

Dual-gene therapy of *SIRT3* and *SIRT4* enhances bone repair in diabetes through mitochondrial network remodeling

Supplementary File

Table S1. Gene primer sequences for real-time quantitative polymerase chain reaction (Human)

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
<i>MFN2</i>	CTCTCGATGCAACTCTATCGTC	TCCTGTACGTGTCTTCAAGGAA
<i>OPA1</i>	TGTGAGGTCTGCCAGTCTTTA	TGTCCTTAATTGGGGTCGTTG
<i>DRP1</i>	CTGCCTCAAATCGTCGTAGTG	GAGGTCTCCGGGTGACAATTC
<i>TIM23</i>	GCTCCTTGCTCCAACAGTCA	AGAAATAACTCCCCAACTGTCCTT
<i>TOM40</i>	ACAAGGATGCAGGACACCAG	CACCCACGATCCAGTTGCTA
<i>PINK1</i>	GCCTCATCGAGGAAAAACAGG	GTCTCGTGTCCAACGGGTC
<i>ROMO1</i>	AAGCTGCTTCGACCGTGTC	CCCGCATTCCGATCCTGAG
<i>NADH</i>	GTGGTGCGTCCAGAGAGAC	GGCCTTCGCCATATCCTTTTCGAGGA
<i>ND1</i>	TACAACTACGCAAAGGCCCC	AGATGTGGCGGGTTTTAGGG
<i>ND2</i>	TAAACTCCAGCACCACGACC	AGCTTGTTTCAGGTGCGAGA
<i>COX1</i>	CCTACTCCTGCTCGCATCTG	AGAATGGGGTCTCCTCCTCC
<i>ATP6</i>	AGCGCCACCCTAGCAATATC	TTGGATTAAGGCGACAGCGA
<i>OCN</i>	ATGAGAGCCCTCACACTCCT	CTTGACACAAAGGCTGCAC
<i>RUNX2</i>	CGCCTCACAAACAACCACAG	ACTGCTTGACGCCTTAAATGAC
<i>OPN</i>	ACATGGCTAAACCCTGACCCA	TTACTTGGAAGGGTCTGTGGGG
<i>COL-1</i>	GAGGCTCTGAAGGTCCCCAG	GCAATACCAGGAGCACCATTG
<i>α-KGDH</i>	CCGGAGACAGGCAGTTGT	CACTGAGAAAAGGGCTCAGCA
<i>IDH</i>	GCCTGGAGTTTAAAAGGCGAT	TCGTTGGTGGCATCAGGATT
<i>CS</i>	CCAGTGCTTCTCCACGAAT	GGAACGGATGCCCTCATCA
<i>NDUFB-3</i>	TCAGATTGCTGTCTAGGTGCT	GCTGTCTTCTGCCTTTGCG
<i>MTCO3</i>	TCACCCCGCTAAATCCCCTA	TGACGTGAAGTCCGTGGAAG
<i>SDHB</i>	GCCCCATGGTATTGGATGCT	AGTTGCTCAAATCCTTATTGAGGT
<i>TFAM</i>	CGCTCCCCCTTCAGTTTGT	CCAACGCTGGGCAATTCTTC
<i>SIRT3</i>	GGCGGCAGGGACGATTATTA	GTA CTGCTGGAGGTTGCTGT
<i>SIRT4</i>	AATTTGTTACGCCCCCTGCT	AGCTCATCTTCATTCTTTGCGA
<i>β-actin</i>	ATCAAGGTGGGTGTCTTTCCT	GCAATGATCTGAGGAGG

