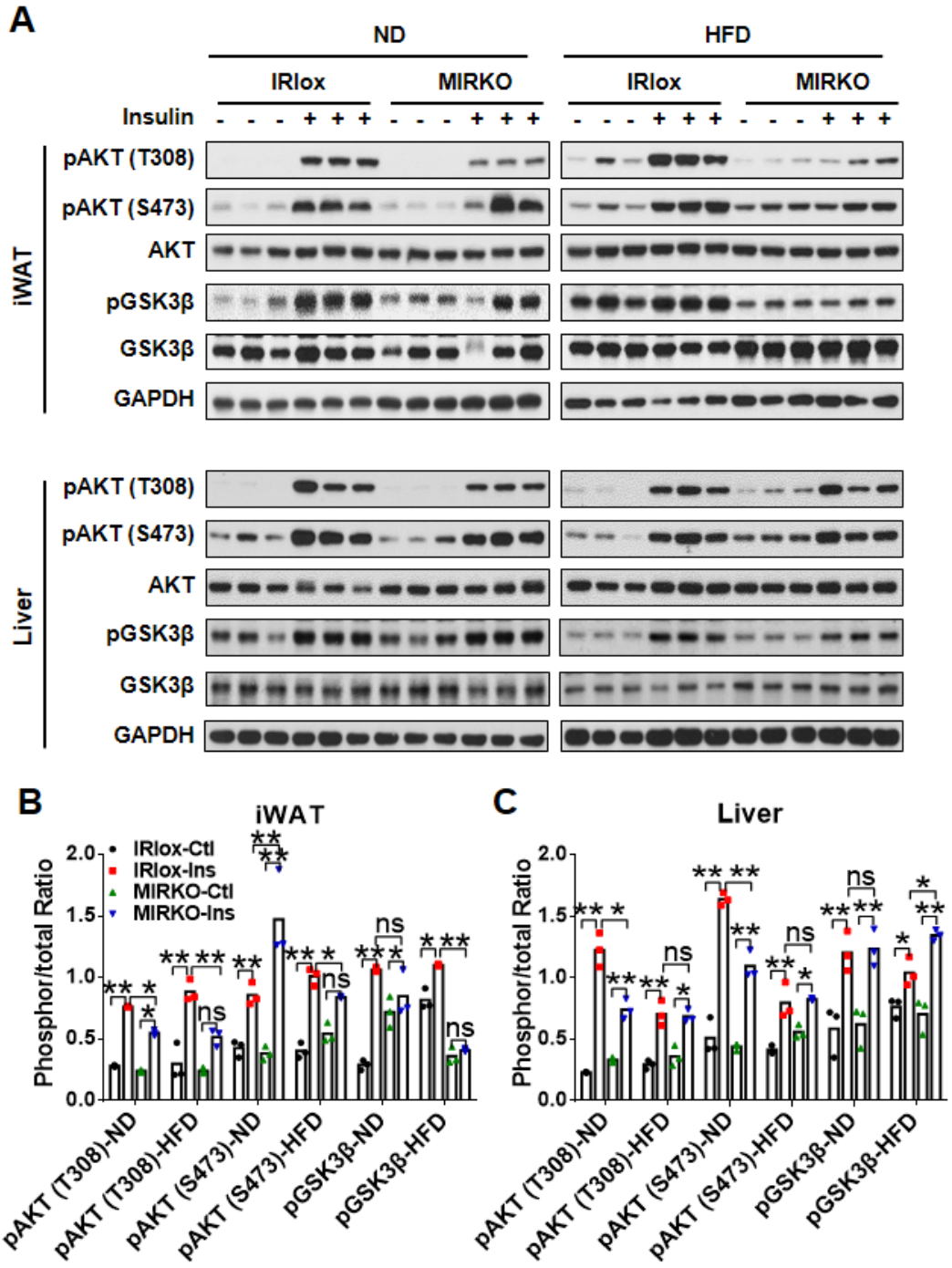
A
ND
HFD
IRlox
MIRKO
IRlox
MIRKO
Insulin
- - - + + + - - - + + + - - - + + + - - - + + +
iWAT
pAKT (T308)
pAKT (S473)
AKT
pGSK3β
GSK3β
GAPDH
Liver
pAKT (T308)
pAKT (S473)
AKT
pGSK3β
GSK3β
GAPDH
B
iWAT
IRlox-Ctl
IRlox-Ins
MIRKO-Ctl
MIRKO-Ins
Phosphor/total Ratio
2.0
1.5
1.0
0.5
0.0
pAKT (T308)-ND
pAKT (T308)-HFD
pAKT (S473)-ND
pAKT (S473)-HFD
pGSK3β-ND
pGSK3β-HFD
C
Liver
Phosphor/total Ratio
2.0
1.5
1.0
0.5
0.0
pAKT (T308)-ND
pAKT (T308)-HFD
pAKT (S473)-ND
pAKT (S473)-HFD
pGSK3β-ND
pGSK3β-HFD

Figure S2. MIRKO aggravates DIO-induced insulin resistance in iWAT and liver. *A*: Immunoblot analysis of AKT phosphorylation at Thr308 and Ser473 and GSK3β (Ser9) phosphorylation in iWAT and liver tissues from MIRKO and IRlox mice at 15 min after i.p. injection of at 1 U/kg, n=3 per group. *B* and *C*: Quantitative analysis of the ratio of phosphorylation and total protein levels of AKT and GSK3β in panel A. Data are expressed as means ± SEM; ns, nonsignificant, **P*<0.05, ***P*<0.01.

