Supplemental Material

Supplemental Methods

PCR Primers:

Acadm (mouse # Mm01323361_mH), CD36(mouse # Mm00432403_m1), Cpt1a (mouse # Mm00550448_m1), Hadh (mouse # Mm00492535_m1), Acot1 (mouse # Mm01622471_s1), PDK4 (mouse # Mm01166879_m1), Dgat1 (mouse # Mm00515643_m1), fabp1 (mouse # Mm00444340_m1), Ucp1 (mouse # Mm01244861_m1), Cidea (mouse # Mm00432554_m1), Prdm16 (mouse # Mm00712556_m1), Dio2 (mouse # Mm00515664_m1), Adrb3 (mouse # Mm02601819_g1), PPARα (mouse # Mm00440939_m1), Atp6 (mouse # Mm03649417_g1), cox1 (mouse #Mm04225243_m1), cycs (mouse #Mm01621048_s1), Nd1 (mouse # Mm04225274_s1), Nrf1 (mouse # Mm00447998_m1), ppargc1a (mouse # Mm01208835_m1), Tfam1 (mouse # Mm00447485_m1), Nppa (mouse # Mm01255747_g1), Ctgf (mouse # Mm01192933_g1), Postn (mouse # Mm01284913_g1), Tgfb (mouse # Mm01178820_m1), Col1a1 (mouse # Mm00801666_g1), Lox (mouse # Mm00495386_m1) or gylceraldehyde-3-phosphate dehydrogenase (GAPDH, mouse #99999915_g1,) by Applied Biosystems.

EM gold sample prepration:

NRVMs fixed in EM grade 4% paraformaldehyde, 0.1% glutaraldehyde in 80 mM phosphate buffer (Sorenson's buffer) with 3 mM magnesium chloride, pH 7.2 where then processed, with all subsequent steps performed at 4^0C until 70% ethanol dehydration. Samples were first rinsed 3 times in buffer containing 3% sucrose, then incubated in 1.5% potassium ferrocyanide reduced 1% osmium tetroxide in 80 mM phosphate buffer, containing 3 mM magnesium chloride followed by two distilled water rinses, dehydrated in a graded series of Ethanol, followed by propylene oxide, and embedded in EPON (Ted Pella, Inc.). Plates are baked at 37^0C for 2-3 days

before an overnight bake at 60^0C. 80-90 nm ultra-thin sections were picked up on formvar coated 200 mesh nickel grids and allowed to dry. Grids were floated on all subsequent steps. All solutions were filtered except for antibodies which were centrifuged at 13K for 5 min. Grids were wetted 3 times on D-H_2O, then placed on 3% sodium metaperiodate for 20 min. After a 15 min rinse in D-H_2O, grids were placed in 50 mM NH_4Cl in TBS for 10 min, followed by 20 min block in blocking solution (3% normal goat serum and 3% bovine serum albumin in TBST). Antibody (anti GFP, Abcam ab6556) incubation was done at appropriate dilutions (1:100) in the same block. Antibody block (no primary) served as negative control. Incubations were carried out at 4^0C overnight. Blocks were equilibrated to room temperature, grids were placed on blocking solution for 10 min, followed by a 1 min rinse in TBS. Gold conjugated secondary antibody (12nm, Colloidal gold 109-185-088, Jackson Immuno Research Labs) was diluted 1:40 in TBS and sections were incubated for 2 hrs at room temperature in a humidity chamber. After a 10 min TBS incubation followed by a quick D-H_2O rinse, grids were hard fixed in 1% glutaraldehyde in 100 mM sodium cacodylate buffer for 5 min. After a brief D-H_2O rinse, grids were stained with 2% uranyl acetate (aq.) for 20 min, rinsed again with D-H20; blot dried and allowed to dry overnight before viewing.