Abbreviations: MFN2: Mitofusin 2; OPA1: Optic atrophy 1; DRP1: Dynamin-related protein 1; TIM23; Translocase of inner mitochondrial membrane 23; TOM40: Translocase of outer mitochondrial membrane 40; PINK1: PTEN-induced kinase 1; ROMO1: Reactive oxygen species modulator 1; NADH: Nicotinamide adenine dinucleotide+hydrogen; ND1: NADH dehydrogenase 1; ND2: NADH dehydrogenase 2; COX1: Cytochrome C oxidase I; ATP6: ATP synthase 6; OCN: Osteocalcin; RUNX2: Runt-related transcription factor 2; OPN: Osteopontin; COL-1: Collagen type I alpha 1 chain; α-KGDH: Alpha-ketoglutarate dehydrogenase; IDH: Isocitrate dehydrogenase; CS: Citrate synthase; NDUFB-3: NADH: Ubiquinone oxidoreductase subunit B3; MTCO3: Mitochondrial cytochrome C oxidase III; SDHB: Succinate dehydrogenase complex iron sulfur subunit B; TFAM: Mitochondrial transcription factor A; SIRT3: Sirtuin 3; SIRT4: Sirtuin 4.

Table S2. Gene primer sequences for real-time quantitative polymerase chain reaction (Mouse)

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
<i>Nd1</i>	CACCCCTTATCAACCTCAA	ATTTGTTTCTGCGAGGGTTG
<i>Nd2</i>	ATCCTCCTGGCCATCGTACT	ATCAGAAGTGAATGGGGCG
<i>Cox1</i>	TCCGAGCCCCAGATATAGCA	TTTCCGGCTAGAGGTGGGTA
<i>Atp6</i>	GCAGTCCGGCTTACAGCTAA	GGTAGCTGTTGGTGGGCTAA
<i>β-actin</i>	GCTCCGGCATGTGCAAAG	CCTTCTGACCCATTCCCACC

Table S3. Antibodies used in the study

Antibody	Dilution	Catalog number	Supplier
Primary antibodies (Rabbit IgG)			
SIRT3	1/1,000	A5419	ABclonal
SIRT4	1/1,000	A15800	ABclonal
DRP1	1/1,500	12957-1-AP	Proteintech
MFN2	1/1,500	FNab05151	FineTest
OPA1	1/1,500	27733-1-AP	Proteintech
TIM23	1/1,500	11123-1-AP	Proteintech
RHOT1	1/1,500	ASE-38697	SAB
ANT2	1/1,500	ab222843	Abcam
TOM40	1/1,000	DF12239-50	Affinity Biosciences
PINK1	1/1,000	PA1-16604	Invitrogen
ROMO1	1/1,000	SE47598	SAB
NADH	1/1,500	41203-1	SAB
SNX9	1/1,000	15721-1-AP	Proteintech
β -actin	1/1,500	ab181602	Abcam
p-IRS1	1/1,000	AF5845	Beyotime
IRS1	1/1,000	17509-1-AP	Proteintech
AKT	1/1,500	ab179463	Abcam
PI3K	1/1,500	ab86714	Abcam
p-AKT	1/1,500	ab38449	Abcam
p-PI3K	1/1,000	ab182651	Abcam
Glut4	1/1,500	A25174	ABclonal
PGC-1 α	1/1,500	2178S	CST
TFAM	1/1,500	ab131607	Abcam
CD206	1/1,000	24595	CST
CD86	1/1,000	19589S	CST
Secondary antibody (Goat IgG)			
Goat anti-rabbit IgG (H+L)	1/2,000	ab205718	Abcam

Abbreviations: SIRT3: Sirtuin 3; SIRT4: Sirtuin 4; DRP1: Dynamin-related protein 1; MFN2: Mitofusin 2; OPA1: Optic atrophy 1; TIM23: Translocase of inner mitochondrial membrane 23; RHOT1: Ras homolog family member T1; ANT2: Adenine nucleotide translocator 2; TOM40: Translocase of outer mitochondrial membrane 40; PINK1: PTEN-induced kinase 1; ROMO1: Reactive oxygen species modulator 1; NADH: Nicotinamide adenine dinucleotide+hydrogen; SNX9: Sorting nexin 9; IRS1: Insulin receptor substrate 1; PGC-1 α : PPARGC1A (Peroxisome proliferator-activated receptor gamma coactivator 1 alpha); TFAM: Mitochondrial transcription factor A; IgG: Immunoglobulin G.

