Supplemental Materials

Roflumilast-mediated phosphodiesterase 4D inhibition reverses diabetes-associated cardiac dysfunction and remodeling: effects beyond glucose lowering

Rui Xu[1, 2, *], Jing Fu[1, 2, *], Yuting Hu[1], Xiaoyan Yang[1,2], Xiang Tao[1,3], Long Chen[3], Kai Huang[3], Qin Fu[1, 2]

[1]Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China;

[2]Key Laboratory for Drug Target Research and Pharmacodynamic Evaluation of Hubei Province, Wuhan, China;

[3]Clinical Center of Human Gene Research, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

* R. Xu and J. Fu are contributed equally to this work.

Address for Correspondence:

Qin Fu, PhD

Department of Pharmacology, School of Basic Medicine, Tongji Medical College

Huazhong University of Science and Technology

Hangkong Road 13th, 430030, Wuhan, China

Tel: +86-27-83692801

Email fuqin@mails.tjmu.edu.cn

SUPPLEMENTAL TABLE

Table 1. Clinical characteristics of donors and heart failure patients with diabetes.

According to the regulations on organ donation in China, the information of donors is confidential.

Patient	Diagnosis	Age	Gender	EF
Donor 1	N/A	N/A	N/A	N/A
Donor 2	N/A	N/A	N/A	N/A
Donor 3	N/A	N/A	N/A	N/A
Donor 4	N/A	N/A	N/A	N/A
Donor 5	N/A	N/A	N/A	N/A
Donor 6	N/A	N/A	N/A	N/A
Patient 1	DM, RHD	51	Female	22%
Patient 2	DM, AVR	50	Female	37%
Patient 3	DM, MVD	55	Male	33%
Patient 4	DM, DCM	54	Male	16%
Patient 5	DM, CAD	44	Male	30%
Patient 6	DM, AVI	62	Male	29%

DM: diabetes mellitus; RHD: rheumatic heart disease; AVR: aortic valve replacement; MVD: mitral valve dysplasia; DCM: dilated cardiomyopathy; CAD: coronary artery disease; AVI: aortic valve insufficiency; N/A: not available.

Table 2. List of primers.

Gene	Species	Primer	Sequences (5'to3')
PDE4A	Mouse	F	CTTCTGCGAGACCTGCTCCA
		R	GAGTTCCCGGTTCAGCATCC
PDE4B	Mouse	F	AATGTGGCTGGGTACTCACA
		R	AAGGTGTCAGATGAGATTTTAAACG
PDE4C	Mouse	F	AGCCAGTGGATTCTGGAGTG
		R	TTTCGAGGTCAAAGCTGCTC
PDE4D	Mouse	F	ACCGCCAGTGGACGGACCGGA
		R	CATGCCACGCTCCCGCTCTCGG
CREB	Mouse	F	TTGTTGTTCAAGCTGCCTCTG
		R	CAGCAGGCTGTGTAGGAAGTG
Sirt1	Mouse	F	GCTGACGACTTCGACGACG
		R	TCGGTCAACAGGAGGTTGTCT
ANP	Mouse	F	TCGTCTTGGCCTTTTGGCT
		R	TCCAGGTGGTCTAGCAGGTTCT
BNP	Mouse	F	CTCCTGAAGGTGCTGTCC
		R	GCCATTTCCTCCGACTTT
Collagen1a1	Mouse	F	TAGGCCATTGTGTATGCAGC
		R	ACATGTTCAGCTTTGTGGACC
Fn1	Mouse	F	ATGTGGACCCCTCCTGATAGT
		R	GCCCAGTGATTTCAGCAAAGG
TGF-β1	Mouse	F	AGCTGCGCTTGCAGAGATTA
		R	AGCCCTGTATTCCGTCTCCT
NOX2	Mouse	F	TTGGGTCAGCACTGGCTCTG
		R	TGGCGGTGTGCAGTGCTATC
$P22^{phox}$	Mouse	F	GTCCACCATGGAGCGATGTG
		R	CAATGGCCAAGCAGACGGTC
SOD2	Mouse	F	CAGACCTGCCTTACGACTATGG
		R	CTCGGTGGCGTTGAGATTGTT
GATA4	Mouse	F	ATCTAAGACGCCAGCAGGTC
		R	CACAGTACTGAATGTCTGGGAC
MEF2a	Mouse	F	TAAGACAATTTCAACGGCAGC
		R	TCGCTTTGTAAAAGTAACCTGTCG
Fbln2	Mouse	F	ACACAGAGCACCGGGGAC
		R	CTCGATGCAGTTCTCCAGCA
MMP2	Mouse	F	AATGTGGCTGGGTACTCACA
		R	AAGGTGTCAGATGAGATTTTAAACG
MMP9	Mouse	F	CAAATTCTTCTGGCGTGTGA
		R	CGGTTGAAGCAAAGAAGGAG
TIMP1	Mouse	F	AGAGACACACCAGAGCAGATAC
		R	GGGAACCCATGAATTTAGCCCT
TIMP2	Mouse	F	GGCTGTGAGTGCAAGATCACT
		R	CGCGCAAGAACCATCACTTC
18s	Mouse	F	TTGACGGAAGGGCACCACCAG
		R	GCACCACCACCCACGGAATCG
ANP	Rat	F	CAACACAGATCTGATGGATTTCA

		R	CCTCATCTTCTACCGGCATC
BNP	Rat	F	GCTGCTGGAGCTGATAAGAGAA
		R	GTTCTTTTGTAGGGCCTTGGTC
Collagen1a1	Rat	F	ATGTTCAGCTTTGTGGAC
		R	GGATGCCATCTTGTCCAG
Fn1	Rat	F	CCACAGCCATTCCTGCGCCA
		R	TCACCCGCACTCGGTAGCCA
TGF-β1	Rat	F	GGGCTACCATGCCAACTTCTG
		R	GAGGGCAAGGACCTTGCTGTA
NOX2	Rat	F	CTGCCAGTGTGTCGGAATCT
		R	TGTGAATGGCCGTGTGAAGT
$P22^{phox}$	Rat	F	CCTCCACTTACTGCTGTCCG
		R	GTAGGTGGCTGCTTGATGGT
SOD2	Rat	F	ACGCGACCTACGTGAACAAT
		R	TAACATCTCCCTTGGCCAGC
18s	Rat	F	TTGACGGAAGGGCACCACCAG
		R	GCACCACCACCCACGGAATCG

