Materials and Methods

Generation of RTEF-1 transgenic mice

A conditional knockout (KO) line of RTEF-1 was generated by crossing homozygous *TEAD4*lox/lox mice (a gift from Dr. Andres Buonanno, NICHHD, NIH) with transgenic mice expressing *Cre* recombinase under control of the endothelial cell-specific Tie2 promoter/enhancer (a gift from Dr. Anthony Rosenzweig, BIDMC)[1]. Mice were screened by PCR to verify germline transmission, using the following primers as described previously[2]:

	Forward Primer	Reverse Primer
P1	CTAGCATTAAGGAATGTCCCGA	
P2		CTCAACATACAGTTTGAAGCAC
P3		CGTATAGCATACATTATACGAAG
P4	GTGTTCTTAGAGGTACAGTCA	

RTEF-1 transgenic mice were generated at the BIDMC Transgenic Core Facility using the vascular endothelial (VE)-cadherin promoter to drive endothelial-specific expression of human RTEF-1[3]. RTEF-1 transgenic mice were genotyped by PCR as described previously[4], and floxed littermates were used as control mice in all of the mouse studies. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, 1996) and was approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Cell culture and transfection

Human microvascular endothelial cells (HMEC-1, CDC) were cultured in MCDB-131, and human embryonic kidney cells 293 (HEK 293) were cultured in DMEM, both supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin, at 37 °C in 95% air and 5% CO2 atmosphere. HEK 293 cells were transfected with DNA (1 μg/10^5 cells) using polyethylenimine (PEI) according to previously optimized transfections at a ratio of 1 (DNA) to 3 (PEI). Wild-type IGFBP-1 (IGFBP-1FL) or TK.IRS3 or VF-2 mutant promoter luciferase construct, control vector, PXJ40 and/or an

increasing amount of RTEF-1 expression vector (generous gifts from Dr. Alexandre Stewart, University of Ottawa, Canada) were transfected into HEK 293 cells. PXJ40/lacZ (0.01 μg) was co-transfected to determine transfection efficiency of approximately 70–80%. After transfection, cells were incubated for 48 h before analysis.

siRNA transfection

siRNAs targeting human RTEF-1 were synthesized by Genepharma, Inc. (Shanghai, China). Knockdown efficiency of the two duplexes of RTEF-1 siRNAs (siRNA-1: 5'-GGG CAG ACC UCA ACA CCA ATT-3', 5'-UUG GUG UUG AGG UCU GCC CAG-3' and siRNA-2: 5'-ACC CAA GAU GCU GUG UAU UTT-3', 5'-AAU ACA CAG CAU CUU GGG UTT-3') or a nontarget control (5'-UUC UCC GAA CGU GUC ACG UTT-3', 5'- ACG UGA CAC GUU CGG AGA ATT-3') was determined by transfection into HMEC-1 cells at a final concentration of 50 nM according to the manufacturer's protocol. Briefly, a master mix of Lipofectamine 2000 (Invitrogen, CA) was diluted with 1ml of OPTI-MEM (Invitrogen, CA) and incubated for 5 min. The Lipofectamine 2000 dilution was added to the DNA/siRNA dilution, incubated for 20 min, and added dropwise to the cells. Five hours after transfection, media was changed and the cells were allowed to recover overnight.

Quantitative real-time PCR analysis

Total RNA was extracted from the apex of mouse hearts from wild type and transgenic mice, as well as the RTEF-1 o/e and GFP stably expressing HMEC-1 cell lines using Trizol (Invitrogen, CA) according to the manufacturer's instructions. A total of 2.0μg of RNA from both endothelial cell lines stably transfected with RTEF-1 or isolated from the apex of wildtype (WT), RTEF-1 o/e and RTEF-1 EC-specific KO transgenic mouse hearts were reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA) with random primers according to the manufacturer's

protocol. Quantitative real-time PCR (QPCR) amplification was done using SYBR Green Master Mix (Applied Biosystems, CA) according to the manufacturer's protocol with the following primers:

RTEF-1	F: 5'-CCACGAAGGTCTGCTCTTTC-3'	R: 5'-AAGTTCTCCAGCACGCTGTT-3'
IGFBP-1	F: 5'-AAATGGAAGGAGCCCTGCC-3'	R: 5'-GGGTAGACACACCAGCAGAGT-3'
IGF-1	F: 5'-TGGTGGATGCTCTTCAGTTC-3'	R: 5'-GACAGAGCGAGCTGACTTG-3'
GAPDH	F: 5'-TGGTGAAGCAGGCATCTGAG-3'	R: 5'-CTCCTGCGACTTCAACAGCA-3'

Real time quantitative PCR was performed in an under SDS 7000 System (Applied Biosystems, CA). For all individual cDNAs, amplification of each specific mRNA sequence was performed in at least 3 independently performed PCR experiments. For each reaction, expression was calculated as $2^{-\Delta Ct}$, where ΔC_t is the difference between the C_t for the gene of interest and the C_t for the housekeeping gene, GAPDH.

ELISA and Blood Glucose analysis

Mice ages 8-10 weeks were maintained on a standard 12:12-h light-dark cycle and received a standard diet. Blood glucose levels were obtained from feed-deprived (overnight), restrained un-anesthetized mice. Blood was obtained via submandibular bleed, and glucose was quantified using a CVS TRUEtrack glucose monitor (Home Diagnostics, FL). Plasma IGFBP-I concentration was measured by ELISA following the instructions of the manufacturer (Boster Biotechnology, Wuhan, China). IGFBP-1 concentration was measured with a maximum absorbance of 450 nm. The limit of sensitivity is at or above 31.2 pg/ml. After submandibular bleeds, feed was returned to the cages and mice were allowed to recover. Insulin resistance (IR) was assessed from fasting insulin and glucose levels by the previously validated homeostasis model assessment (HOMA-IR)[5].

Metabolic Studies

GTT and ITT assays were performed in 5-month-old conscious WT and transgenic mice (n = 6-8 for each genotype and diet). For GTT, mice were fasted overnight and given free access to water. Glucose (1 g/kg

body weight) was injected intraperitoneally. Blood glucose was measured by tail bleeding using CVS TRUEtrack glucometer (Home Diagnostics, FL) at 0, 30, 60, and 120 minutes after glucose injection. For ITT, mice were injected with insulin (0.75 U/kg body weight; Eli Lilly and Co. MI) intraperitoneally, and blood glucose was measured at the same timepoints for GTT.

Promoter Activity and Chromatin Immunoprecipitation (ChIP)

HEK293 cells were transfected with following constructs:

Construct	Sequence
FL IGFBP-1.Luc	-116 GCTCACAAG**CAAAACAA**AC**TTATTTTG**AACACGGGG -81
Mutant IGFBP-1.Luc	-116 GCTAGCAAGCACCGACGACCAGGCCTGAACACGGGG -81
TK81.IRS3	**IRSA-IRSA-IRSA**-Thym-Kin -81

with or without RTEF-1, indicated in the figure legends, using PEI (Polysciences), and incubated for 24 hours prior to measurement of luciferase activity. Luciferase activity was determined using the dual luciferase assay system (Promega). Chromatin immunoprecipitation (ChIP) was performed with the ChIP-IT Express Kit (Active Motif, Carlsbad, CA) in accordance with the manufacturer's instructions. The IGFBP-1 and actin promoters were amplified with the primer pairs: 5'-CCCTAACAACGGGACAAACA-3' and 5'-TTG CAC CAG GAG GTT AAT GA-3', and 5'-TGCACTGTGCGGCGAAGC-3' and 5'-TCGAGCCATAAAAGGCAA-3', respectively. The IGFBP-1 primers were designed to the insulin response sequence.

Immunoblot Analysis

RTEF-1 and GFP-stably transfected HMEC-1 cells were lysed in cold RIPA buffer (Boston Bio-Products, MA). Conditioned medium was removed and concentrated using centrifugal ultracel-10k filters (Millipore, MA). Protein concentrations were determined with the DC Protein Standard Assay (Bio-Rad, Munich, Germany). Samples were subjected to SDS–PAGE, transferred to nitrocellulose membranes (Whatman, Springfield Mill, UK) and subsequently blocked in TBS–Tween 20 containing 5% non-fat milk for 1h. The membranes were incubated with the indicated primary antibodies: polyclonal anti-

IGFBP-1 antibody with 1:500 dilution, (Millipore, MA); polyclonal anti-RTEF-1 antibody with 1:10000 dilution, (Genemed Synthesis, CA), monoclonal anti-vinculin with 1:65000 dilution followed by incubation with horseradish peroxidase-conjugated secondary antibodies anti-rabbit IgG with 1:3000 dilution, (Calbiochem, CA) or anti-mouse IgG with 1:2000 dilution (Vector Labs, CA). Blots were developed using the chemiluminescence detection system according to the instructions of the manufacturer (Thermo Fisher, PA). Densitometric analysis was done using the NIH software program, Image J.