Table S4. Small interfering RNA

Gene	Sense 5' to 3'	Antisense 5' to 3'
<i>SIRT3</i>	AAUGCCACUGGGUGUGCUGAU	CAGCACACCCAGUGGCAUUC
<i>SIRT4</i>	AGUCAACGCAAAGCUCAUCUU	GAUGAGCUUUGCGUUGACUUU

Abbreviations: SIRT3: Sirtuin 3; SIRT4: Sirtuin 4.

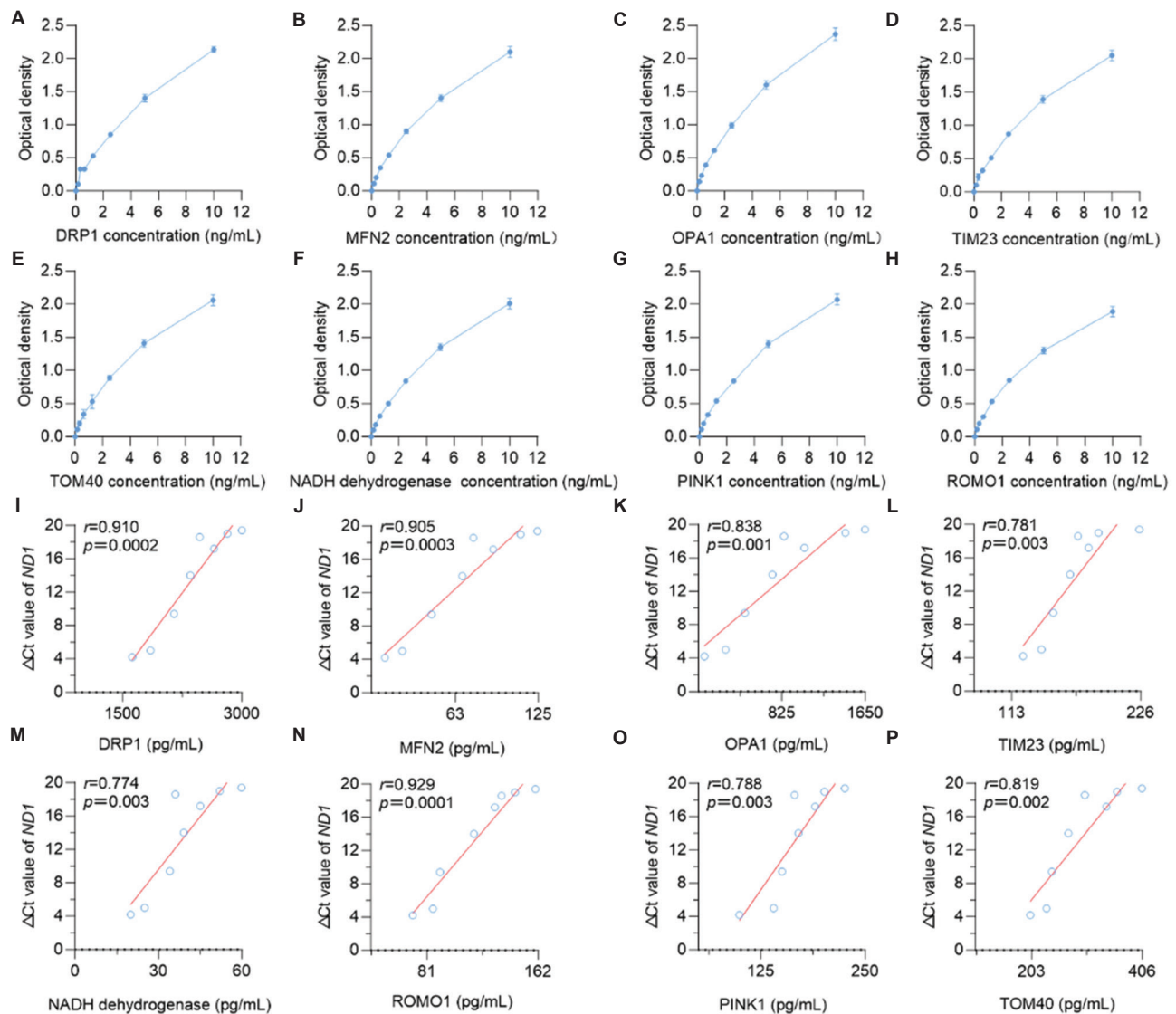


Figure S1. Enzyme-linked immunosorbent assay detection standard curves of eight mitochondrial network marker proteins and correlation analysis with mitochondrial DNA (mtDNA) content. (A-H) Standard curves of eight marker proteins ($n = 5$); the correlation between (I-P) eight marker proteins and mtDNA content ($n = 3$).

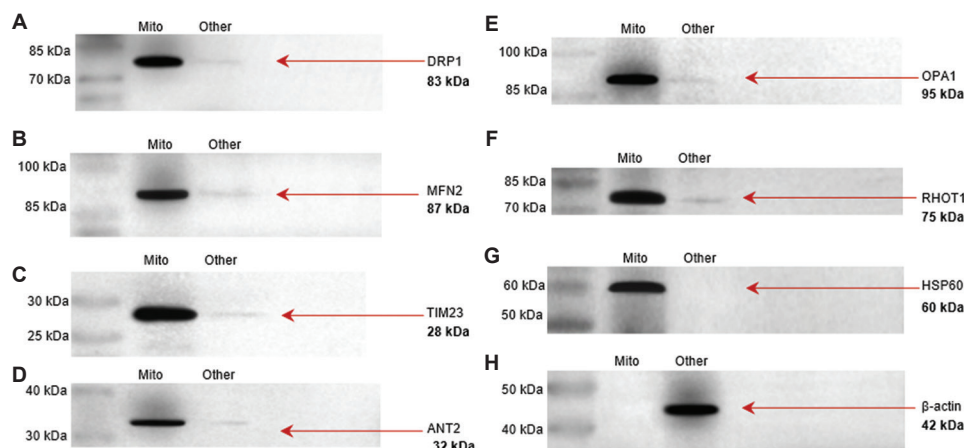


Figure R1. Original images for Western blotting for DRP1, MFN2, TIM23, ANT2, OPA1, RHOT1, HSP60, and β-actin in Figure 1A. (A) Western blotting for DRP1 (83 kDa). (B) Western blotting for MFN2 (87 kDa). (C) Western blotting for TIM23 (28 kDa). (D) Western blotting for ANT2 (32 kDa). (E) Western blotting for OPA1 (95 kDa). (F) Western blotting for RHOT1 (75 kDa). (G) Western blotting for HSP60 (60 kDa). (H) Western blotting for β-actin (42 kDa).

Abbreviations: ANT2: Adenine nucleotide translocator 2; DRP1: Dynamin-related protein 1 (DNM1L); HSP60: Heat shock protein 60 (HSPD1); MFN2: Mitofusin 2; OPA1: Optic atrophy 1; RHOT1: Rhot1; TIM23: Translocase of inner mitochondrial membrane 23.