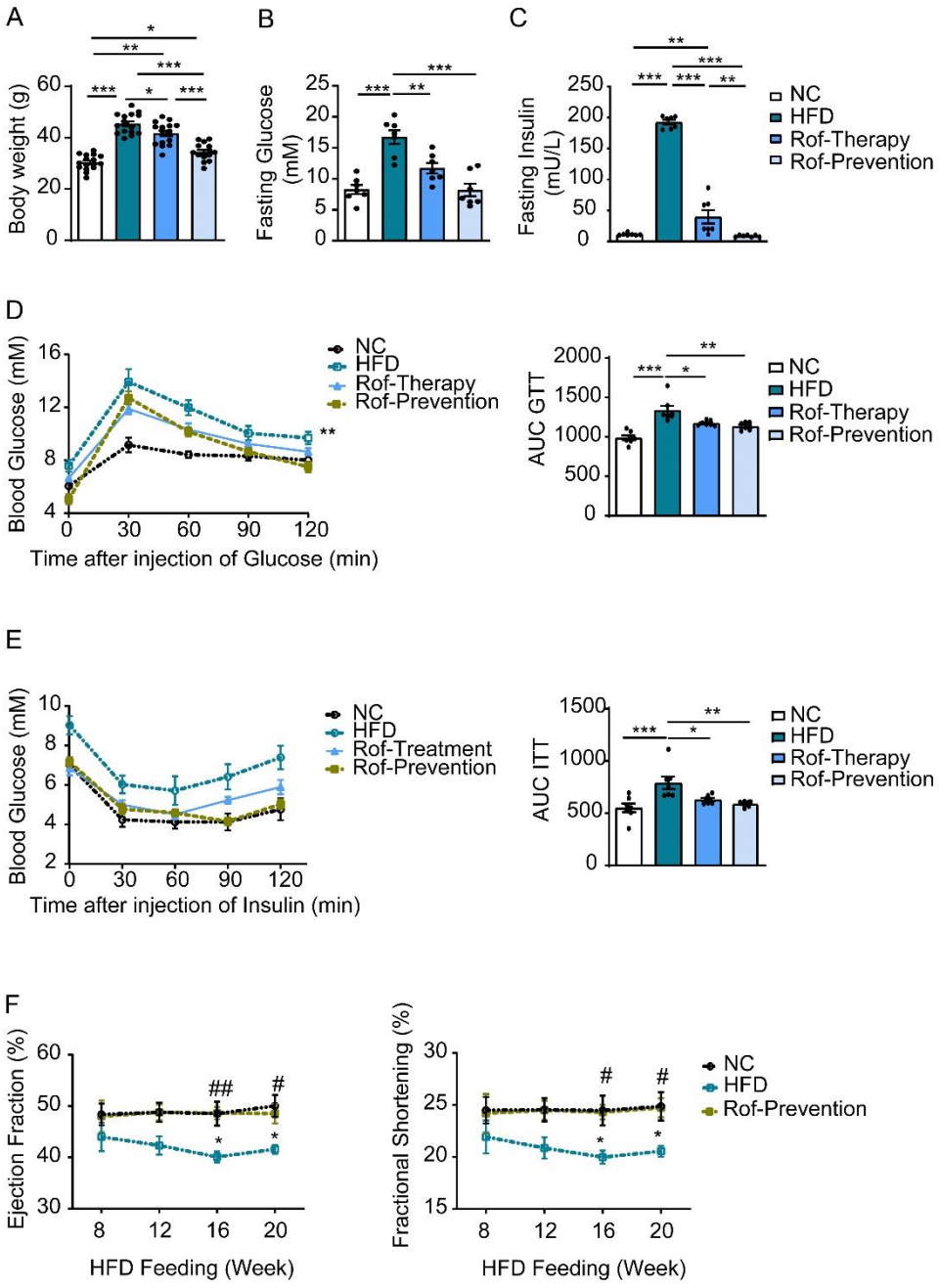
Table 3. List of antibodies.

Antibody	Company	Catalog#
Akt	Cell Signaling	9272
collagen 1	Proteintech	14695-1-AP
CREB	Bimake	A5014
cTNT	Abcam	ab8295
Fibronectin1	Proteintech	15613-1-AP
FoxO3a	Cell Signaling	2497
GAPDH	Proteintech	60004-1-Ig
GFP	Santa Cruz	sc-8334
PDE4D	Proteintech	12918-1-AP
PDE4D3	FabGennix	431AP
PDE4D5	FabGennix	451AP
PDE4D9	FabGennix	491AP
phospho-Akt (Ser473)	Cell Signaling	4051
phospho-CREB (Ser133)	Cell Signaling	9198
phospho-FoxO3a (Ser253)	Cell Signaling	9466
phospho-PDE4D (Ser190)	Abcam	ab59212
phospholamban	Affinity Bioreagent	MA3-922
phospho-phospholamban (Ser16)	Millipore	07-52
phospho-Smad2 (Ser250)	Bimake	A5192
SERCA2	Bimake	A5708
Sirt1	Abclonal	A19667
Smad2	Bimake	A5117
TGF-β1	Santa Cruz	sc-65378
Vimentin	proteintech	10366-1-AP
Pan Acetyl-Lysine *	Abclonal	A2391