Supplemental Figures and Legends

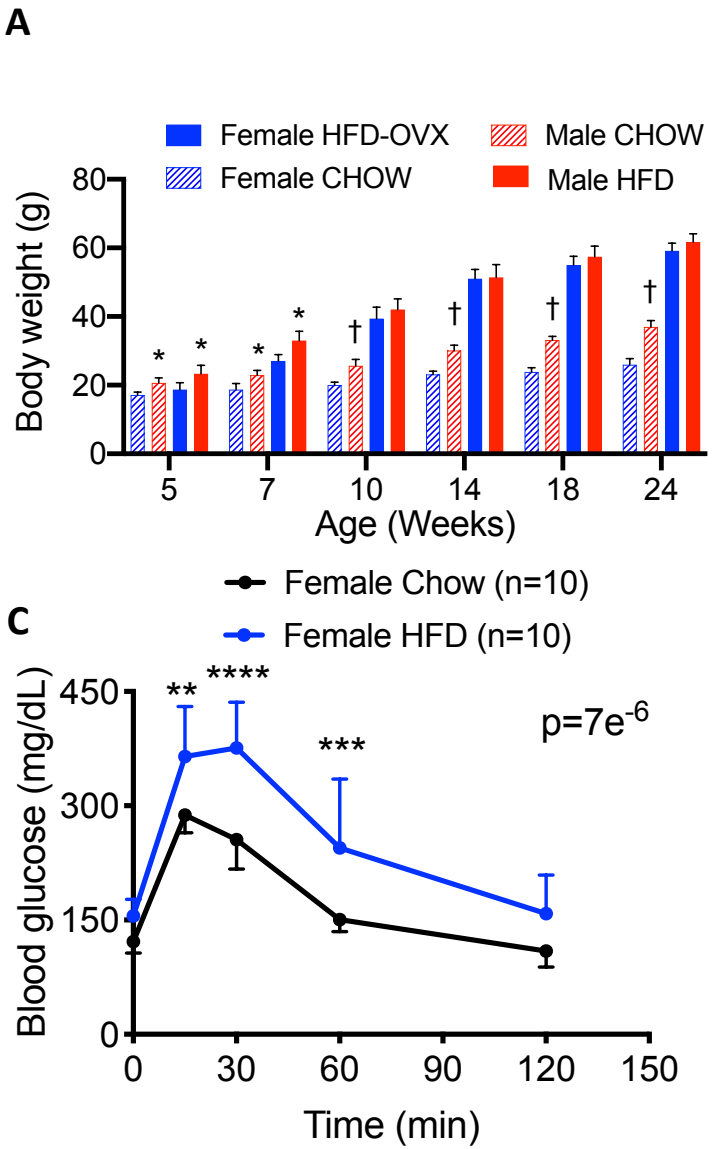

A
Female HFD-OVX
Male CHOW
Female CHOW
Male HFD
Body weight (g)
80
60
40
20
0
5
7
10
14
18
24
Age (Weeks)
B
Chow
HFD(High Fat Diet)
HFD +OVX
C
Female Chow (n=10)
Female HFD (n=10)
Blood glucose (mg/dL)
450
300
150
0
**
****
***
p=7e-6
0
30
60
90
120
150
Time (min)
D
Control Diet
HFD-OVX
50uM
50uM

Figure S1

Supplemental Figure S1. Model of severe obesity and cardiac pressure-load (CMS) in female C57BL6/N mice. A) Total body mass increase over 6-month period for ovariectomized (OVX) females and males with control or high-fat diet. N=9/group, mean ±SD. Female weights post OVX become equal to males. * $p<0.005$, † $p<0.0005$ vs same diet in females. 2WANOVA, Sidak's multiple comparisons test (SMCT). **B)** Example of severe abdominal adiposity particularly in OVX females with DIO. This was representative of nearly all the mice in these cohort. **C)** Depressed glucose tolerance in OVX+HFD versus female chow-diet controls (n=10/group, 2WANOVA, SMCT; ** $p=0.002$, *** $=0.0001$, and **** $6.7e^{-7}$ between groups). **D)** Representative micrographics of liver in normal and DIO showing marked steatosis in OVX mice measured after 13 weeks of DIO and prior to mTAC or drug/placebo treatment (replicated x8).

