Supplementary Information

MicroRNA-29 ameliorates fibro-inflammation and insulin resistance in HIF1α-deficient obese adipose tissue by inhibiting endotrophin generation

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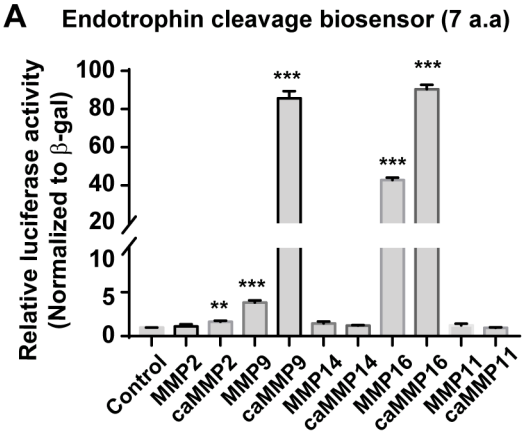
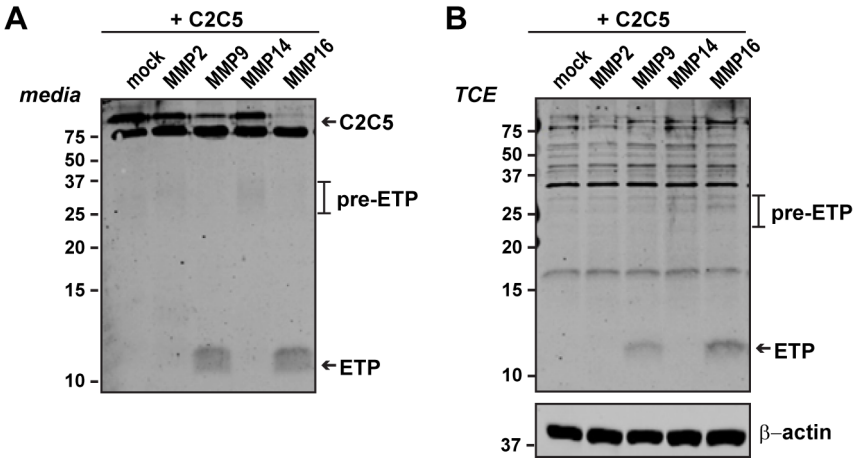
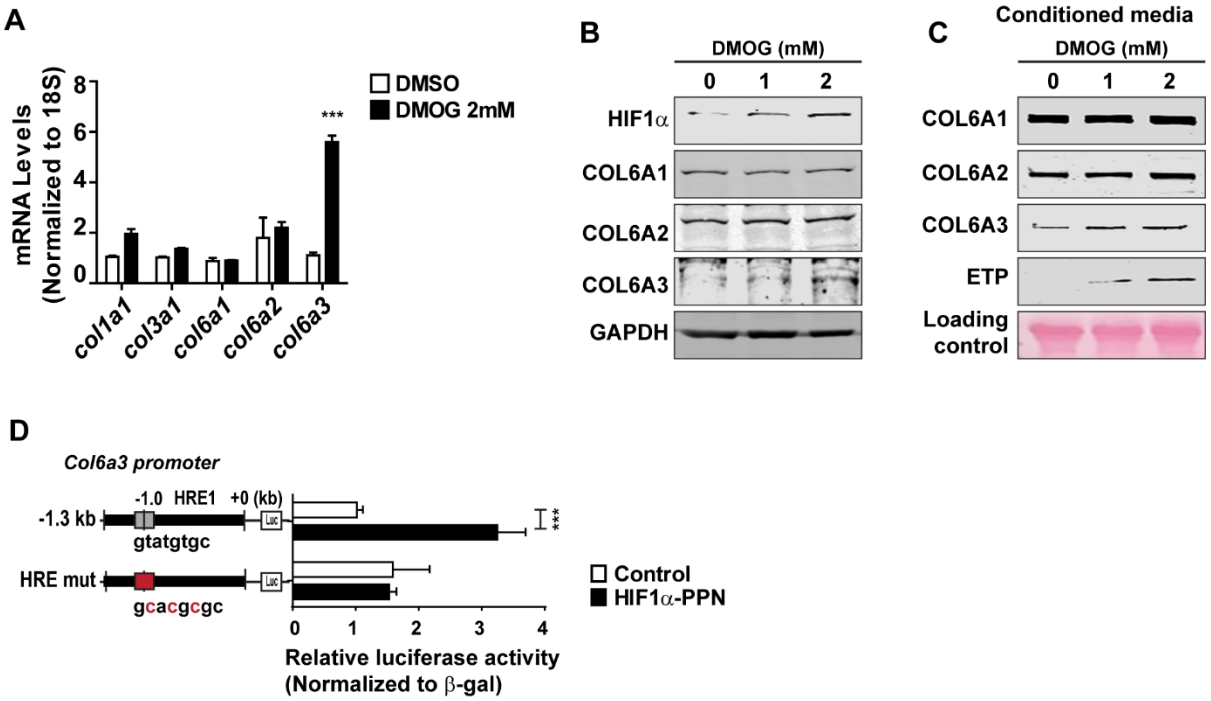