* Immunoprecipitation

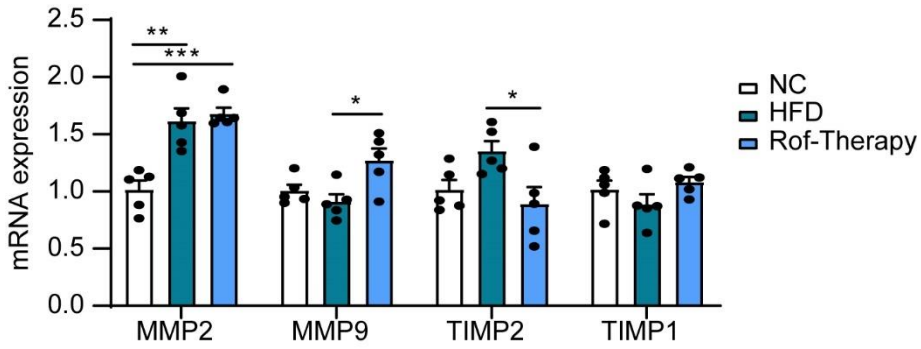
Abclonal (Boston, MA, USA)
Bimake (Houston, TX, USA)
FabGennix (Frisco, TX, USA)
Santa Cruz (Santa Cruz, CA, USA)
Abcam (Boston, MA, USA)
Affinity Bioreagent (Golden, CO, USA)
Cell Signaling (Danvers, MA, USA)
Millipore (Billerica, MA, USA)
Proteintech (Chicago, IL, USA)

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

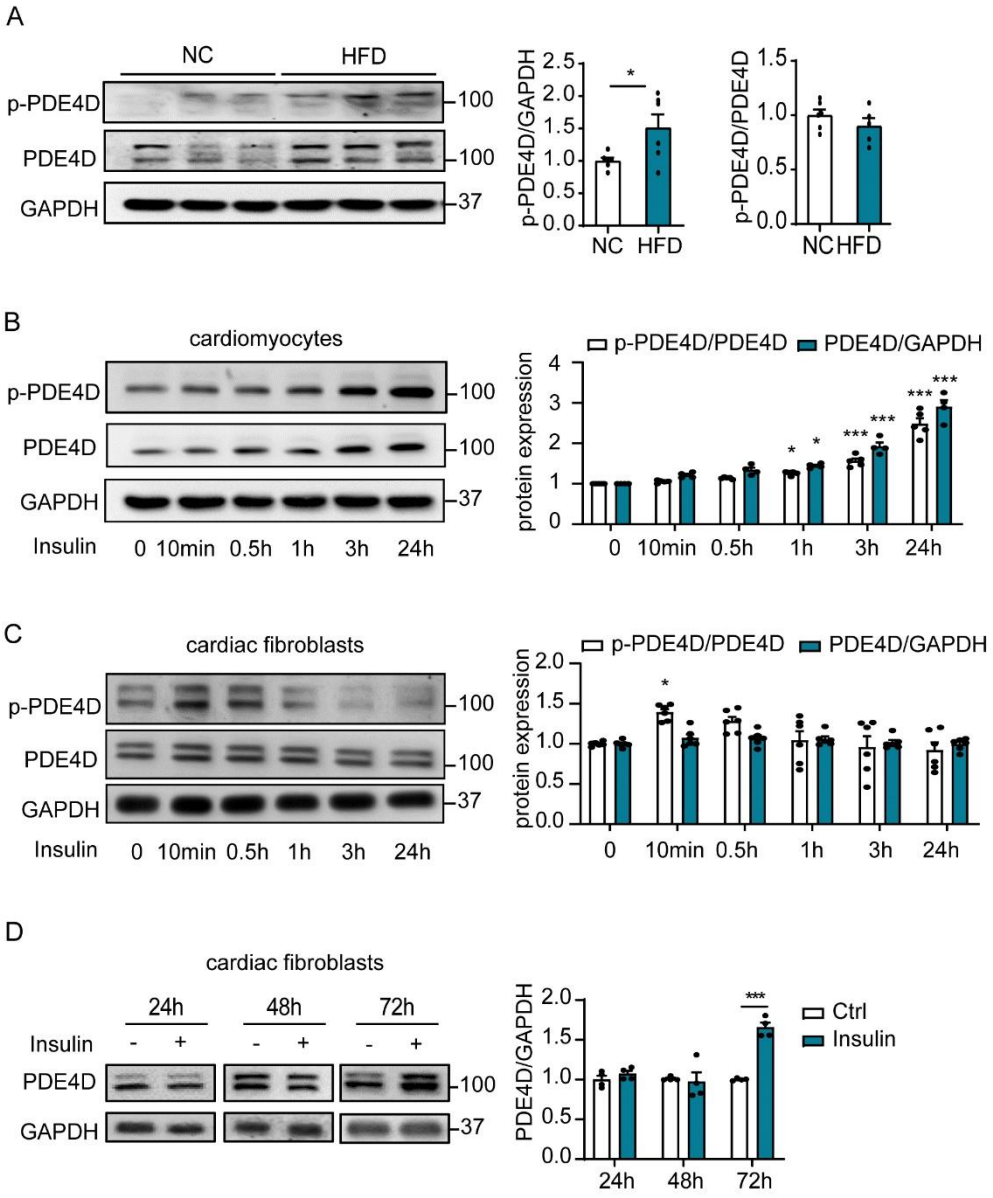


Supplemental Figure 1. PDE4 Inhibitor Roflumilast Improves HFD-Induced Hyperglycemia, Hyperinsulinemia and Cardiac Dysfunction. Mice fed with normal chow or HFD were co-treated with roflumilast for 20 weeks (Rof-prevention) or treated with roflumilast for 4 weeks (Rof-therapy) post 16 weeks feeding. **A**, Body weight was measured. n=15-16 mice per group. **B** and **C**, fasting blood glucose (**B)** and **s**erum insulin (**C)** were

measured after overnight fasting. n=7 mice per group. **D**, Oral glucose tolerance testing (GTT) and areas under curve of each group are shown. n=7 mice per group. **E,** Insulin tolerance testing (ITT) and areas under curve of each group are shown. n=7 mice per group. *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA and Tukey's multiple comparison test. **F,** Time-course analysis of cardiac contractile function-Ejection Fraction and Fractional Shortening were measured by echocardiography, n=7-10 mice per group. All data are presented as mean±SEM. **D left and F,** *P<0.05, **P<0.01, NC vs. HFD; # P<0.05 HFD vs. Rof-Prevention by 2-way ANOVA and Tukey's multiple comparison test.