Immunofluorescence

Hearts were removed from transgenic and littermate control mice, embedded in O.C.T. compound (Sakura Finetek USA Inc.) and frozen at -80^{o}C. Tissues were sectioned with a Cryostat CM Model 3050S-3-1-1 (Leica, Wetzlar, Germany), fixed in 4% paraformaldehyde, and stained with antibodies against RTEF-1 (Genemed, CA) with 1:750 dilution and CD31 (Sigma) with a 1:500 dilution followed by incubation with goat anti-rabbit FITC and goat anti-mouse TRITC (Santa Cruz Inc, CA) secondary antibodies, respectively, at a dilution of 1:500. Immunofluorescence stained sections were visualized with a DM5000B upright microscope (Leica, Germany).

Blood Pressure Measurements

Blood pressure was measured non-invasively on conscious mice using a volume pressure recording tail-cuff system (CODA^{TM} system by Kent Scientific Corporation).Two measurements were taken per mouse and were averaged after 20 cycles subsequent to 8 acclimation cycles; all false readings were excluded. The CODA system analyzes six measurements: systolic, diastolic pressure, mean pressure, rate, blood flow and blood volume[6-8] (BIDMC, Metabolic Core Facility). Additionally, blood pressure was confirmed using telemetry. Briefly, the catheter was secured in place with a 6-0 silk suture and arterial blood pressure was measured by inserting a 1.4 Fr high-fidelity pressure catheter (SPR-671, Millar catheters) in a carotid artery. The catheter was calibrated before each experiment. Heart rate and aortic pressure were

recorded at 2 kHz and analyzed using a built-in analytic program in PowerLab software Chart 5 (AD Instruments, CO; BIDMC, Cardiophysiology Core Facility).

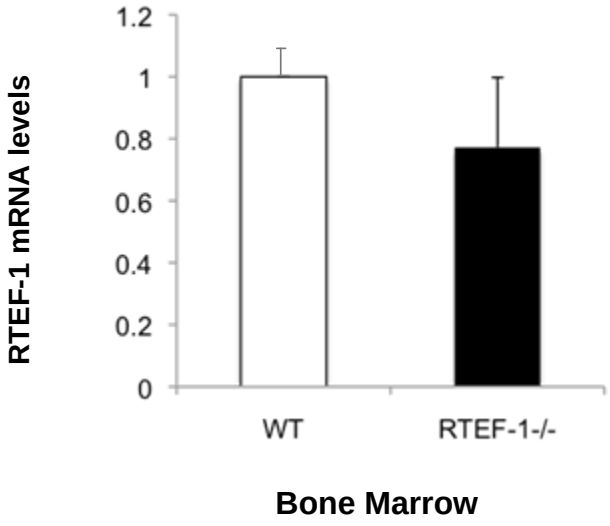
Statistical analysis

Data was obtained from at least three independent cell cultures or animals, as denoted in the figure legends. Data are presented as means ± SEM. The trapezoidal rule was used to determine the area under the curve (AUC). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as (fasting glucose level × fasting insulin level)/22.4. The level of statistical significance was determined using Student's two-tailed t-test when differences between the means of two populations were considered. Comparison of multiple time points between groups was made using a 1-way or 2-way repeated measures ANOVA.