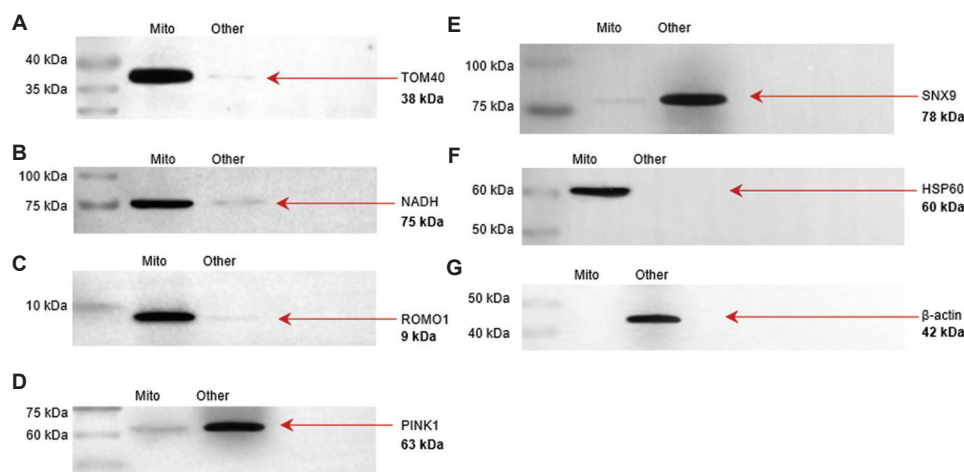


Figure R2. Original images for Western blotting for TOM40, NADH, ROMO1, PINK1, SNX9, HSP60, and β-actin in Figure 1B. (A) Western blotting for TOM40 (38 kDa). (B) Western blotting for NADH (75 kDa). (C) Western blotting for ROMO1 (9 kDa). (D) Western blotting for PINK1 (63 kDa). (E) Western blotting for SNX9 (78 kDa). (F) Western blotting for HSP60 (60 kDa). (G) Western blotting for β-actin (42 kDa). Abbreviations: HSP60: Heat shock protein 60; NADH: Nicotinamide adenine dinucleotide (reduced form); PINK1: PTEN-induced kinase 1; ROMO1: Reactive oxygen species modulator 1; SNX9: Sorting nexin 9; TOM40: Translocase of outer mitochondrial membrane 40.

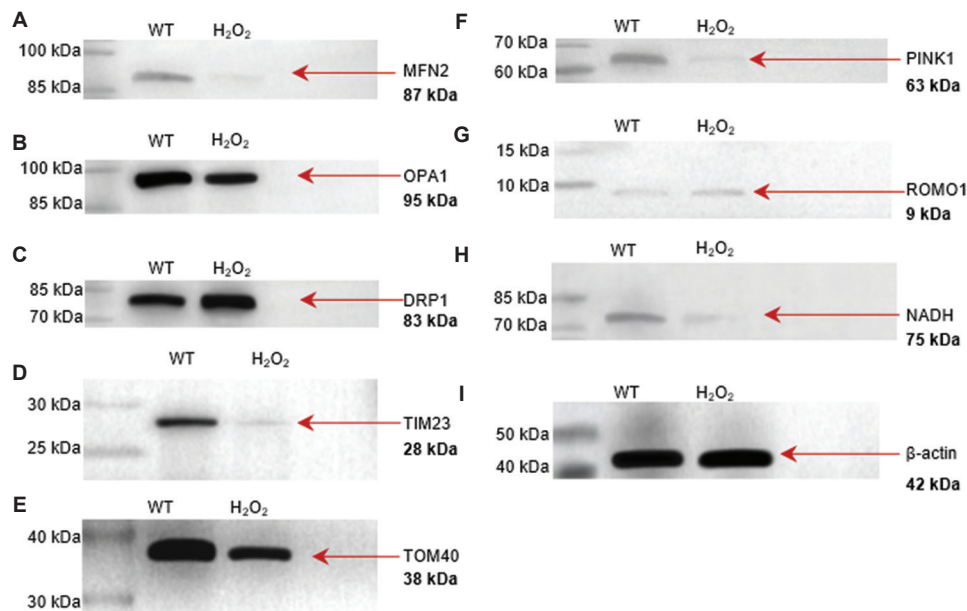


Figure R3. Original images for Western blotting for MFN2, OPA1, DRP1, TIM23, TOM40, PINK1, ROMO1, NADH, and β -actin in Figure 1M. (A) Western blotting for MFN2 (87 kDa). (B) Western blotting for OPA1 (95 kDa). (C) Western blotting for DRP1 (83 kDa). (D) Western blotting for TIM23 (28 kDa). (E) Western blotting for TOM40 (38 kDa). (F) Western blotting for PINK1 (63 kDa). (G) Western blotting for ROMO1 (9 kDa). (H) Western blotting for NADH (75 kDa). (I) Western blotting for β -actin (42 kDa).