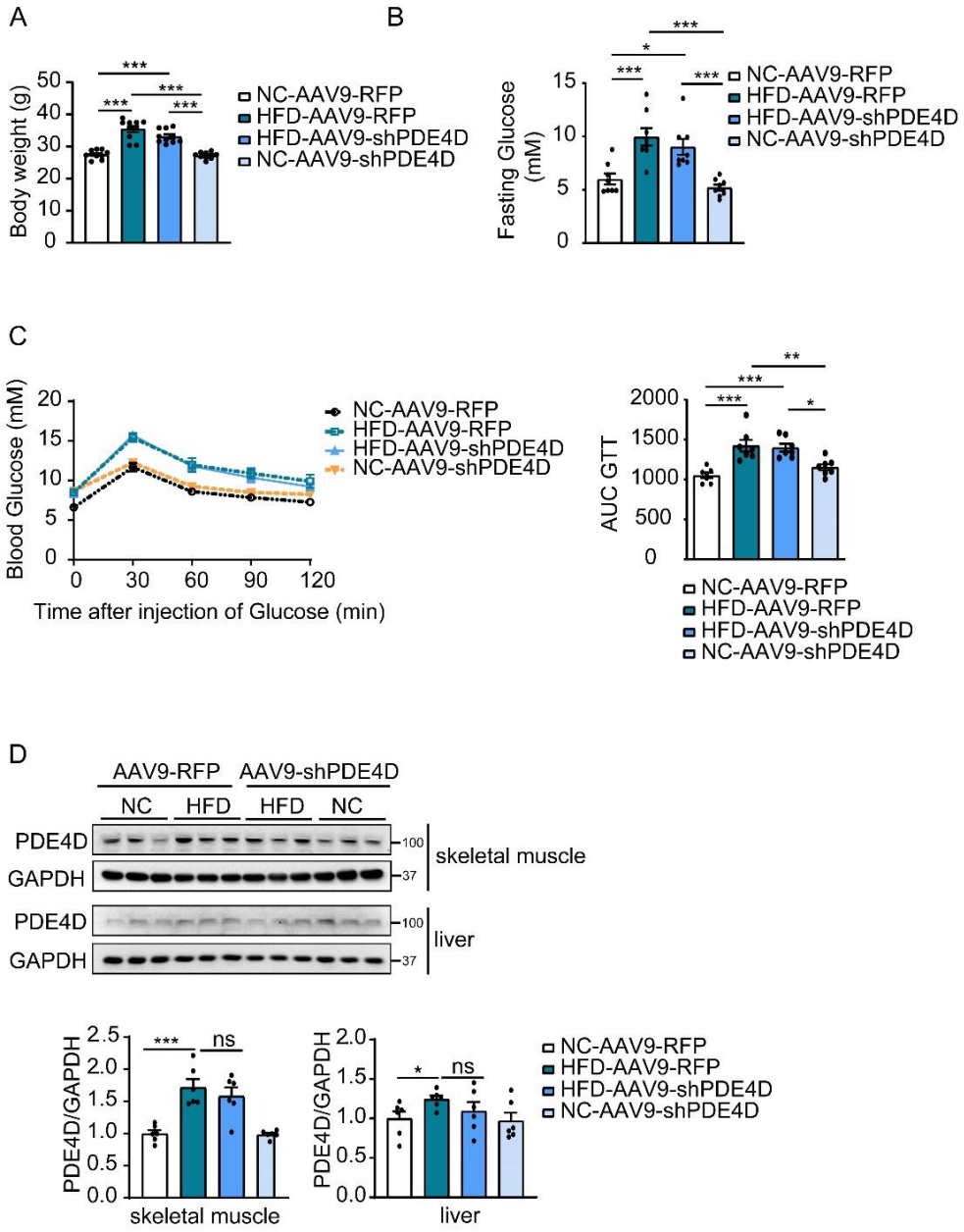


Supplemental Figure 2. The effects of PDE4 Inhibitor Roflumilast on MMPs and TIMPs expression in HFD heart. Transcript levels of indicated genes involved in cardiac ECM remodeling (MMP2, MMP9, TIMP2 and TIMP1), n=5 mice per group. All data are presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA and Tukey's multiple comparison test. HFD, high-fat diet; NC, normal chow; Rof, roflumilast; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases.



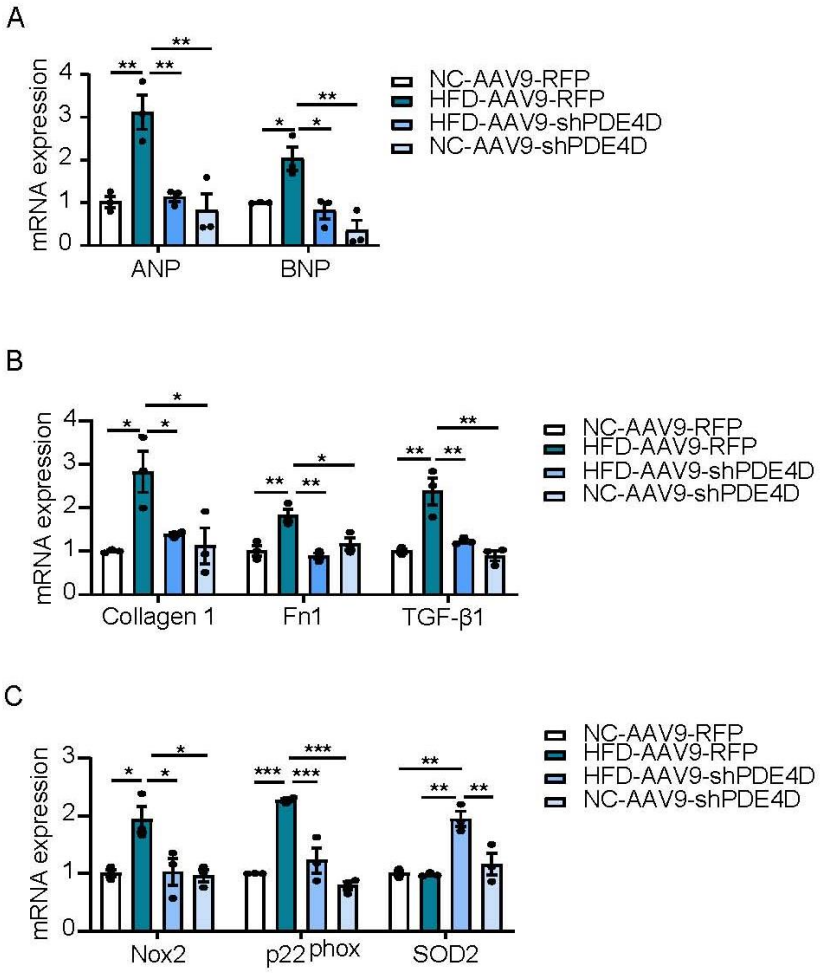
Supplemental Figure 3. The expression of phospho-PDE4D and PDE4D is increased in HFD mouse hearts or insulin-treated cardiomyocytes and cardiac fibroblasts. A, Western blotting analysis of phosopho-PDE4D and PDE4D expression in indicated hearts after 20 weeks' chow or HFD feeding, n=5-6 mice per group. B, Western blotting analysis of phosopho-PDE4D and PDE4D expression with insulin (100nM) treatment for different time in neonatal rat cardiomyocytes, n=4-5 samples per group. C, Western blotting analysis