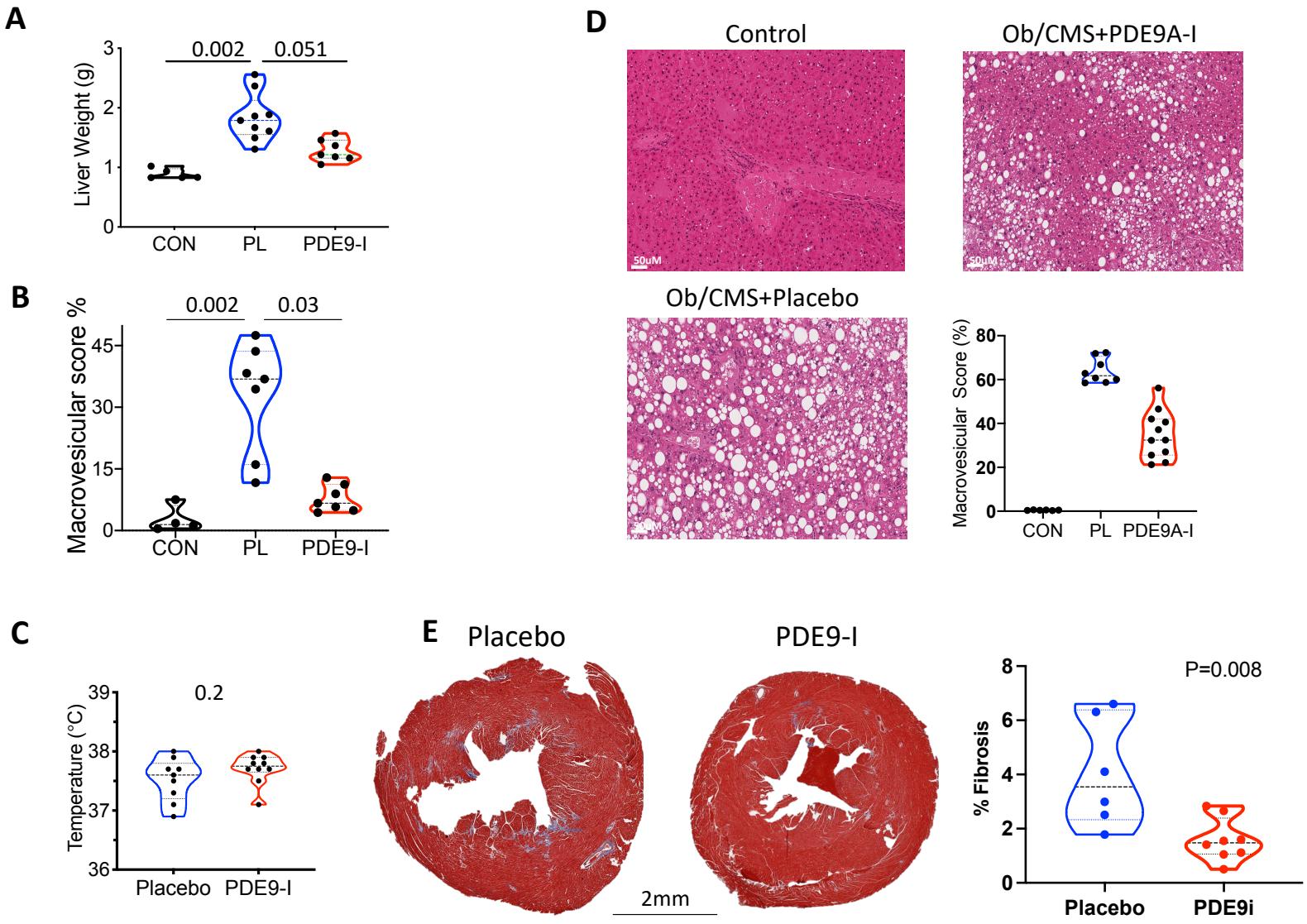

A
Liver Weight (g)
0.002
0.051
CON
PL
PDE9-I
B
Macrovesicular score %
0.002
0.03
CON
PL
PDE9-I
C
Temperature (°C)
0.2
Placebo
PDE9-I
D
Control
Ob/CMS+PDE9A-I
Ob/CMS+Placebo
Macrovesicular Score (%)
CON
PL
PDE9A-I
E
Placebo
PDE9-I
2mm
% Fibrosis
P=0.008
Placebo
PDE9i