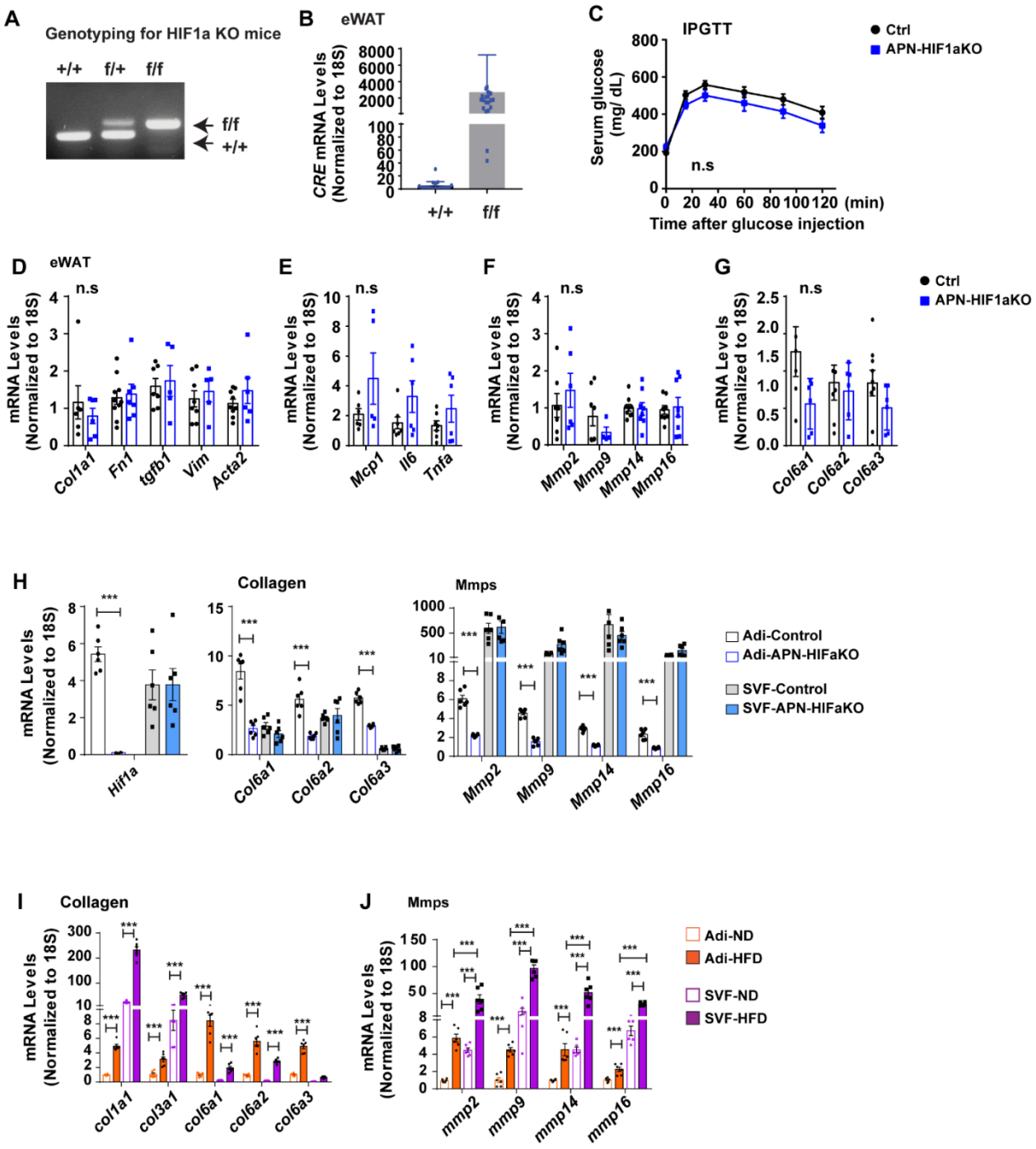
Supplementary Figure 1. Multiple MMPs are involved in the generation of endotrophin targeting LMVST cleavage site existing slightly upstream of C5 domain of COL6A3. (A) Luciferase activities of ETP-7AA cleavage biosensor, including LMVST cleavage site, were determined using various MMPs (pro MMPs [MMP] and catalytic MMPs [caMMP]). HEK293T cells were transfected with pro- and catalytic MMPs expressing vector. Statistical significance was evaluated by unpaired Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$.



Supplementary Figure 2. Generation of pre-endotrophin and endotrophin cleaved from C2C5 construct in SK-HEP1 hepatocyte cell line. (A-B) The protein levels of pre-endotrophins (pre-ETPs) and endotrophin (ETP) cleaved from C2C5 by various MMPs were determined by immunoblots with anti-ETP antibodies. C2C5 and indicated MMPs were transiently transfected into SK-HEP1 cells, and subsequently both conditioned media **(A)** and cell lysate **(B)** were subjected for analysis. β-actin was used as loading control.



Supplementary Figure 3. The levels of *Col6a3* transcription and COL6A3 processing to release endotrophin were elevated in pseudohypoxic condition. (A) The mRNA levels of collagens in 3T3-L1 adipocytes were determined by qRT-PCR in the presence or absence of DMOG (2mM) treatment. Statistical significance was evaluated by *2-way ANOVA*. $^{***}p < 0.001$. (**B-C**) The protein levels of HIF-1α and COL6 chains were determined by immunoblots. 3T3-L1 adipocytes were treated with different concentrations of DMOG for 36 hours and tissue lysates **(B)** and conditioned media **(C)** were subjected for analysis. GAPDH and pouceau staining was used as loading control, respectively. **(D)** Luciferase activity of mouse *Col6a3* promoter. HEK293T cells were transiently transfected with luciferase reporter containing the wild-type (−1.3 kb) or HRE mutant (HRE mut) *Col6a3* promoter with or without HIF1a-PPN overexpression for 48 hours. Luciferase activities were normalized with β-galactosidase activity. Statistical significance was evaluated by unpaired Student's *t*-test. $^{***}p < 0.001$.



Supplementary Figure 4. (A-B) Generation of APN-HIF1aKO mice. (A) Genotyping for the wild type (+/+), HIF1a flox/+ (f/+) and HIF1a KO flox/flox (f/f) mice. Band size of wild type (+/+) and HIF1a KO flox/flox (f/f) is 151 and 193bp, respectively. **(B)** The levels of CRE gene expression were determined by qRT-PCR in eWAT APN-HIF1aKO (APN-HIF1$\alpha^{f/f}$) and their littermate control (HIF1$\alpha^{f/f}$). **(C-G) Metabolic phenotypes for APN-HIF1aKO mice. (C)** Insulin sensitivity of 11-weeks HFD-fed APN-HIF1aKO (n=10) and their littermate control (n=12) was determined by IPGTT. (**D-G**) The mRNA levels of genes involved in fibrosis (**D**) and inflammation (**E**), *Mmps* (**F**), and *Col6* chains (**G**) in the eWATs of 12-weeks HFD-fed

APN-HIF1aKO (n=10) and their littermate control (n=10). **(H-J) qRT-PCR analysis for *Collagens* and *Mmps* in adipocyte and stromal-vascular fractions (SVF) of eWAT.** (**H**) The mRNA levels of *Hif1a*, *Col6*, and *Mmps* in adipocytes and SVFs of eWAT from 20-weeks of HFD fed APN-HIF1aKO mice (n=6) compared to their littermate controls (n=6). **(I-J)** The mRNA levels of *Collagens* **(I)** and *Mmps* **(J)** were analyzed by qRT-PCR with adipocytes versus SVF isolated from eWAT of 20-weeks HFD-fed wild type mice (n=6) compared to that of ND-fed lean controls (n=6). Statistical significance was evaluated by two-way ANOVA. $^{***}p < 0.001$. n.s stands for no significance.