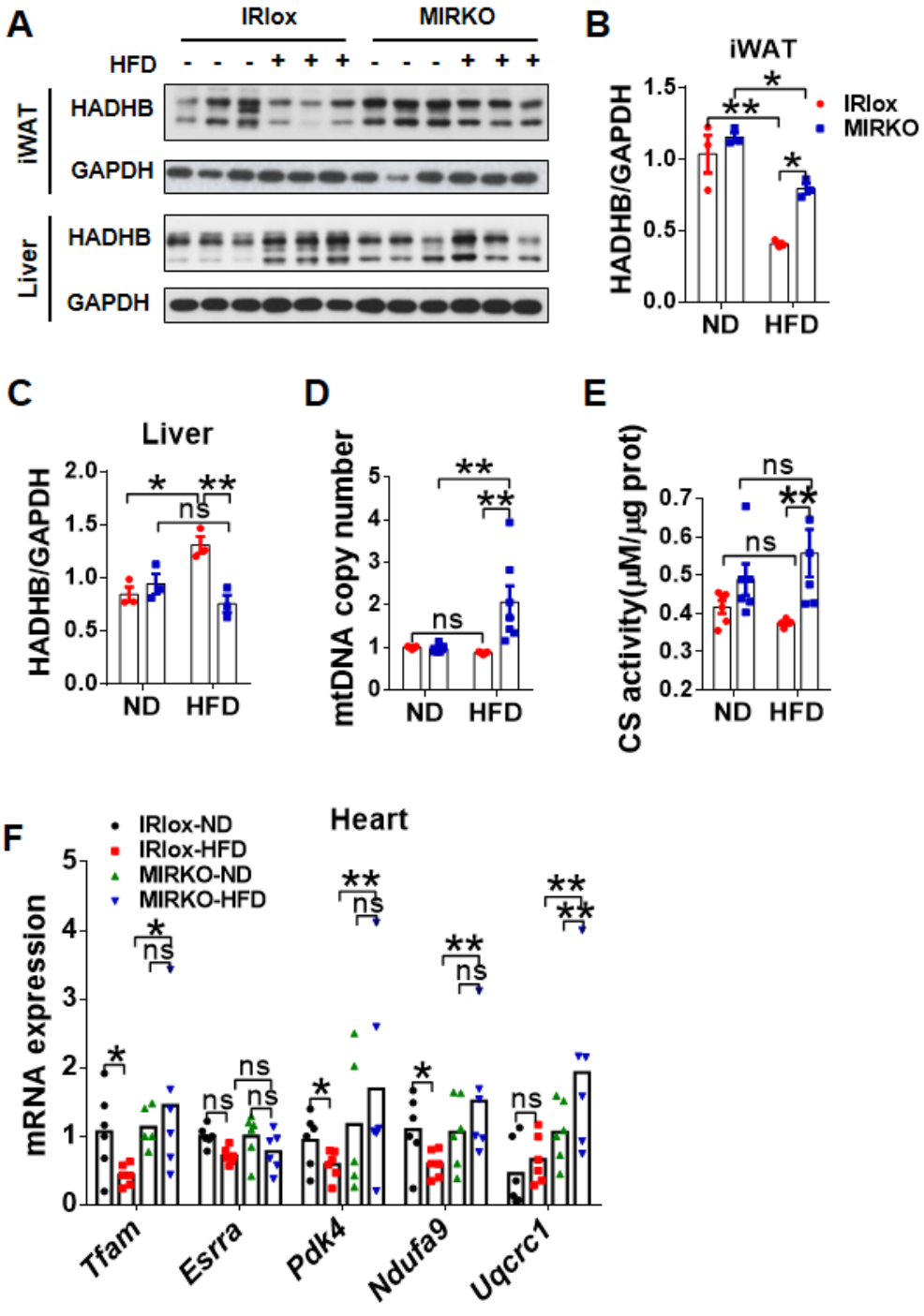
A
IRlox
MIRKO
HFD
- - - + + + - - - + + +
iWAT
HADHB
GAPDH
Liver
HADHB
GAPDH
B
iWAT
HADHB/GAPDH
IRlox
MIRKO
ND
HFD
C
Liver
HADHB/GAPDH
ND
HFD
D
mtDNA copy number
ND
HFD
E
CS activity(μM/μg prot)
ND
HFD
F
Heart
IRlox-ND
IRlox-HFD
MIRKO-ND
MIRKO-HFD
mRNA expression
Tfam
Esrra
Pdk4
Ndufa9
Uqcrc1

Figure S3. **MIRKO promotes mitochondrial biogenesis and respiration in the heart.** *A*: Immunoblot analysis of mitochondrial trifunctional protein β-subunit (HADHB) in iWAT and liver tissues isolated from MIRKO and IRlox control mice in response to DIO, n=3 per group. *B*-*C*: Quantitative analysis of the protein expression of HADHB in iWAT (*B*) and liver (*C*) tissues. *D*: RT-qPCR analysis of mtDNA copy number in heart tissues from MIRKO and IRlox control mice in response to DIO, n=6 per group. *E*: Evaluation of the level of citrate synthase (CS) activity in heart tissues, n=6 per group. *F*: RT-qPCR analysis mRNA