Figure S2

Supplemental Figure S2. Hepatic steatosis in OVX and male mice with ob/CMS model. A) Quantification of hepatic mass and **B)** percent area occupied by lipid macro-vesicles in OVX mice with DIO/mTAC model related to examples shown in Figure 1f. PL-placebo, CON- chow diet, KW with DMCT p-values shown. **C)** Core temperature in OVX ob/CMS mice treated with placebo versus PDE9-I. **D)** Example liver histology and summary analysis of macro-vesicular lipid accumulation in male mice with ob/CMS model. Kruskal Wallis (KW) with Dunn's multiple comparison test (DMCT) P values displayed. **e)** Example left ventricle cross-sectional histology stained with Masson Trichrome stain from OVX-DIO/mTAC model with summary results for percent fibrosis/total area for placebo (n=6) versus PDE9-I (n=8) treated mice. P value from Mann Whitney (MW) test. As with all violin plots, median and quartiles are shown.

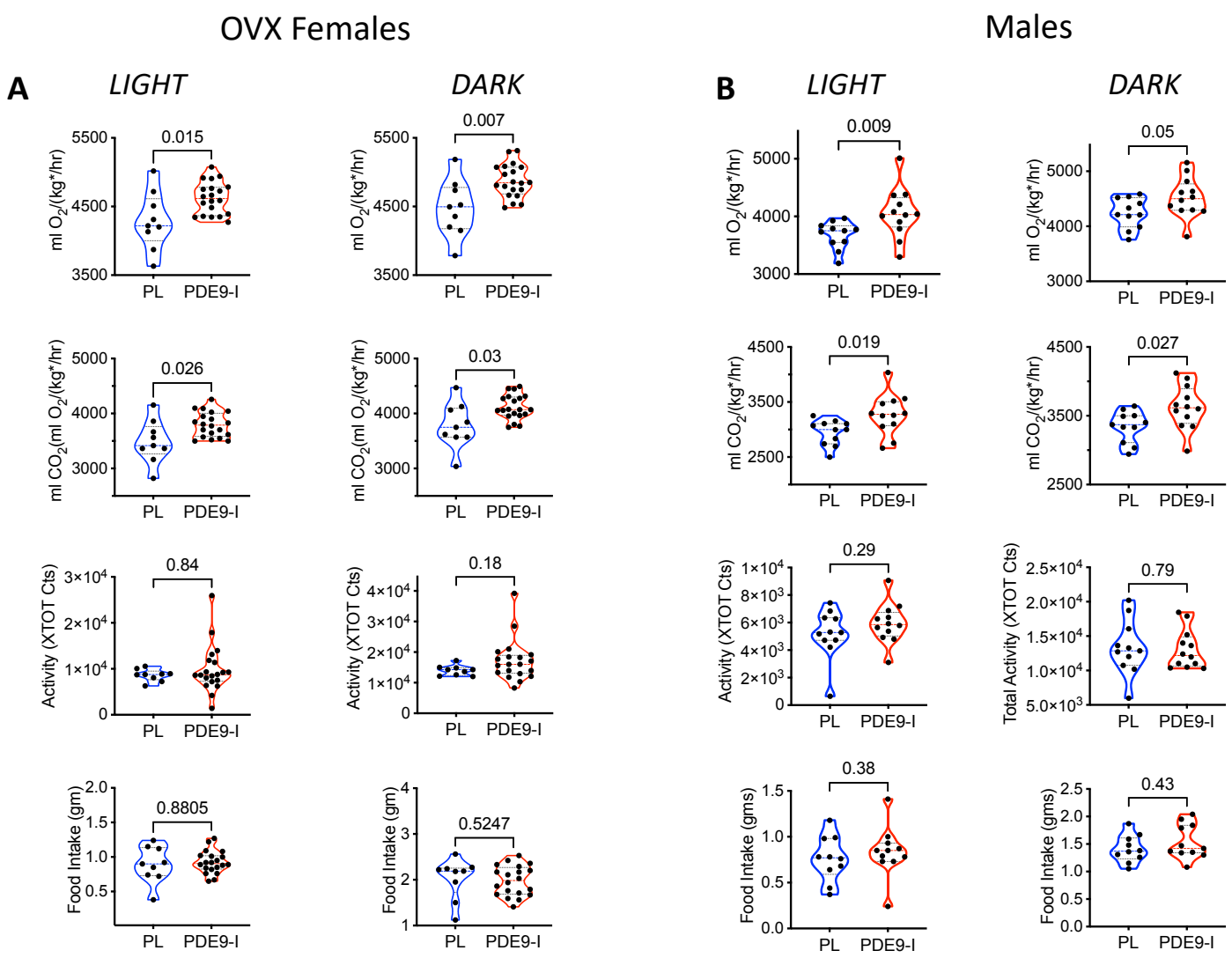

OVX Females
Males
A
B
LIGHT
DARK
LIGHT
DARK
ml O2/(kg*/hr)
ml CO2(ml O2/(kg*/hr)
ml CO2/(kg*/hr)
Activity (XTOT Cts)
Total Activity (XTOT Cts)
Food Intake (gm)
Food Intake (gms)
PL
PDE9-I
0.015
0.007
0.009
0.05
0.026
0.03
0.019
0.027
0.84
0.18
0.29
0.79
0.8805
0.5247
0.38
0.43

Figure S3

Supplemental Figure S3. Circadian effects of PDE9-I on today body O_2 consumption and CO_2, activity and food in OVX and Male mice with DIO/mTAC.

Each time period was 12 hours. PL – placebo. **A)** Data for OVX-female mice (n=9 Placebo, n=20 PDE9-I). **B)** data for Male mice (n=10 Placebo, n=9 PDE9-I). Activity is greater during the dark period as expected. For both groups in both time periods, PDE9-I increases O_2 consumption and CO_2 production without significantly altering activity or food intake. P values displayed are for MW test between the two treatment groups.