of phosopho-PDE4D and PDE4D expression with insulin (100nM) treatment for different time in neonatal rat cardiac fibroblasts, n=6 samples per group. D, Western blotting analysis of PDE4D expression in neonatal rat cardiac fibroblasts treated with insulin (100nM) for indicated time, n=4 samples per group. All data are presented as mean±SEM. A and D, *P<0.05, ***P<0.001 by unpaired t test. B and C, *P<0.05, ***P<0.001 vs. 0 min by 1-way ANOVA and Tukey's multiple comparison test. HFD, high-fat diet; NC, normal chow; Rof, roflumilast.

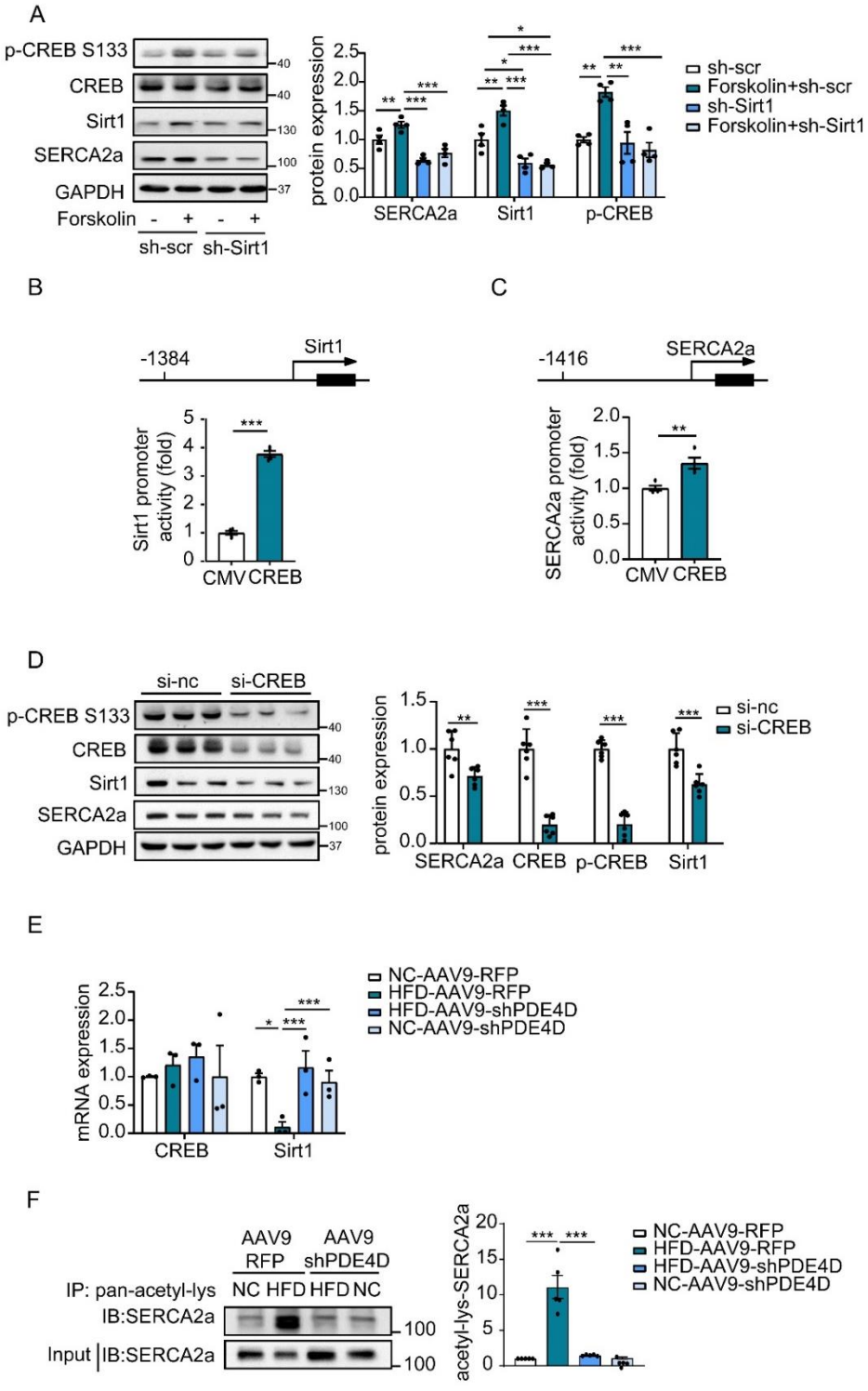


Supplemental Figure 4. Cardiac PDE4D Suppression Prevents Cardiac Dysfunction Independent of Glucose Metabolism in HFD Mice. Mice were fed with normal chow or HFD subjected to injections with AAV9-RFP or AAV9-shPDE4D for 5 weeks. **A** and **B,** Body weight **(A)** and fasting blood glucose **(B)** were measured in indicated groups. n=8-10 mice per group. **C,** Oral glucose tolerance testing (GTT) and areas under curve of each group are shown. n=7 mice per group. **D,** Western blotting analysis of PDE4D expression in skeletal muscle (upper and left) and liver (bottom and right), n=6 mice per group. All data are presented as mean±SEM. **A** through **C**, *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA and Tukey's

multiple comparison test. **D**, *P<0.05, ***P<0.001 by unpaired t test.