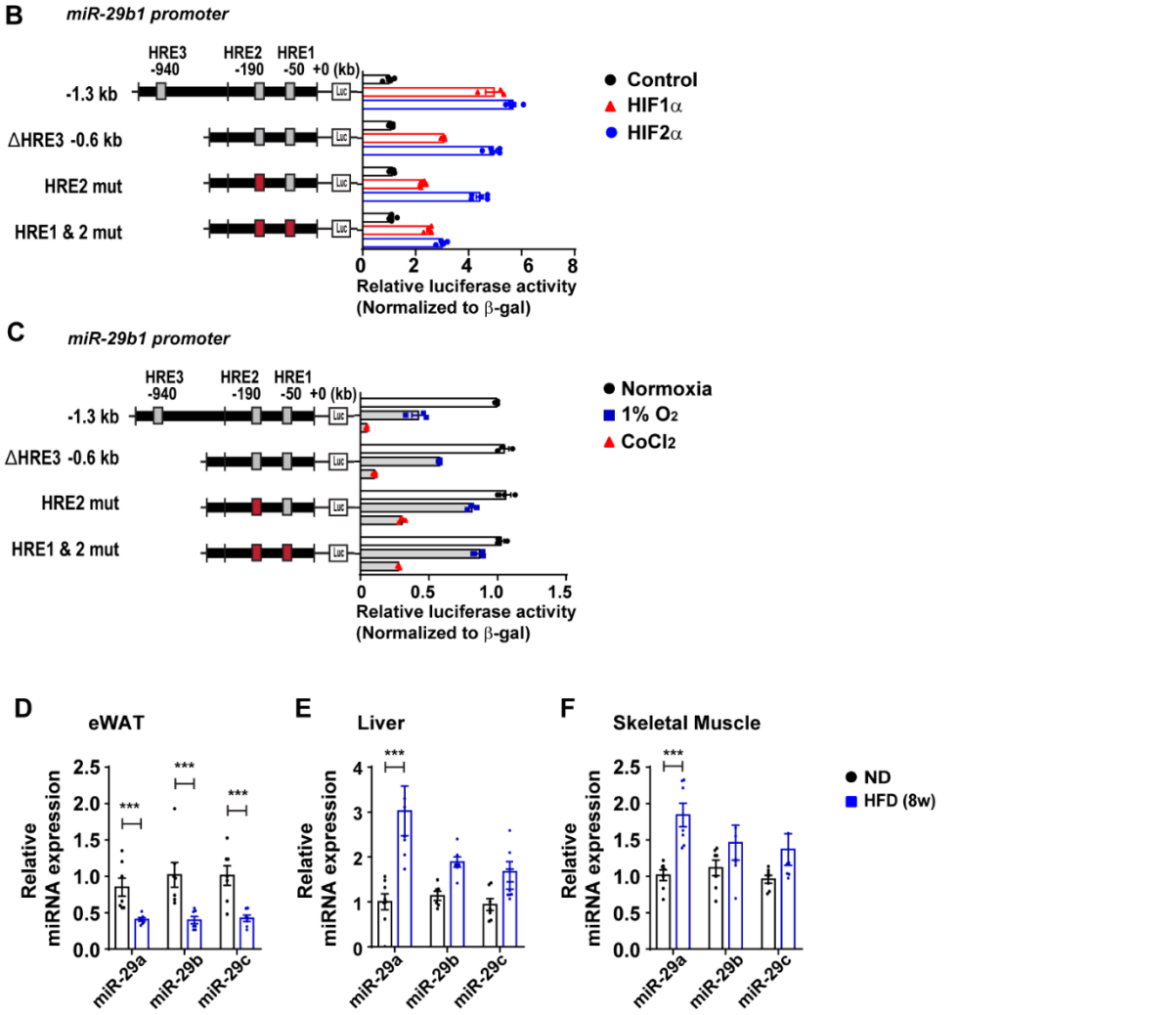
A Hypoxia-linked miRNA candidates target the *Col6a3* levels

miRNA candidates
hsa-miR-29b-3p
hsa-miR-29c-3p
hsa-miR-133a-3p
hsa-miR-181a-5p
hsa-miR-181c-5p
hsa-miR-877-5p
hsa-miR-605-5p

G Predicted miR-29 binding sites of the target genes

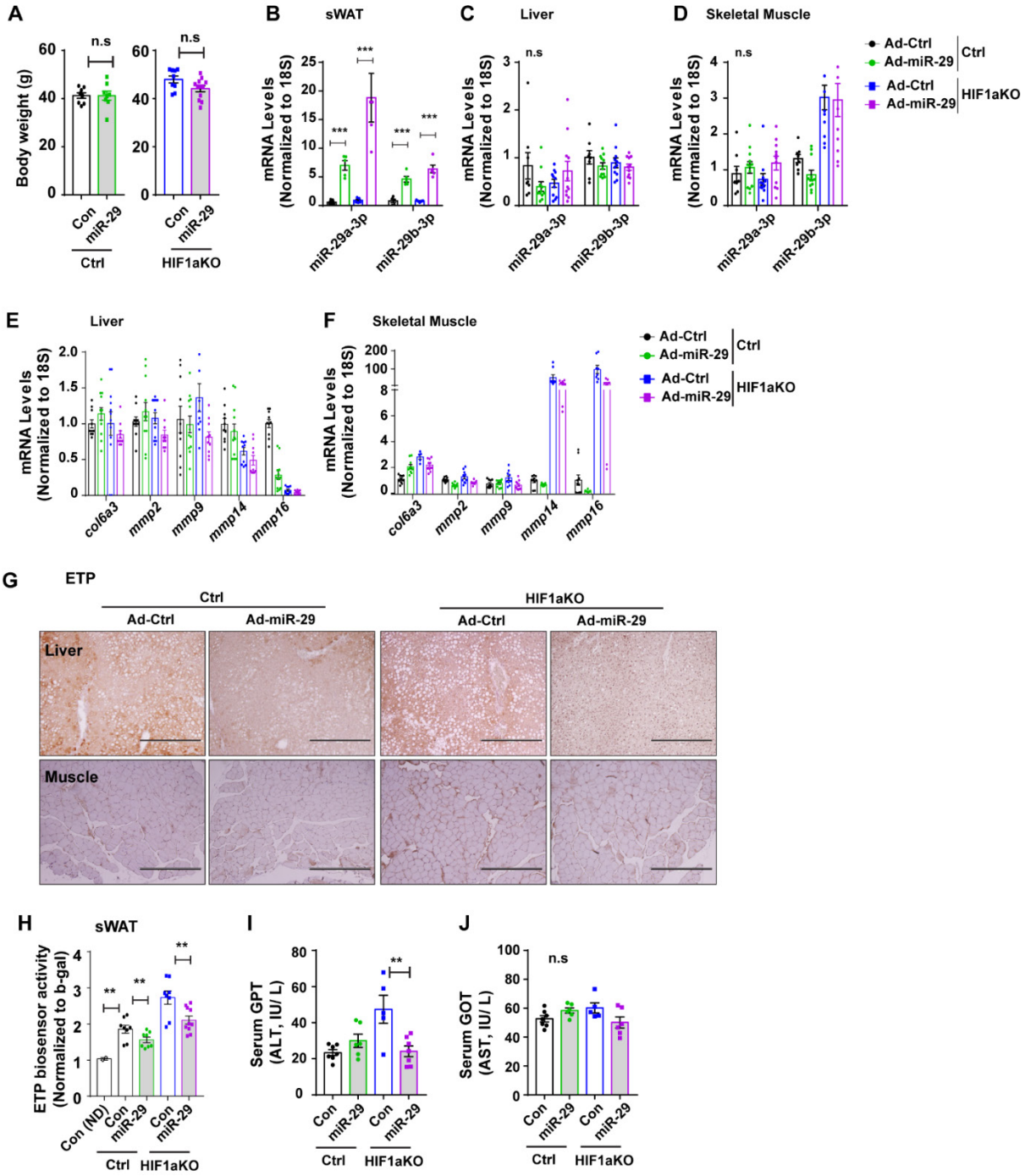
Target gene	Position in the UTR	3'UTR sequences
mouse *Col6a3*	163-170	- CGUGACCUGUCAAUAUGGUGCUA-
human *Col6a3*	166-173	- CAUGACCUAUCAGUAUGGUGCUA-
mouse *mmp2*	256-264	- UCCUCUGGGCUGCCCUGGUGCUC-
human *mmp2*	299-305	- UGCUUUGGGCUGCCCUGGUGCUG-
mouse *mmp9*	86-92	- CCCAUCCGAGGGAAAGGUGCUAG-
human *mmp9*	XXX	- GAGGAGUGGAGGUGGGCUGGGCC-
mouse *mmp16*	6425-6431	- GCCUAGGAUUGGCUAUGGUGCUG-
	8496-8503	- AAAGGCUAAAUAAAAUGGUGCUA
	8545-8551	- UCAGGCAAAAUUUUG--UGGUGCUU-
human *mmp16*	6581-6587	- UCCUAGGAUAGGAUAUGGUGCUG-
	8815-8822	- AUAAGGCUAAAUAAAUGGUGCUA-
	8864-8870	- UCAGGCAAAAUUUUG--UGGUGCUU-