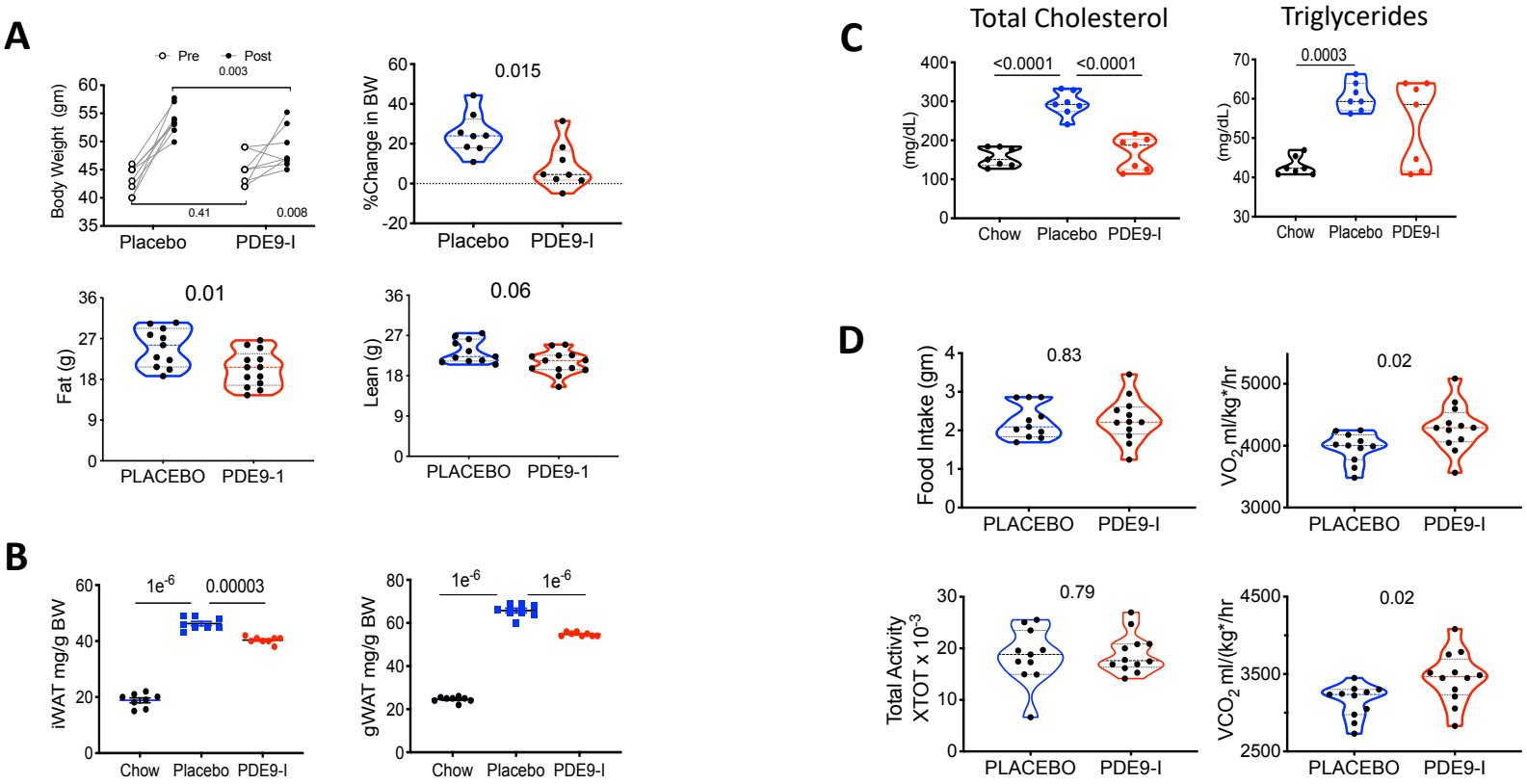
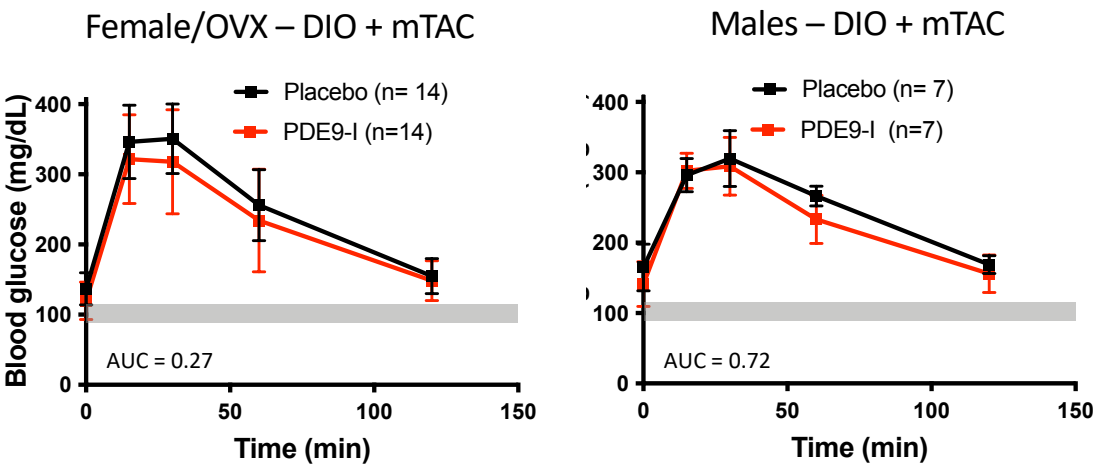

A
Pre
Post
0.003
Body Weight (gm)
0.41
0.008
Placebo
PDE9-I
%Change in BW
0.015
Placebo
PDE9-I
Fat (g)
0.01
PLACEBO
PDE9-1
Lean (g)
0.06
PLACEBO
PDE9-1
B
iWAT mg/g BW
1e-6
0.00003
Chow
Placebo
PDE9-I
gWAT mg/g BW
1e-6
1e-6
Chow
Placebo
PDE9-I
C
Total Cholesterol
(mg/dL)
<0.0001
<0.0001
Chow
Placebo
PDE9-I
Triglycerides
(mg/dL)
0.0003
Chow
Placebo
PDE9-I
D
Food Intake (gm)
0.83
PLACEBO
PDE9-I
VO2 ml/kg*/hr
0.02
PLACEBO
PDE9-I
Total Activity
XTOT x 10-3
0.79
PLACEBO
PDE9-I
VCO2 ml/(kg*/hr
0.02
PLACEBO
PDE9-I