References:

1. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol*. 2001;230:230-42.
2. Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A. Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. *Development*. 2007;134:3827-36.
3. Gory S, Vernet M, Laurent M, Dejana E, Dalmon J, Huber P. The vascular endothelial-cadherin promoter directs endothelial-specific expression in transgenic mice. *Blood*. 1999;93:184-92.
4. Xu M, Jin Y, Song Q, Wu J, Philbrick MJ, Cully BL, An X, Guo L, Gao F, Li J. The Endothelial-Dependent Effect of RTEF-1 in Pressure Overload Cardiac Hypertrophy - Role of VEGF-B. *Cardiovasc Res*.
5. Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care*. 2000;23:57-63.
6. Feng M, Whitesall S, Zhang Y, Beibel M, D'Alecy L, DiPetrillo K. Validation of volume-pressure recording tail-cuff blood pressure measurements. *Am J Hypertens*. 2008;21:1288-91.
7. Rateri DL, Moorleghen JJ, Balakrishnan A, Owens AP, 3rd, Howatt DA, Subramanian V, Poduri A, Charnigo R, Cassis LA, Daugherty A. Endothelial cell-specific deficiency of Ang II type 1a receptors attenuates Ang II-induced ascending aortic aneurysms in LDL receptor-/- mice. *Circ Res*;108:574-81.

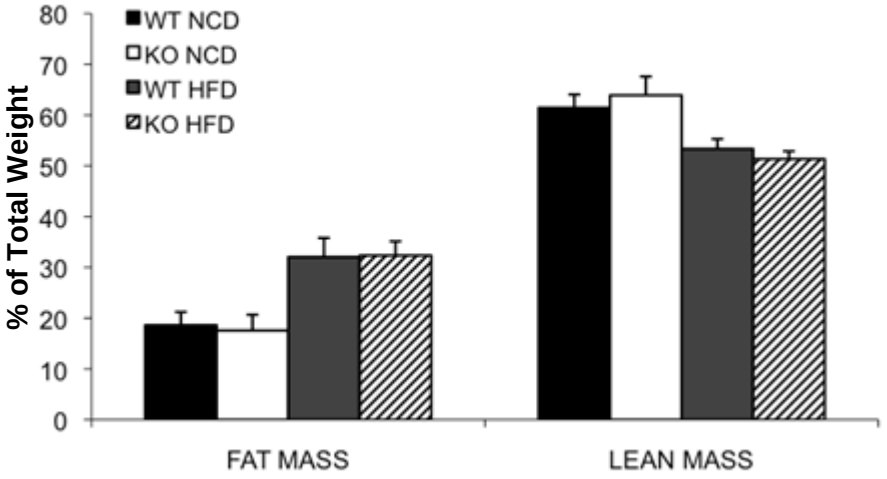
8. Whitesall SE, Hoff JB, Vollmer AP, D'Alecy LG. Comparison of simultaneous measurement of mouse systolic arterial blood pressure by radiotelemetry and tail-cuff methods. *Am J Physiol Heart Circ Physiol*. 2004;286:H2408-15.



Online Figure I.

Bone marrow was extracted from both WT and RTEF-/- mice to determine if RTEF-1 levels were decreased in hematopoietic cells.

A.