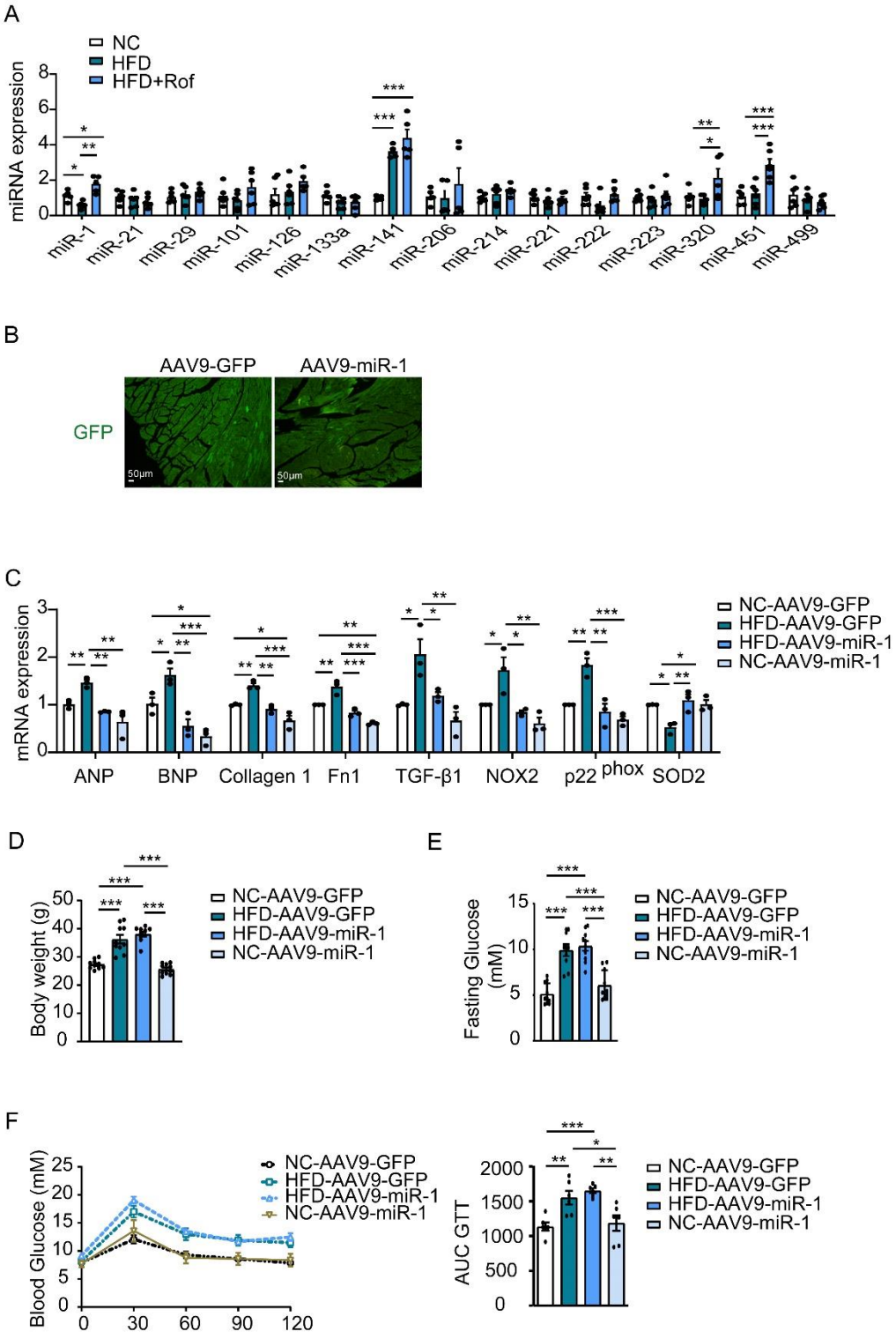


Supplemental Figure 5. Cardiac PDE4D Suppression Reverses HFD-Induced Cardiac Remodeling. A through **C,** Transcript levels of indicated genes involved in cardiac hypertrophy (ANP and BNP) **(A)**, fibrosis (Collagen 1, Fn1 and TGF-β1) **(B)**, and oxidative stress (NOX2, $p22^{phox}$ and SOD2) **(C)** in NC or HFD mice injected with AAV9-shPDE4D or AAV9-RFP, n=3 mice per group. All data are presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA and Tukey's multiple comparison test.



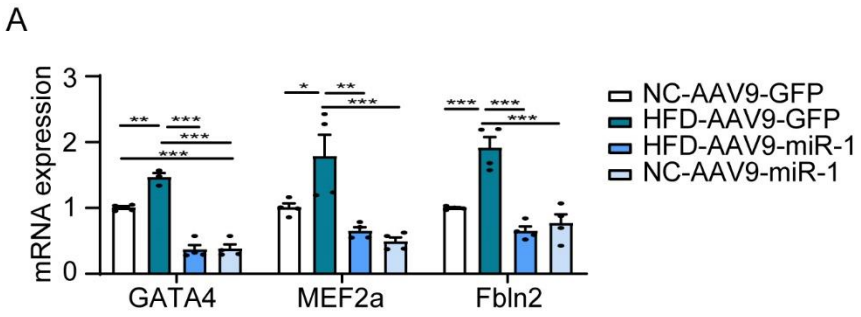
Supplemental Figure 6. PDE4D Inhibition Preserves SERCA2a Expression in Cardiomyocytes via cAMP/PKA/CREB/Sirt1 Signaling Pathway. A, Western blotting analyses of the expression of phosphorylation of CREB, Sirt1and SERCA2a in NRVMs were transfected with control scramble (sh-scr) or Sirt1-shRNA (sh-Sirt1) treated with or without