*miR-29 binding sites



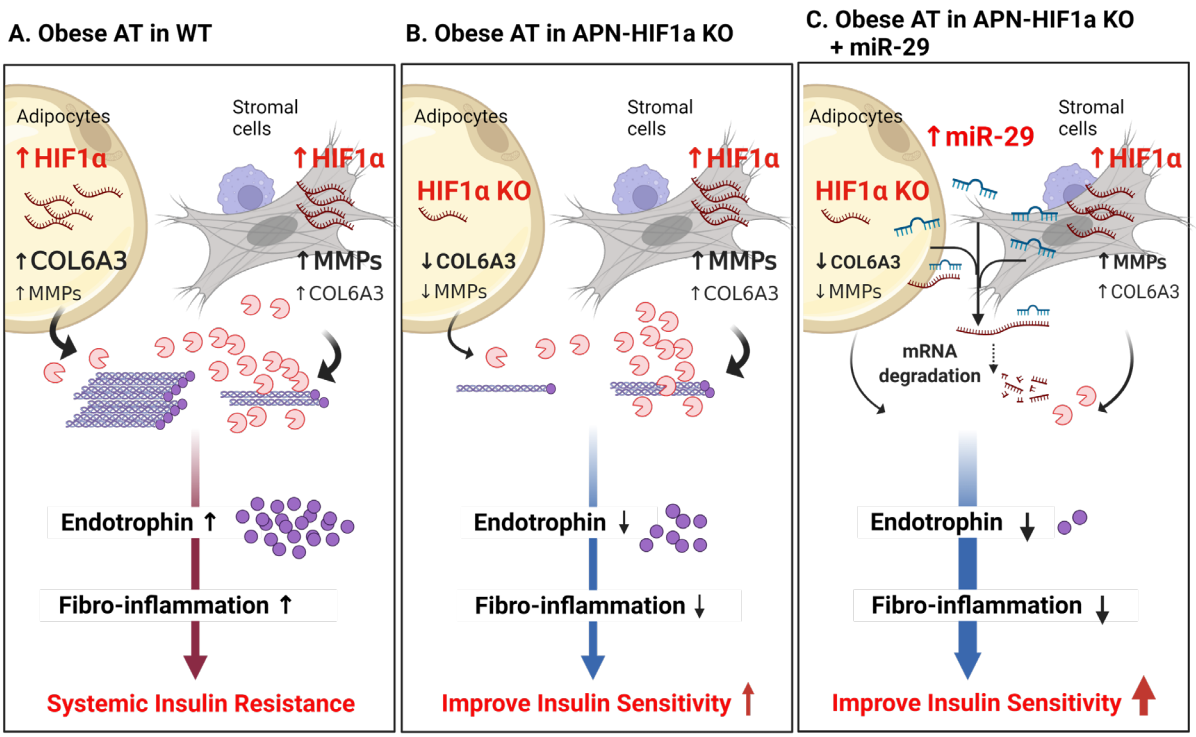
Supplementary Figure 5. Hypoxia-linked miR-29s are differentially regulated in various metabolic tissues in obesity. (A) Lists of hypoxia-linked miRNA candidates that target the *Col6a3* gene. **(B-C)** Luciferase activity of miR-29b1 promoter harboring three HREs (-1.3kb), HRE3 deleted (ΔHRE3 -0.6kb), HRE2 mutant (HRE2 mut), and HRE1 and 2 mutant (HRE1&2

mut) co-expressed with either HIF1α-PPN or HIF2α overexpression **(B)**. Cells were cultured in hypoxic conditions such as low oxygen (1% O_2) and CoCl2 (100μM) and determined the miR-29b1 promoter activity **(C)**. **(D-F)** The levels of miR-29s were determined by qRT-PCR in eWAT **(D)**, liver **(E)**, and skeletal muscle **(F)** from the wild-type mice fed on HFD for 8-weeks compared to ND (n=7 per groups). Statistical significance was evaluated by two-way ANOVA. $^{***}p < 0.001$. **(G)** Predicted miR-29 binding sites at 3'UTR regions of *MMPs* and *Col6a3* gene.



Supplementary Figure 6. Metabolic effects of Ad-miR-29 overexpression in AT of HFD-induced obese APN-HIF1aKO mice. (A) Body weight of APN-HIF1aKO and their littermate control injected with Ad-Ctrl or Ad-miR-29 after 8-weeks of HFD feeding. **(B-D)** The levels of miR-29a-3p and miR-29b-3p were determined in sWAT **(B)**, liver **(C)**, and skeletal muscle **(D)** from Ad-Ctrl or Ad-miR-29 injected APN-HIF1aKO mice compared to those of littermate controls (n=6-8 per groups). **(E-F)** The mRNA levels of *Mmps* and *Col6a3* in liver **(E)** and skeletal muscle **(F)** were determined by qRT-PCR (n=6-8 per groups). **(G)** The protein levels

of endotrophin in liver and skeletal muscle were determined by immunohistochemistry (IHC). Scale bar: 400μm. **(H)** Luciferase activity of ETP-12AA cleavage biosensor in sWAT extracts from HFD-fed APN-HIF1aKO and their littermate controls injected with either Ad-Ctrl or Ad-miR-29 (n=7-8 per groups). **(I-J)** Serum levels of GPT **(I)** and GOT **(J)** in HFD-induced obese APN-HIF1aKO mice and littermate controls injected with Ad-Ctrl or Ad-miR-29. Statistical significance was evaluated by unpaired Student's *t-test*. $^{**}p < 0.01$; $^{***}p < 0.001$. n.s stands for no significance.