Abbreviations: DRP1: Dynamin-related protein 1; MFN2: Mitofusin 2; NADH: Nicotinamide adenine dinucleotide (reduced form); OPA1: Optic atrophy 1; PINK1: PTEN-induced kinase 1; ROMO1: Reactive oxygen species modulator 1; TIM23: Translocase of inner mitochondrial membrane 23 (TIMM23); TOM40: Translocase of outer mitochondrial membrane 40 (TOMM40).

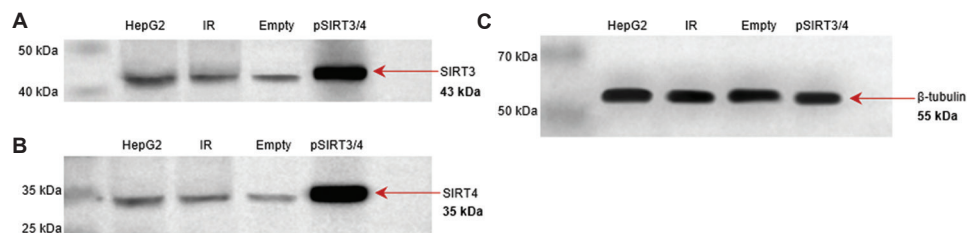


Figure R4. Original images for Western blotting for SIRT3, SIRT4, and β -tubulin in Figure 2B. (A) Western blotting for SIRT3 (43 kDa). (B) Western blotting for SIRT4 (35 kDa). (C) Western blotting for β -tubulin (55 kDa).

Abbreviations: IR: Insulin resistance; SIRT3: Sirtuin 3; SIRT4: Sirtuin 4.

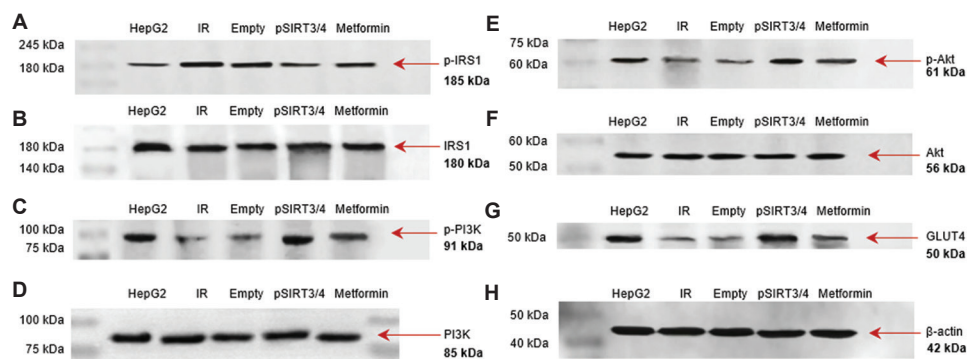


Figure R5. Original images for Western blotting for p-IRS1, IRS1, p-PI3K, PI3K, p-Akt, Akt, GLUT4, and β -actin in Figure 2G. (A) Western blotting for p-IRS1 (185 kDa). (B) Western blotting for IRS1 (180 kDa). (C) Western blotting for p-PI3K (91 kDa). (D) Western blotting for PI3K (85 kDa). (E) Western blotting for p-Akt (61 kDa). (F) Western blotting for Akt (56 kDa). (G) Western blotting for GLUT4 (50 kDa). (H) Western blotting for β -actin (42 kDa).

Abbreviations: Akt: Protein kinase B; GLUT4: Glucose transporter type 4; IRS1: Insulin receptor substrate 1; p-Akt: Phosphorylated protein kinase B; p-IRS1: Phosphorylated insulin receptor substrate 1; p-PI3K: Phosphorylated phosphoinositide 3-kinase; PI3K: Phosphoinositide 3-kinase.