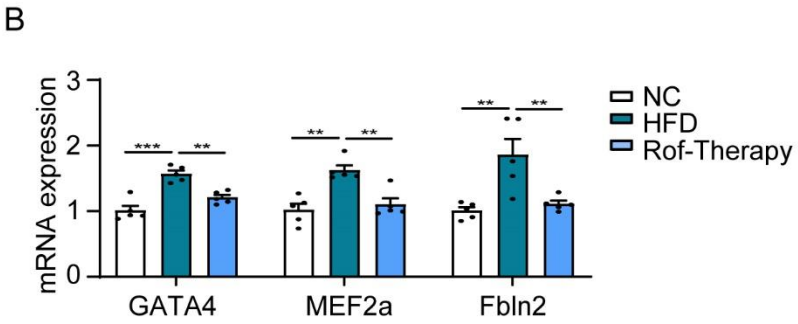
PKA activator Forskolin (10μM, 24h), n=4 samples per group. **B** and **C,** Luciferase activity in 293T cells co-transfected with constructed Sirt1 (100ng; -1384/+277 relative to the transcription start site) promoter reporter plasmid **(B)** or constructed SERCA2a (100ng; -1416/+251 relative to the transcription start site) promoter reporter plasmid **(C)** or pRL-TK (10ng) and CREB plasmid (200ng) or control scramble (CMV), n=4 samples per group. **D,** Western blotting analyses of the expression of phosphorylation of CREB, Sirt1and SERCA2a in NRVMs transfected with siRNA negative control (si-nc) or CREB siRNA (si-CREB), n=4 samples per group. **E**, Transcript levels of CREB and Sirt1 in hearts from NC and HFD mice subjected to injection with AAV9-RFP or AAV9-shPDE4D, n=3 mice per group. **F**, Anti-Pan Acetyl-Lysine IP was followed by Western blotting. The protein levels of SERCA2a in the precipitates were determined with protein-specific primary antibodies in indicated groups. n=5 mice per group. All data are presented as mean±SEM. **A, E** and **F,** *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA and Tukey's multiple comparison test. **B** through **D,** **P<0.01, ***P<0.001 by unpaired t test.

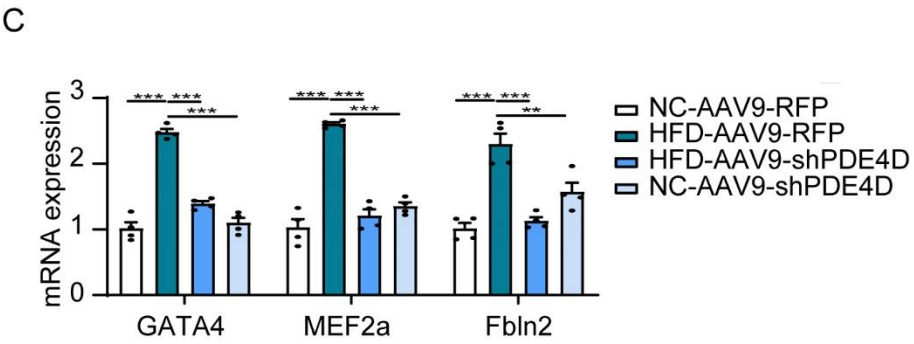


Supplemental Figure 7. Cardiac miR-1 Overexpression Rescues Cardiac Remodeling and Dysfunction Independent of Glucose Metabolism in HFD Mice. A, qRT-PCR analysis of the expression of indicated microRNAs involved in diabetic cardiomyopathy in hearts from

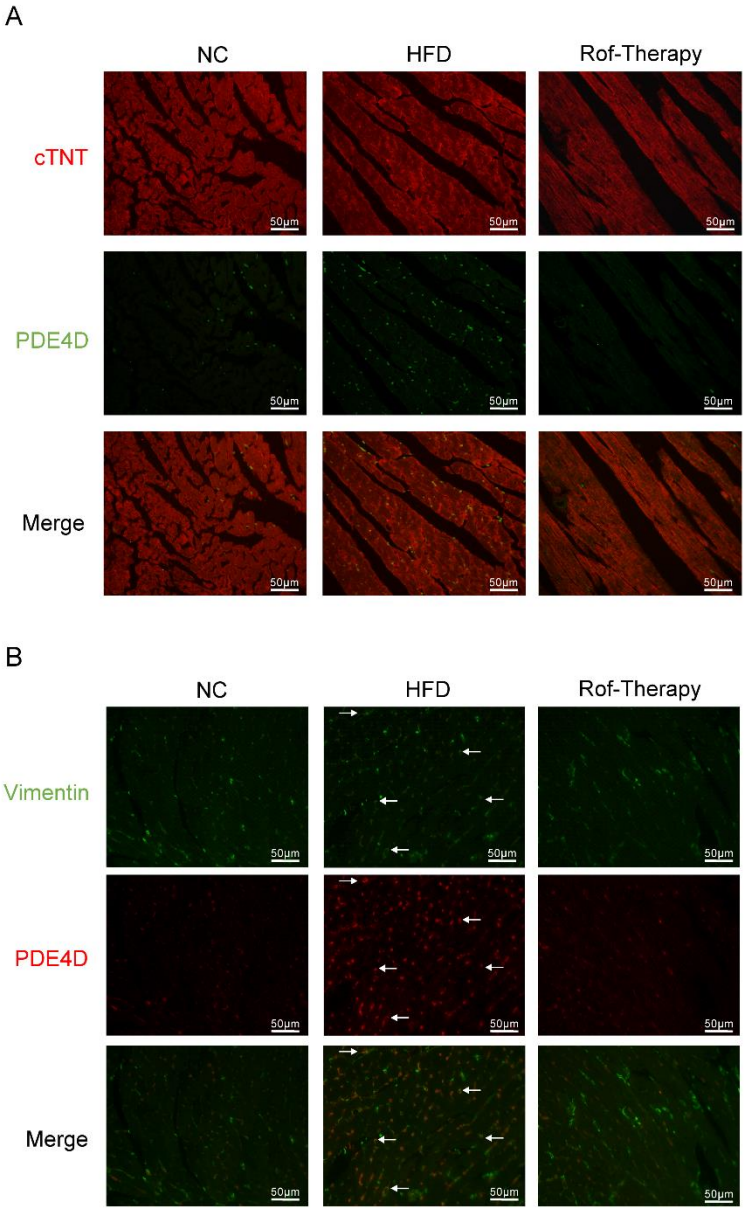
NC or HFD mice treated with roflumilast for 4 weeks, n=5-6 mice per group. **B,** Representative images of GFP expression in indicated hearts. Scale bar, 50 μm. **C** through **F,** Mice were fed with normal chow or HFD injected with AAV9-GFP or AAV9-miR-1 for 4 weeks. **C,** qRT-PCR analysis of cardiac hypertrophy, fibrosis, and oxidative stress-related genes expression in indicated hearts, n=3 mice per group. **D** and **E,** Body weight and fasting blood glucose were measured in indicated groups. n=9-10 mice per group. **E,** Oral glucose tolerance testing (GTT) and areas under curve were measured in indicated groups, n=6 mice per group. All data are presented as mean±SEM. **A**, **C** through **F,** *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA and Tukey's multiple comparison test.