levels of key biomarkers involved in mitochondrial function (*Tfam*, *Esrra*, *Pdk4*, *Ndufa9*, *Uqcrc1*) in the heart tissues from MIRKO and IRlox control mice in response to DIO, n=6 per group. Data are expressed as means ± SEM; ns, nonsignificant, **P*<0.05, ***P*<0.01.

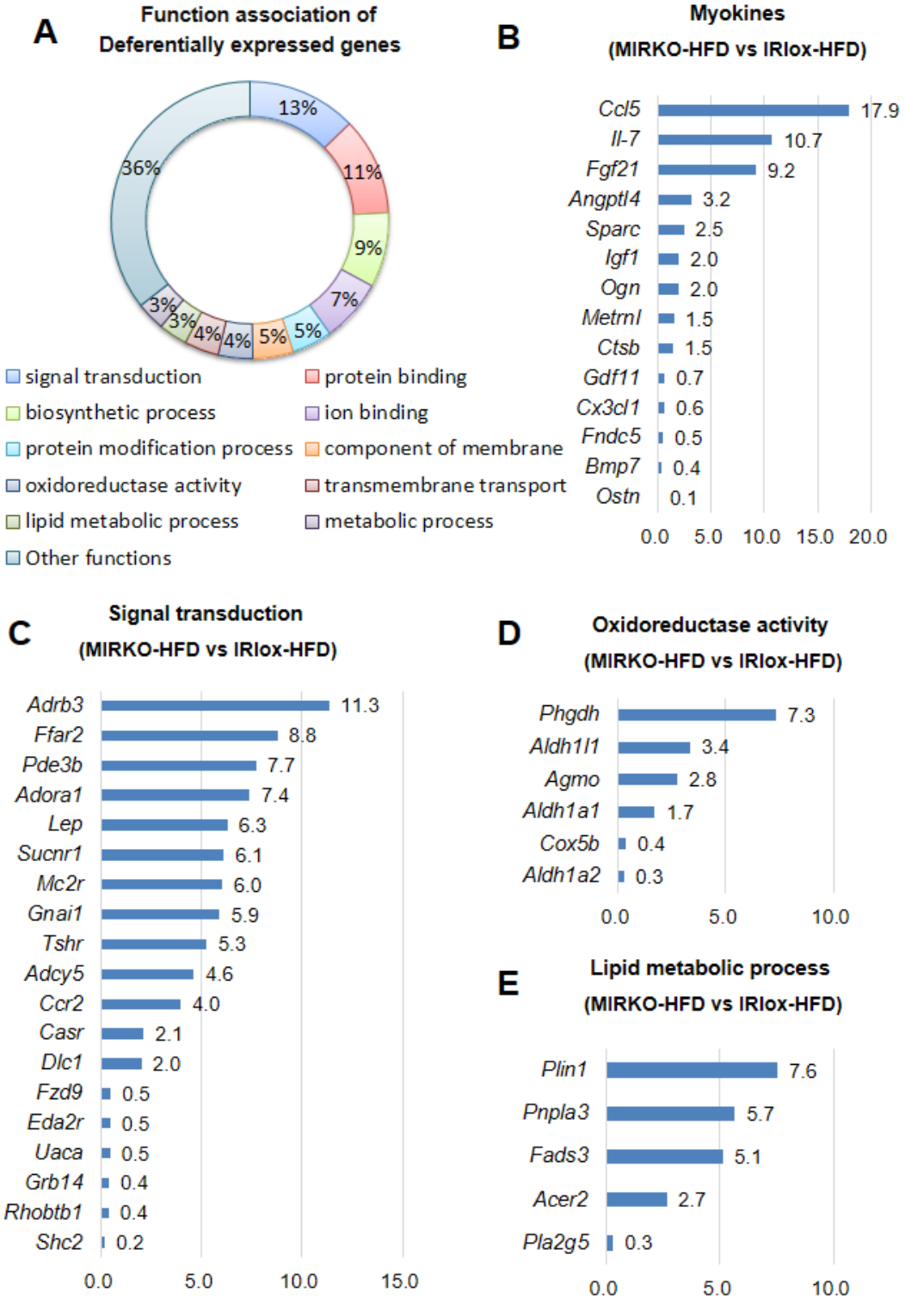
A
Function association of
Deferentially expressed genes
13%
11%
9%
7%
5%
5%
4%
4%
3%
3%
36%
signal transduction
protein binding
biosynthetic process
ion binding
protein modification process
component of membrane
oxidoreductase activity
transmembrane transport
lipid metabolic process
metabolic process
Other functions
B
Myokines
(MIRKO-HFD vs IRlox-HFD)
Ccl5 17.9
Il-7 10.7
Fgf21 9.2
Angptl4 3.2
Sparc 2.5
Igf1 2.0
Ogn 2.0
Metrnl 1.5
Ctsb 1.5
Gdf11 0.7
Cx3cl1 0.6
Fndc5 0.5
Bmp7 0.4
Ostn 0.1
0.0 5.0 10.0 15.0 20.0
C
Signal transduction
(MIRKO-HFD vs IRlox-HFD)
Adrb3 11.3
Ffar2 8.8
Pde3b 7.7
Adora1 7.4
Lep 6.3
Sucnr1 6.1
Mc2r 6.0
Gnai1 5.9
Tshr 5.3