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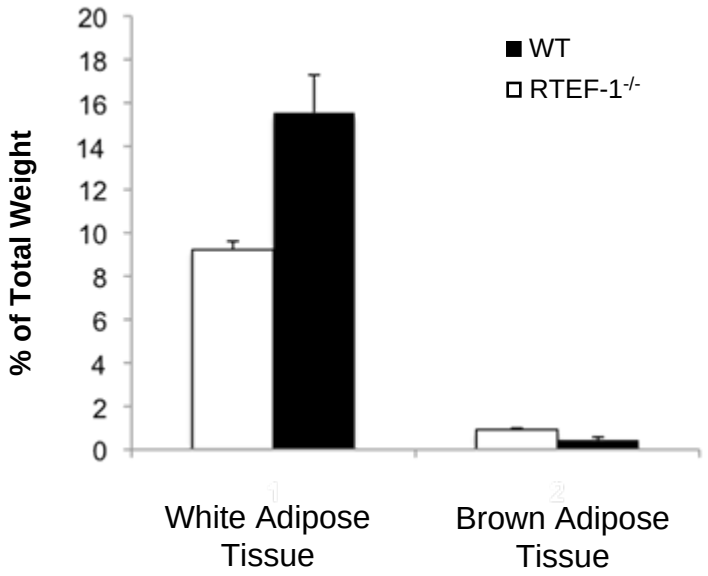
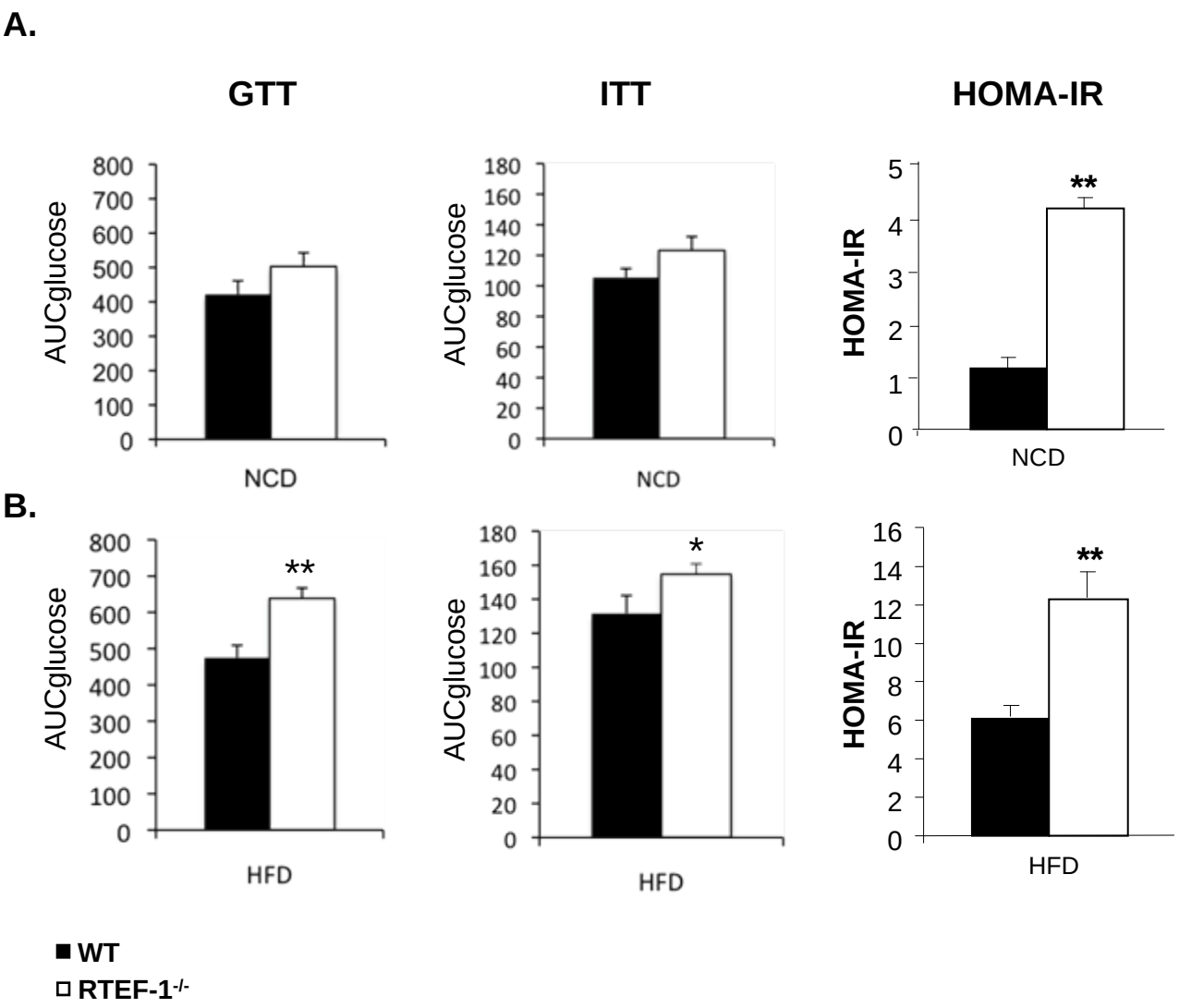


Online Figure II.