Supplementary Figure 7. Schematic representation of endotrophin generation and its effect on insulin sensitivity in obese AT. **(A) ATs in obese wild-type mice.** COL6A3 and MMPs are abundantly secreted from adipocytes and stromal cells in obese ATs, respectively. In obese AT in WT mice, endotrophin in highly generated, and which is associated with fibro-inflammation and insulin resistance. **(B) ATs in obese APN-HIF1aKO mice**. COL6A3 levels are significantly decreased whereas MMPs are still expressed from stromal cells in ATs in APN-HIF1aKO mice, leading to decrease of the generation of endotrophin, and improve fibro-inflammation and systemic insulin sensitivity following chronic HFD challenged conditions. **(C) miR-29 overexpressing ATs in APN-HIF1aKO mice in obesity**. AdmiR-29 overexpression in ATs of APN-HIF1aKO mice significantly improved metabolic phenotypes due to efficient suppression of the levels of *Col6a3* and *Mmps* such as *Mmp2*, *Mmp9* and *Mmp16* in both adipocytes and stromal cells responsible for endotrophin generation, leading to significant improvement of fibro-inflammation and systemic insulin sensitivity.

Supplementary Table 1. List of primer sequences used for mouse genotyping.

Target gene	Sequence	Band size (bp)
Hif1α flox	F-5'- CCATAACGCTCTTTAAAAAA -3' R-5'- CCCAGCACTGAAAACTGTCT -3'	wild type (WT): 151bp (one band) flox/+: 151, 193bp (two bands) flox/flox: 193bp (one band)
Adiponectin-CRE	Adipo-cre F-5'- ACGGACAGAAGCATTTTCCA -3' Adipo-cre R-5'- GGATGTGCCATGTGAGTCTG -3' Adipo-WT F-5'- CTAGGCCACAGAATTGAAAGATCT -3' Adipo-WT R-5'- GTAGGTGGAAATTCTAGCATCATCC -3'	wild type (WT,-/-): 324bp (one band) Adiponectin-CRE: 200, 324bp (two band)

F: Forward primer. R: reverse primer.

Supplementary Table 2. Chemicals, Peptides, and Recombinant Proteins.

Name	Company	Catalog
Mmu-miR-29a-3p	abm	MPM01081
Mmu-miR-29b-3p	abm	MPM01085
Mmu-miR-29c-3p	abm	MPM01086
Hsa-miR-29a-3p	abm	MPH02387
Hsa-miR-29b-3p	abm	MPH02391
Universal 3'miRNA Reverse primer	abm	MPH00000
miRNA All-in-one cDNA synthesis kit	abm	G899
miR-29a,b,c-3p mimic	Genolution	
G-fectin transfection reagent	Genolution	
MMP-9 Inhibitor I (AG-L-66085)	Santa-Cruz	sc-311437
Thapsigargin	SigmaAldrich	T9033
Recombinant Mouse TNF-alpha protein	R&D systems	aa 80-235
Palmitic acid	SigmaAldrich	258725
Scrambled siRNA	Genolution	
DMOG	SigmaAldrich	D3695
Cobalt(II) chloride	SigmaAldrich	232696
Coelenterazine	SigmaAldrich	C2230

Supplementary Table 3. Construct sequences of ETP cleavage biosensor.

Name	Sequence	Comment	Vector
ETP cleavage biosensor	ATGGACAGCAAAGGTTCGTCGCAGAAAGGGTCCCGCCTGC TCCTGCTGCTGGTGGTGTCAAATCTACTCTTGTGCCAGGGT GTGGTCTCCGCTAGCATGGCAACTGTTCCTGAACTCAACTG TGAAATGCCACCTTTTGACAGTGATGAGAATGACCTGTTCT TTGAAGTTGACGGACCCCAAAAGATGAAGGGCTGCTTCCA AACCTTTGACCTGGGCTGTCCTGATGAGAGCATCCAGCTTC AAATCTCGCAGCAGCACATCAACAAGAGCTTCAGGCAGGC AGTATCACTCATTGTGGCTGTGGAGAAGCTGTGGCAGCTAC CTGTGTCTTTCCCGTGGACCTTCCAGGATGAGGACATGAGC ACCTTCTTTTCCTTCATCTTTGAAGAAGAGCCCATCCTCTGT GACTCATGGGATGATGATGATAACCTGAGTTCAACCATCA ATCTAATGGTGAGCACAGAACCACTCGAGCCCATTAGACA ACTGCACTACAGGCTCCGAGATGAACAACAAAAAAGCCTC GTGCTGTCGGACCCATATGAGCTGAAAGCTCTCCACCTCAA TGGACAGAATATCAACCAACAAGTGATATTCTCCATGAGCT TTGTACAAGGAGAACCAAGCAACGACAAAATACCTGTGGC CTTGGGCCTCAAAGGAAAGAATCTATACCTGTCCTGTGTAA TGAAAGACGGCACACCCACCCTGCAGCTGGAGAGTGTGGA TCCCAAGCAATACCCAAAGAAGAAGATGGAAAAACGGTTT GTCTTCAACAAGATAGAAGTCAAGAGCAAAGTGGAGTTTG AGTCTGCAGAGTTCCCCAACTGGTACATCAGCACCTCACAA GCAGAGCACAAGCCTGTCTTCCTGGGAAACAACAGTGGTC AGGACATAATTGACTTCACCATGGAATCCGTGTCTTCCTCT AGAGGATCCACTAGTGGCGAGGCCAAGCCCACCGAGAACA ACGAAGACTTCAACATCGTGGCCGTGGCCAGCAACTTCGC GACCACGGATCTCGATGCTGACCGCGGGAAGTTGCCCGGC AAGAAGCTGCCGCTGGAGGTGCTCAAAGAGATGGAAGCCA ATGCCCGGAAAGCTGGCTGCACCAGGGGCTGTCTGATCTG CCTGTCCCACATCAAGTGCACGCCCAAGATGAAGAAGTTC ATCCCAGGACGCTGCCACACCTACGAAGGCGACAAAGAGT CCGCACAGGGCGGCATAGGCGAGGCGATCGTCGACATTCC TGAGATTCCTGGGTTCAAGGACTTGGAGCCCATGGAGCAG TTCATCGCACAGGTCGATCTGTGTGTGGACTGCACAACTGG CTGCCTCAAAGGGCTTGCCAACGTGCAGTGTTCTGACCTGC TCAAGAAGTGGCTGCCGCAACGCTGTGCGACCTTTGCCAGC AAGATCCAGGGCCAGGTGGACAAGATCAAGGGGGCCGGTG GTGACGATTACAAGGATGACGACGATAAGGGAGGAGGAG GAAGTCATCATCACCATCACCAC	Black: prolactin signal (pRL) Red: Pro-IL-1β Blue: ETP Cleavage sites (12A.A) Green: Linker Purple: Gluc Orange: Flag, 6xHis	pRA

Supplementary Table 4. List of primer sequences used for PCR Cloning.