Adcy5 4.6
Ccr2 4.0
Casr 2.1
Dlc1 2.0
Fzd9 0.5
Eda2r 0.5
Uaca 0.5
Grb14 0.4
Rhobtb1 0.4
Shc2 0.2
0.0 5.0 10.0 15.0
D
Oxidoreductase activity
(MIRKO-HFD vs IRlox-HFD)
Phgdh 7.3
Aldh1l1 3.4
Agmo 2.8
Aldh1a1 1.7
Cox5b 0.4
Aldh1a2 0.3
0.0 5.0 10.0
E
Lipid metabolic process
(MIRKO-HFD vs IRlox-HFD)
Plin1 7.6
Pnpla3 5.7
Fads3 5.1
Acer2 2.7
Pla2g5 0.3
0.0 5.0 10.0

Figure S4. **Functional characterization of differentially expressed genes** (DEGs). *A:* 198 DEGs were obtained from heart samples from MIRKO and IRlox mice on HFD. The biological functions associated with these DEGs were determined by GO SLIM annotation analysis (see Methods section) and are expressed as percentage. *B-E*: Annotation of differentially expressed genes in the heart tissues between MIRKO and IRlox mice on HFD, including myokines (B), signal transduction (C), oxidoreductase activity (D), and lipid metabolic process (E), summarized as fold of change in x-axis of each corresponding bar chart.