A. Body composition was determined by NMR in WT vs. RTEF-1-/- mice on both NCD and HFD diets.

B. White and brown mouse adipose tissues were harvested and measured as a percentage of total body weight.

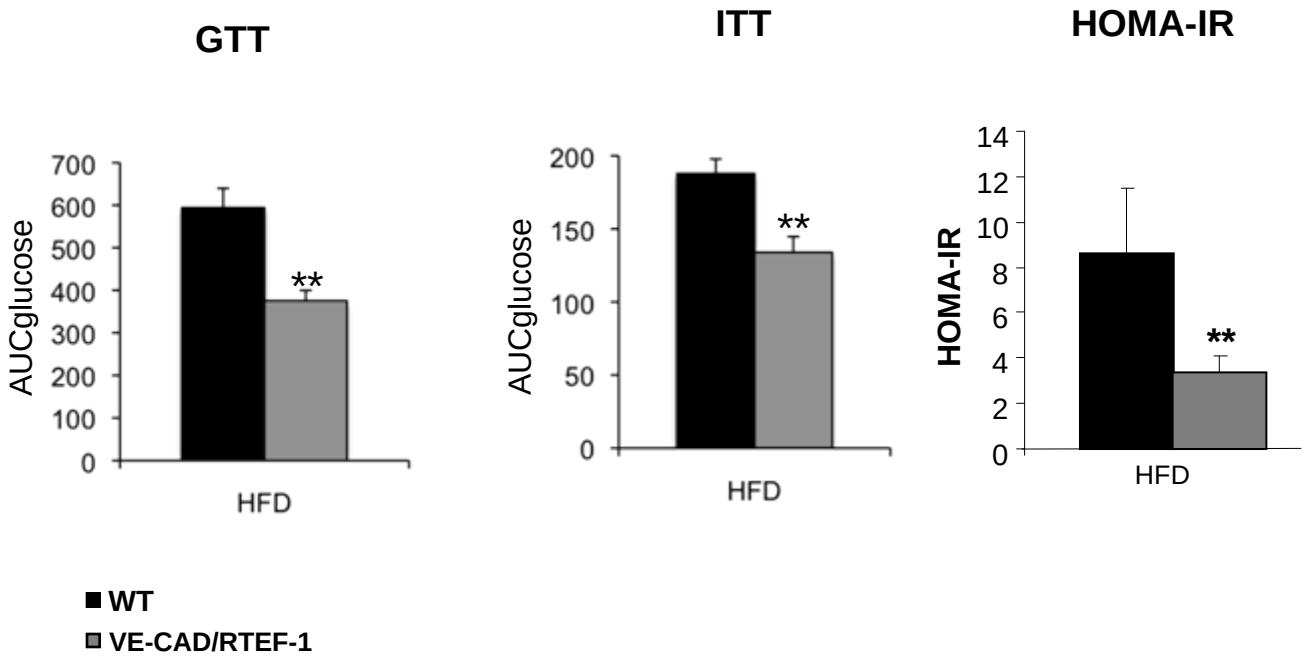


Online Figure III A & B.

A. Area under the curve (AUC) for GTT and ITTs was calculated for NCD WT and RTEF-1-/- mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and RTEF-1-/- mice on a NCD.

B. Area under the curve (AUC) for GTT and ITTs was calculated for NCD WT and RTEF-1-/- mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and RTEF-1-/- mice on a HFD. **C.** Area under the curve (AUC) for GTT and ITTs was calculated for WT and VE-CAD/RTEF-1 mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and VE-CAD/RTEF-1 mice on a HFD.

C.



Online Figure IIIC.

Area under the curve (AUC) for GTT and ITTs was calculated for WT and VE-CAD/RTEF-1 mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and VE-CAD/RTEF-1 mice on a HFD.

	Control	RTEF-1 -/-
SBP	104.05 ± 1.7	99.3 ± 2.6
DBP	69.4 ± 0.2	67.6 ± 2.1
LVSP	105.45 ± 6.5	99.5 ± 6.6
LV EDP	6.3 ± 0.28	7.8 ± 0.49 *
dP/dt_{MAX}	6993 ± 1033	7291 ± 562
dP/dt_{MIN}	-6331.5 ± 47.37	-7022 ± 185

Online Figure IV.

Mice were anesthetized and blood pressure and LV function measured in both control and RTEF-$1^{-/-}$ mice using a Millar pressure system. The parameters included 1) Systolic blood pressure (SBP), 2) Diasolic blood pressure (DBP), 3) LV systolic pressure (LVSP), 4) LV ending diasolic pressure (LV EDP), 5) maximum dP/dt (dP/dt_{max}) and 6) minimum dP/dt_{min}) are shown. Data were analyzed using SPSS analysis software.* $p< 0.05$.