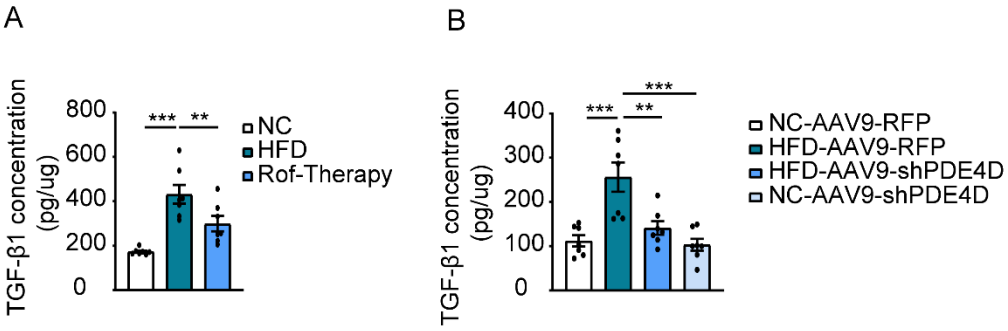




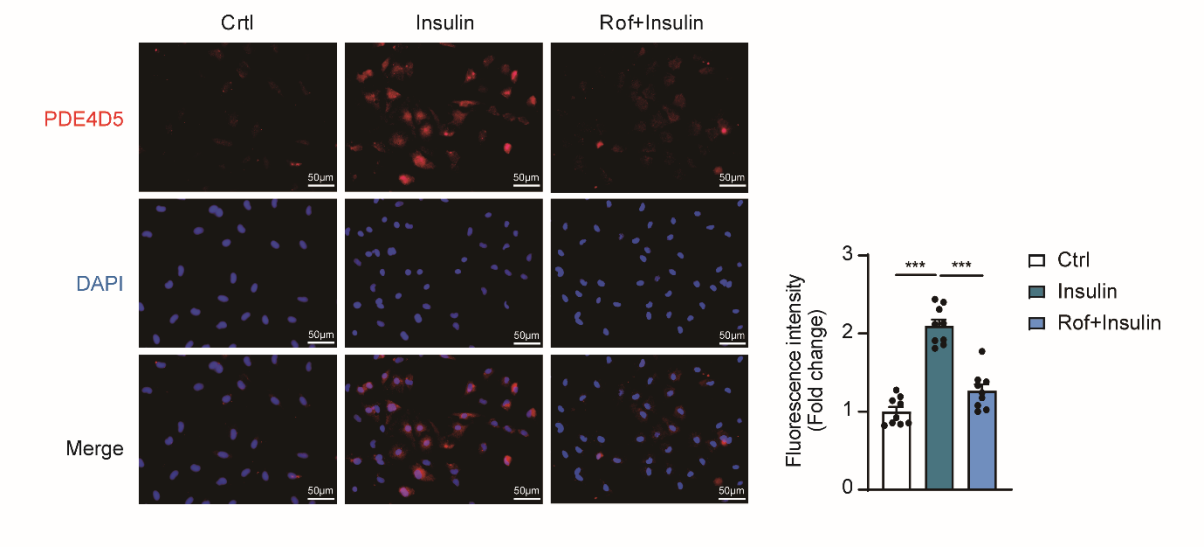
Supplemental Figure 8. miR-1 Target Genes are upregulated in HFD-Induced Cardiac Remodeling. A through **C,** Transcript levels of miR-1 target genes involved in HFD-induced cardiac hypertrophy (GATA4 and MEF2a) and fibrosis (MEF2a and Fbln2) in hearts from NC or HFD mice subjected by treatment with AAV9-miR-1 **(A)**, roflumilast **(B)** and AAV9-shPDE4D5 **(C)**. n=3-5 mice per group. *P<0.05, **P<0.01, ***P<0.001 by 1‐way ANOVA and Tukey's multiple comparison test.



Supplemental Figure 9. PDE4D Inhibition Reduces PDE4D Expression in Cardiomyocytes and Cardiac Fibroblasts in HFD Mice. Representative immunofluorescence images of PDE4D and cTNT (cardiomyocytes marker; A) or PDE4D and vimentin (cardiac fibroblasts marker; B) in indicated mice heart. Scale bar, 50μm. White arrow indicates co-expression of PDE4D with vimentin.



Supplemental Figure 10. PDE4D Inhibition Decreases TGF-β1 Activity in Heart Tissues from HFD Mice. A, Summary data of TGF-β1 activity in heart tissue from NC or HFD mice treated with roflumilast for 4 weeks, n=7 mice per group. **B,** TGF-β1 activity in heart tissue from NC and HFD mice injected with AAV9-RFP or AAV9-shPDE4D, n=7 mice per group. All data are presented as mean±SEM. **P<0.01, ***P<0.001 by 1-way ANOVA and Tukey's multiple comparison test.



Supplemental Figure 11. Roflumilast Reduces Insulin-induced PDE4D5 Expression in Cardiac Fibroblasts. Representative immunofluorescence images and analyses of the expression of PDE4D5 in NRCFs treated with insulin (100 nM, 72h) in the presence or absence of roflumilast (100 nM), from N = 9 fields of view from three independent experiments. Scale bar, 50μm. All data are presented as mean±SEM. ***P<0.001 by 1-way ANOVA and Tukey's multiple comparison test.