Supplemental Table 1. List of utilized primers for qRT-PCR

Gene	Species	Forward Primer
Col1a1	Forward	5'-GAGCGGAGAGTACTGGATCG-3'
	Reverse	5'-GTTCG GGCTGATGTACCAGT-3'
Col3a1	Forward	5'-ACCA AAAGGTGATGCT GGAC-3'
	Reverse	5'-GACCTCGTGCTCCAGTTAGC-3'
Anf	Forward	5'-GTGTACA GTGCGGTGTCCAA-3'
	Reverse	5'-ACCTCATCTTCTACCGGATC-3'
Bnp	Forward	5'-GCTGCTTTGGGCACAAGATAG-3'
	Reverse	5'-GGAGCTCTTCC TACAACAACTT-3'
β-Mhc	Forward	5'-AGGGCGACCTCAACGAGAT-3'
	Reverse	5'-CAGCAGACTC TGGAGGCTCTT-3'
Tnf	Forward	5'-CCCTCACACTCAGATCATCTTCT-3'
	Reverse	5'-GCTACGACGTGGGCTACAG-3'
Nfkb	Forward	5'-ATGGCAGACGATGATCCCTAC-3'
	Reverse	5'-TGTTGACAGTGGTATTTCTGGTG-3'
Il-1b	Forward	5'-GACCTTCCAGGATGAGGACA-3'
	Reverse	5'-AGGCCACAGGTATTTTGTCG-3'
Il-6	Forward	5'-GCTACCAAACTGGATATAATCAGGA-3'
	Reverse	5'-CCAGGTAGCTATGGTACTCCAGAA-3'
Il-4	Forward	5'-GGTCTCAACCCCCAGCTAGT-3'
	Reverse	5'-GCCGATGATCTCTCTCAAGTGAT-3'
ND1	Forward	5'-TGACCCATAGCCATAA TATGATTT-3'
	Reverse	5'-CTCTACGTTAAACCCTGATACTAA-3'
Cyclophilin A	Forward	5'-ACACGCCATAATGGCACTCC-3'
	Reverse	5'-CAGTCTTGGCAGTGCAGAT-3'
Tfam	Forward	5'-CAAAAAGACCTCGTTCAGCA-3'
	Reverse	5'-CTTCAGCCATCTGCTCTTCC-3'
Esrra	Forward	5'-GGAGGACGGCAGAAGTACAA-3'
	Reverse	5'-CAGGTTCAACAACCAGCAGA-3'
Pdk4	Forward	5'-GCTTGCCAATTTCTCGTCTC-3'
	Reverse	5'-CTTCTCCTTCGCCAGGTTCT-3'
Ndufa9	Forward	5'-ATCCCTTACCCTTTGCCACT-3'
	Reverse	5'-CCGTAGCACCTCAATGGACT-3'
Uqcrc1	Forward	5'-TGCCAGAGTTTCCAGACCTT-3'
	Reverse	5'-CCAAATGAGACACCAAAGCA-3'
Cd36	Forward	5'-TCCTTGTAGGAGATGGTGTTCC-3'
	Reverse	5'-CCATTGGTGATGAAAAAGCA-3'
Cpt1b	Forward	5'-TGCCTTTACATCGTCTCCAA-3'
	Reverse	5'-AGACCCCGTAGCCATCATC-3'
Cpt2	Forward	5'-CCAGCTGACCAAAGAAGCA-3'
	Reverse	5'-GCAGCCTATCCAGTCATCGT-3'
Fabp3	Forward	5'-GACGGGAAACTCATCCTGAC-3'
	Reverse	5'-TCTCCAGAAAAATCCCAACC-3'
Pgc-1α	Forward	5'-GTAAATCTGCGGGATGATGG-3'
	Reverse	5'-AGCAGGGTCAAAATCGTCTG-3'
Acadm	Forward	5'-ACTGACGCCGTTCAGATTTT-3'
	Reverse	5'-GCTTAGTTACACGAGGGTGATG-3'
Mstn	Forward	5'-CTTCTGCCAAGAGCGCCTCCAC-3
	Reverse	5'-GCCCAAAGTCTCTCCGGGACCT-3
Erfe	Forward	5'-CTGAAGCTTGGCCTGCCAGGAC-3'
	Reverse	5'-CAGCCACAGGTGCGGTGTACTG-3'

Fstl1	Forward	5’-TGCCGTCACAGAGAAGGGGGAG-3’
	Reverse	5’-AGGCTGGCCACCCTCATTTCCT-3’
Gdf11	Forward	5’-AGCAAACTGCGGCTCAAGGAGG-3’
	Reverse	5’-CAGCAGAGCCTCTTGGGTTGGC-3’
Ostn	Forward	5’-TGTGATGCTGTGGGGCTCAGGA-3’
	Reverse	5’-AGCCTCTGGAACTGGAGAGCCG-3’
Igf2	Forward	5’-GGAGGGGAGCTTGTTGACACGC-3’
	Reverse	5’-AAACCTGGGGAGGGAGTGGAGC-3’
Gapdh	Forward	5’-AATGGTGAAGGTCGGTGTG-3’
	Reverse	5’-GTGGAGTCATACTGGAAC ATGTAG-3’