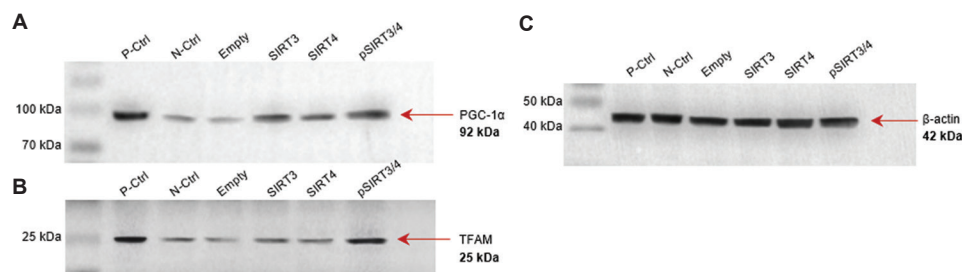


Figure R6. Original images for Western blotting for PGC-1 α , TFAM, and β -actin in Figure 4O. (A) Western blotting for PGC-1 α (92 kDa). (B) Western blotting for TFAM (25 kDa). (C) Western blotting for β -actin (42 kDa).

Abbreviations: PGC-1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM: Mitochondrial transcription factor A.

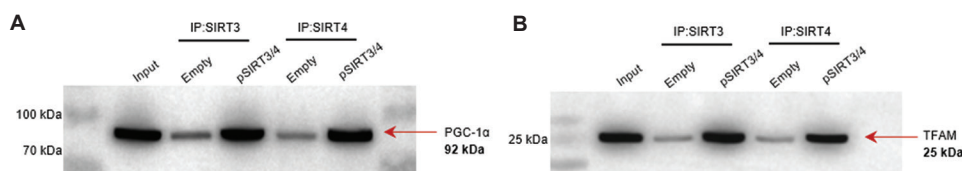


Figure R7. Original images for Western blotting for PGC-1 α and TFAM in Figure 4Q. (A) Western blotting for PGC-1 α (92 kDa). (B) Western blotting for TFAM (25 kDa).

Abbreviations: PGC-1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM: Mitochondrial transcription factor A.

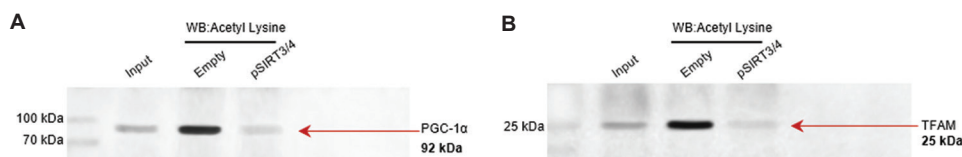


Figure R8. Original images for Western blotting for PGC-1 α and TFAM in Figure 4R. (A) Western blotting for PGC-1 α (92 kDa). (B) Western blotting for TFAM (25 kDa).

Abbreviations: PGC-1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM: Mitochondrial transcription factor A.

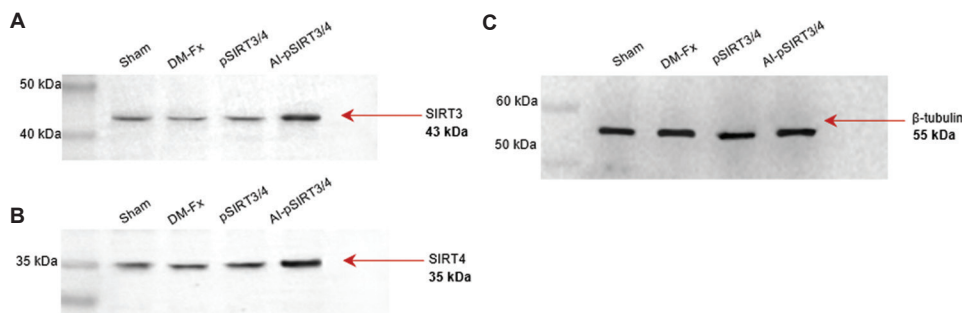


Figure R9. Original images for Western blotting for SIRT3, SIRT4, and β -tubulin in Figure 6E. (A) Western blotting for SIRT3 (43 kDa). (B) Western blotting for SIRT4 (35 kDa). (C) Western blotting for β -tubulin (55 kDa).

Abbreviations: SIRT3: Sirtuin 3; SIRT4: Sirtuin 4.