Genes	Species	Primer sequences	Restriction enzymes	vector
pri-miR-29b1	Human	F-5'- GCTCTCCCATCAATAACAAATTCAGTGAC -3' R-5'- CTTCTTCCAGTTTCTAAGTTGAATTTCACTG -3'	Bgl2, Xho1	pAd-Track
Col6a3 C2C5	Human	F-5'- ATGGACATGGCTTTCATCTTAGATAG -3' R-5'- GGTTCCCATCACACTGATGACT -3'	Sal1, Xho1	pRA
Col6a3 promoter (2kb)	Mouse	F-5'- GTATGCACTCGGTATGTCAGTATCAG -3' R-5'- AGCAGCCTTTGGGGAAAGG -3'	Kpn1, Xho1	PGL4 .10
miR29b1 promoter (1.3kb)	Mouse	F-5'- GTTTCCTCGGGTAGGACAGC -3' R-5'- GAAGAAGCTTTGTCGTCTGTTTTTGT -3'	Xho1, Bgl2	PGL4 .10
MMP2	Human	F-5'- ATGGAGGCGCTAATGGC -3' R-5'- GCAGCCTAGCCAGTCG -3'	Sal1, Xba1	pRA

Mmp9	Mouse	F-5'- ATGAGTCCCTGGCAGCCCC -3' R-5'- AGGGCACTGCAGGAGGT -3'	Sal1, Xba1	pRA
MMP11	Human	F-5'- ATGGCTCCGGCCGCCTGG -3' R-5'- GAGGAAAGTGTTGGCAGGCT -3'	Sal1, Xba1	pRA
MMP14	Human	F-5'- ATGTCTCCCGCCCCAAG -3' R-5'- GACCTTGTCCAGCAGGGA -3'	Sal1, Xba1	pRA
MMP16	Human	F-5'- ATGATCTTACTCACATTCAGCACTGG -3' R-5'- CACCCACTCTTGCATAGAGC -3'	Sal1, Xba1	pRA
Catalytic MMP2	Human	F-5'- TACAACTTCTTCCCTCGCAAGC -3' R-5'- GTCAATGTCAGGAGAGGCCC -3'	Sal1, Xba1	pRA
Catalytic Mmp9	Mouse	F-5'- AAAGGCCTCAAGTGGGACC -3' R-5'- ACGACCATACAGATACTGGATGC -3'	Sal1, Xba1	pRA
Catalytic MMP11	Human	F-5'- TTCGTGCTTTCTGGCGGGCGCTGGGAG -3' R-5'- GGAGGTGACAGTGGGCC -3'	Sal1, Xba1	pRA
Catalytic MMP14	Human	F-5'- TACGCCATCCAGGGTCTCA -3' R-5'- CCTGGGTTGAGGGGGC -3'	Sal1, Xba1	pRA
Catalytic MMP16	Human	F-5'- GGACAGAAATGGCAGCACAAG -3' R-5'- TGGACCATATATCTTCTGGATGCCC -3'	Sal1, Xba1	pRA
Col6a3 3'UTR	Mouse	F-5'- CACTCAACCTCGGGAGGAAAC -3' R-5'- GTTCTAGGCACAGGGTTTGC -3'	Xba1	PGL3 Control
MMP2 3'UTR	Human	F-5'- TGCTGACTGTACTCCTCCCA -3' R-5'- CCAGTGCCCTCTTGAGACAG -3'	Xba1	PGL3 Control
MMP9 3'UTR	Human	F-5'- TATATCTAGACCGTCCTGCTTTGGCAGT-3' R-5'- TATATCTAGAACAAAGGTGAGAAGAGAGGGC-3'	Xba1	PGL3 Control
MMP16 3'UTR	Human	F-5'- TGCATTGTTCTCATTACACTCAACACT -3' R-5'- AGCATGACCCCTTGACCTTT -3'	Xba1	PGL3 Control
Mmp9 shRNA	Mouse	F-5'- CCGGCCCTCTGAATAAAGACGACATCTCGAGATGT CGTCTTTATTCAGAGGGTTTTTG -3' R-5'- AATTCAAAAACCCTCTGAATAAAGACGACATCTCG AGATGTCGTCTTTATTCAGAGGG -3'	EcoR1, Age1	PLKO.1
MMP16 shRNA	Human	F-5'- CCGGCGTGATGTGGATATAACCATTCTCGAGAATG GTTATATCCACATCACGTTTTTG -3' R-5'- AATTCAAAAACGTGATGTGGATATAACCATTCTCG AGAATGGTTATATCCACATCACG -3'	EcoR1, Age1	PLKO.1
Hif1a shRNA	Mouse	F-5'- CCGGTGGATAGCGATATGGTCAATGCTCGAGCATT GACCATATCGCTATCCATTTTTG -3' R-5'- AATTCAAAAATGGATAGCGATATGGTCAATGCTCG AGCATTGACCATATCGCTATCCA -3'	EcoR1, Age1	PLKO.1

F: Forward primer. R: reverse primer.

Supplementary Table 5. Antibodies

Name	Company	Catalog	Species
COL6A1	Santa Cruz	sc-20649	Rabbit
COL6A2	Santa Cruz	sc-292186	Rabbit
Mouse ETP	COVANCE	Tx621	Rabbit
Human ETP	COVANCE	Tx933	Rabbit
HIF1α	CST	D1S7W	Rabbit
GAPDH	Santa Cruz	sc-32233	Mouse
Anti-FLAG	Santa Cruz	sc-166384	Mouse
β-actin	Santa Cruz	sc-47778	Mouse
Anti-V5	Santa Cruz	sc-81594	Mouse
F4/80	Santa Cruz	sc-25830	Rabbit
Endomucin	Santa Cruz	Sc-65495	Rat

Supplementary Table 6. List of primer sequences used for qRT-PCR analysis.

Genes	Species	Primer sequences
Col6a1	Mouse	F-5'- GATGAGGGTGAAGTGGGAGA -3' R-5'- CAGCACGAAGAGGATGTCAA -3'
Col6a2	Mouse	F-5'- ATGTGAGGGAGACCTGTGGA -3' R-5'- TGTGCCTGTTTCTGACTTGG -3'
Col6a3	Mouse	F-5'- CAGAACCATTGTTTCTCACT -3' R-5'- AGGACTACACATCTTTTCAC -3'
Col1a1	Mouse	F-5'- CCTCAGGGTATTGCTGGACAAC -3' R-5'- CAGAAGGACCTTGTTTGCCAGG -3'
Col3a1	Mouse	F-5'- GACCAAAAGGTGATGCTGGACAG -3' R-5'- CAAGACCTCGTGCTCCAGTTAG -3'
18S	Mouse	F-5'- AGGGTTCGATTCCGGAGAGG -3' R-5'- CAACTTTAATATACGCTATTGG -3'
Hif1a	Mouse	F-5'- GATTCGCCATGGAGGGC -3' R-5'- AGACTCTTTGCTTCGCCGAG -3'
Vegfa	Mouse	F-5'- AGCCGAGCTCATGGACGGGT -3' R-5'- TGGCGGGCTCCTCTCCCTTC -3'
Glut1	Mouse	F-5'- GGTGTGCAGCAGCCTGTGTA -3' R-5'- CAACAAACAGCGACACCACAGT -3'
Pai1	Mouse	F-5'- AGTCTTTCCGACCAAGAGCA -3' R-5'- ATCACTTGCCCCATGAAGAG -3'
Pdk1	Mouse	F-5'- GCAGCAGAGAGTAAACTGTTTG -3' R-5'- TGGTCACCTGACCTCTCG -3'
Tgfb1	Mouse	F-5'- GACCCTGCCCCTATATTTGGA -3' R-5'- CCGGGTTGTGTTGGTTGTAGA -3'

Fn	Mouse	F-5'- ATGTGGACCCCTCCTGATAGT -3' R-5'- GCCCAGTGATTTCAGCAAAGG -3'
Vim	Mouse	F-5'- GCCAGCAGTATGAAAGCGTG -3' R-5'- ACCTGTCTCCGGTACTCGTT -3'
Acta2	Mouse	F-5'- GTCCCAGACATCAGGGAGTAA -3' R-5'- TCGGATACTTCAGCGTCAGGA -3'
Lox	Mouse	F-5'- TCTTCTGCTGCGTGACAACC -3' R-5'- GAGAAACCAGCTTGGAACCAG -3'
Mmp2	Mouse	F-5'- CAAGTTCCCCGGCGATGTC -3' R-5'- TTCTGGTCAAGGTCACCTGTC -3'
Mmp9	Mouse	F-5'- CTGGACAGCCAGACACTAAAG -3' R-5'- CTCGCGGCAAGTCTTCAGAG -3'
Mmp14	Mouse	F-5'- CAGTATGGCTACCTACCTCCAG -3' R-5'- GCCTTGCCTGTCACTTGTAAA -3'
Mmp16	Mouse	F-5'- CTGACAAGATCCCTCCACCTAC -3' R-5'- GTGTTGAAGTTCCCATCACAGA -3'
MMP2	Human	F-5'- CCCACTGCGGTTTTCTCGAAT -3' R-5'- CAAAGGGGTATCCATCGCCAT -3'
MMP9	Human	F-5'- GGGACGCAGACATCGTCATC -3' R-5'- TCGTCATCGTCGAAATGGGC -3'
MMP14	Human	F-5'- CGAGGTGCCCTATGCCTAC -3' R-5'- CTCGGCAGAGTCAAAGTGG -3'
MMP16	Human	F-5'- GATTCAGCCATTTGGTGGGAGG -3' R-5'- CCCTTTCCAGACTGTGATTGGC -3'
Mcp1	Mouse	F-5'- CTGGATCGGAACCAAATGAG -3' R-5'- CGGGTCAACTTCACATTCAA -3'
Il6	Mouse	F-5'- TCGTGGAAATGAGAAAAGAGTTG -3' R-5'- AGTGCATCATCGTTGTTCATACA -3'
Tnfα	Mouse	F-5'- CATCTTCTCAAAATTCGAGTGACAA -3' R-5'- TGGGAGTAGACAAGGTACAACCC -3'
VEGFa-ChIP	Mouse	F-5'- CTGAATGCTAGGGTGGTTTGAATCACCATG -3' R-5'- GAGCCCTTGTCTGATCTGCAGTCATCAG -3'
Col6a3-ChIP	Mouse	F-5'- AAGGTGATGGGTAGAAGTGGC -3' R-5'- CAACCATCTAGGTCCAGGGG -3'

F: Forward primer. R: reverse primer.

Supplementary Materials

Adenovirus

Adenovirus-expressing pri-miR-29b-1~29a were cloned using the AdEasy system (Agilent, Santa Clara, CA) according to the manufacturer's protocol. pri-miR-29b-1~29a was amplified from genomic DNA using PCR and cloned into the pAdTrack shuttle vector (Addgene, Watertown, MA). Pri-miR-29b-1~29a and control pAdTrack plasmids were linearized with *Pme*I and transformed into BJ5183-AD-1 competent cells for recombination with the adenoviral backbone. Confirmed recombinant plasmid was linearized by *Pac*I and transfected into AD-293 (Agilent Technologies) cells to generate recombinant adenoviruses. Harvested adenovirus was amplified by infecting AD-293 cells and purified via CsCl density gradient (1). Purified Ad-GFP or Ad-miR29 (1×10^9 Pfu/ml, 40 μl), mixed with PBS up to 200 μl, were directly injected into the subcutaneous fat pads of mice. Primers for pri-miR-29b-1~29a PCR are listed in **Supplementary Table 4.**

Western blot

Cells were lysed, total protein was subjected to 8% or 15% SDS-PAGE, and transferred onto nitrocellulose membranes. The nitrocellulose membrane was blocked with 5% skim milk, and immunoblotting was performed using mouse anti-COL6A1 (Santa Cruz), anti-COL6A2 (Santa Cruz), anti-human ETP (Covance), anti-mouse ETP (Covance), anti-HIF1α (CST), anti-GAPDH (Santa Cruz), anti-β Actin (Santa Cruz), anti-FLAG (Santa Cruz), anti-V5 (Santa Cruz). Membranes were then incubated with secondary antibodies labeled with an infrared dye emitting at 800 nm (Li-Cor Bioscience, Lincoln, NE). The data were analyzed using Odyssey software (version 2.1, Li-Cor Bioscience). Details pertaining to the antibodies used in this study are provided in **Supplementary Table 5**.

Histology

For immunohistochemical studies, ATs were excised and fixed in PBS-buffered 10% formalin

for 1 day and washed with 50% ethanol and distilled H_2O. Following paraffin embedding, 4-μm sections were deparaffinized. After antigen retrieval with citrate buffer and blocking with 5 % bovine serum albumin and 0.3 % H_2O_2, sections were stained with a primary antibody against ETP, biotinylated secondary antibody (Life Technologies, cat#65-6140, Carlsbad, CA), and streptavidin/HRP (1:250) (Agilent Dako, cat#P0397, Santa Clara, CA). Secondary antibodies were detected using the DAB+ Substrate Chromogen System (Agilent Dako) following the manufacturer's protocol. The slides were counterstained with filtered Harris hematoxylin. All images were acquired using the FSX100 microscope (Olympus, Tokyo, Japan). The adipocyte area (μm^2) was measured using Image J. The antibodies used in this study are listed in **Supplementary Table 5.**

Immunoprecipitation

HEK293T cells, transient transfected with indicated expressing vectors mentioned in **Fig. 3H** were lysed with IP lysis buffer containing 50mM Tris-HCl (pH 8.0), 150mM NaCl and 1% NP-40 plus proteases inhibitor and centrifuged at 13000rpm for 15 min. After protein quantification, 800μg of cell lysates were incubated with 20ul of PBS pre-washed Anti-FLAG Affinity gel (Cat# B23102) 2 hours at 4°C rotate shaker. After 5 rounds of centrifugation (200g, 30 sec) with IP lysis buffer washing, beads were resuspended in 50ul SDS sample loading buffer and boiled at 99°C for 8 min. 25ul were loaded for western blotting analysis.

Measurement of serum TG, cholesterol, NEFA, GPT and GOT

Measurement of serum TG (AM157S-K), cholesterol (AM 202-K), NEFA (279-75401), GPT (AM102-K) and GOT (AM103-K) were performed in accordance with the manufacturer's protocol.

ETP recombinant protein

The native form of ETP was purified with conditioned media collected from mouse ETP-

overexpressing HEK293T cells using fast protein liquid chromatography with size exclusion.

Cell culture

3T3-L1 cell differentiation was induced as previously described (2). In brief, 3T3-L1 pre-adipocyte cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C and 5% CO_2. At 100% confluence, growth medium was removed, and an identical volume of differentiation medium containing insulin (5 μg/ml), dexamethasone (1.0 μM), and methylisobutyl-xanthine (IBMX, 0.5 mM) in DMEM with 10% FBS was added at 37˚C and 10% CO_2 for 48 h. Every 48 h for 8 days, the differentiation medium was replaced with adipocyte maintenance medium containing insulin (1.0 μg/ml) in DMEM with 10% FBS at 37˚C and 10% CO_2. Differentiated 3T3-L1 cells were cultured in a hypoxic chamber (Don Whitley Scientific, Bingley UK, 1% O_2) or under normoxia (21% O_2) at 37˚C for the indicated times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using Trizol (Favorgen, Ping-Tung, Taiwan). RT-qPCR was performed on an ABI 7500 using SYBR TOPreal™ qPCR premix kit (enzynomics, Daejeon, Korea). To determine miR-29 levels, miRNA cDNA was synthesized using an miRNA cDNA Synthesis kit (abm). Mmu-miR-29a-3p, -29b-3p, -29c-3p primers, and Hsa-miR-29a, -29b-3p (abm) were used to quantify the expression level of miR-29 members, and 18s rRNA was used as the internal control. Data were normalized using the $2^{-\Delta\Delta Ct}$ method for relative quantification. The catalog numbers of miR-29 primers are listed in **Supplementary Table 1 and 6**.

Chromatin immunoprecipitation-qPCR (ChIP-qPCR)

The chromatin shearing step was performed using Covaris' Kit as per manufacturer instructions, with minor modifications. Briefly, cells were fixed with 1% formaldehyde at room temperature for 8 min and then quenched with 0.125 M glycine. After washing, cells were resuspended in

lysis buffer and incubated on ice for 10 min. Nuclei were collected, and chromatin shearing was performed using a Covaris S220 sonicator to obtain ~500-bp DNA fragments. Sheared chromatin samples were diluted in dilution buffer (0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) and precleared using protein A/G beads (Santa Cruz, sc-2003), rabbit serum, and salmon sperm DNA for 1 h at 4℃. Precleared samples were incubated with HIF1α antibodies (1:400, CST, D1S7W) overnight at 4℃ and then precipitated with protein A/G beads for 1 h at 4℃. The immunoprecipitated DNA complex was sequentially washed with low salt buffer (0.1% SDS, 1% Triton-X 100, 20 mM Tris-HCl pH 8.1, 2 mM EDTA, and 10 mM NaCl), high salt buffer (0.1% SDS, 1% Triton-X 100, 20 mM Tris-HCl, pH 8.1, 2 mM EDTA, and 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1), and TE buffer twice (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). Washed samples were eluted (1% SDS and 100 mM $NaHCO_3$) and incubated with RNase A and proteinase K, followed by reverse cross-linking at 65 ℃ overnight. DNA samples were purified using the Column-based Kit (Bionics, Seoul, Korea) and analyzed via qPCR. Primers used for ChIP-qPCR analysis are listed in **Supplementary Table 6.**

ELISA

Mouse serum was added into a 96-well ELISA plate (cat#32296, SPL Life Science) with 50 mM carbonate buffer (pH 9.2) overnight at 4℃. After removing antigen solution, 200 μl blocking buffer (1% BSA in PBS) was added into each well and incubated for 1 h at room temperature. After washing with PBS, samples were incubated with primary antibody (anti-mouse ETP, 12 μg/ml) and control IgG (rabbit IgG, 12 μg/ml) overnight at 4℃. Samples were then incubated with a secondary HRP-conjugated antibody (1:1000) for 1 h at room temperature after washing thrice with PBS-T. Finally, samples were detected at 590 nm using TECAN (infinite M200) after incubating TMB (cat#T0440, Sigma) for about 30 min.

Immunofluorescence

For immunofluorescence studies, ATs were excised and fixed in PBS-buffered 10% formalin for a day and washed with 50% ethanol as well as distilled H_2O. Following paraffin embedding,

4-μm sections were deparaffinized. After antigen retrieval with citrate buffer and blocking with 5% BSA and 0.3% H_2O_2, sections were stained with a primary antibody against HIF1α (1:400, CST, D1S7W) and endomucin (1:100, Santa Cruz, Sc-65495). Alexa Fluor-488- (1:500, anti-rabbit) and -647-conjugated secondary antibodies (1:500, anti-rat) were used. Slides were mounted using fluorescent mounting medium. Images were obtained using FV1000 (Olympus).

RNA-sequencing

Total RNA samples of HFD-fed APN-HIF1aKO and their littermate control injected with Ad-Ctrl or Ad-miR-29 (n=3 per groups) eWATs were performed RNA-sequencing (Novogene). Analysis of gene expression patterns were performed using the normalized FPKM value of each gene. Genes with similar expression patterns were clustered by using K-means clustering algorithm with R package ClusterR (version 1.2.5). Genes that show a decreasing expression pattern in the order of Ctrl-Con, (Ctrl-miR29 / HIF1aKO-Con) and HIF1aKO –miR-29 are used to perform functional enrichment analysis. Heatmap of genes whose functions were related to Fibrosis, inflammation, and Insulin resistance with a decreasing expression trend are visualized by R package pheatmap (version 1.0.12).

References

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