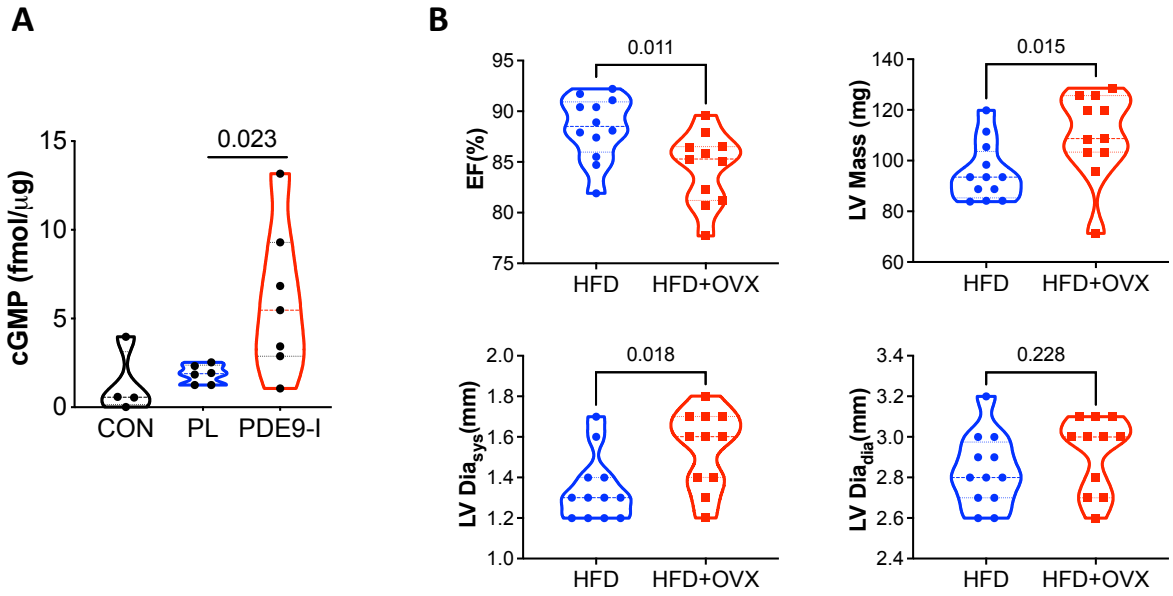
Figure S4

Supplemental Figure S4. PDE9-I reduces obesity and improves metabolic profile in ob/CMS male mice. A) Total body weight before and after treatment in placebo vs PDE9-I treatment groups. (n=8, 2WANOVA, SMCT); percent change from same data, MW; MR-derived total body fat and lean mass for both groups (n=11/13, MW) **B)** iWAT and gWAT from males in both treatment arms. (n=8/group, 1WANOVA, Tukey multiple comparisons test (TMCT). **C)** End-of study serum cholesterol and triglycerides (n=7/group, 1WANOVA, TMCT). **D)** Indirect calorimetry results from same experiment (n=11/12 per group, unpaired t-test). kg* = (lean mass + fat mass * 0.2).



Supplemental Figure S5. Impact of PDE9-I on glucose tolerance test.

Glucose tolerance tests in DIO + mTAC mice from OVX and male groups treated with placebo (vehicle) versus PDE9-I. Data are mean ± SD. Each data set is analyzed by repeated measures mixed effects model: effect of time, drug, and their interaction were: OVX: $P<10^{-11}$, 0.2, 0.57 and Male: $P<10^{-11}$, 0.15, 0.37.



Supplemental Figure S6. PDE9-I increases myocardial cGMP. Effect of OVX on resting heart function and size. A) . A) Myocardial cGMP assessed in OVX mice on chow (CON) or with ob/CMS model treated with placebo (PL) or PDE9-I. KW, P-value from DMCT. **B)** M-mode derived left ventricular ejection fraction (EF), and end-systolic (LV Dia_{sys}) and end-diastolic (LV Dia_{dia}) dimensions, and estimated wall mass. In mice with DIO, OVX resulted in a slight but significant decline in EF with increased end-systolic dimensions, and greater LV mass. This accompanied greater obesity. P values are from MW test.

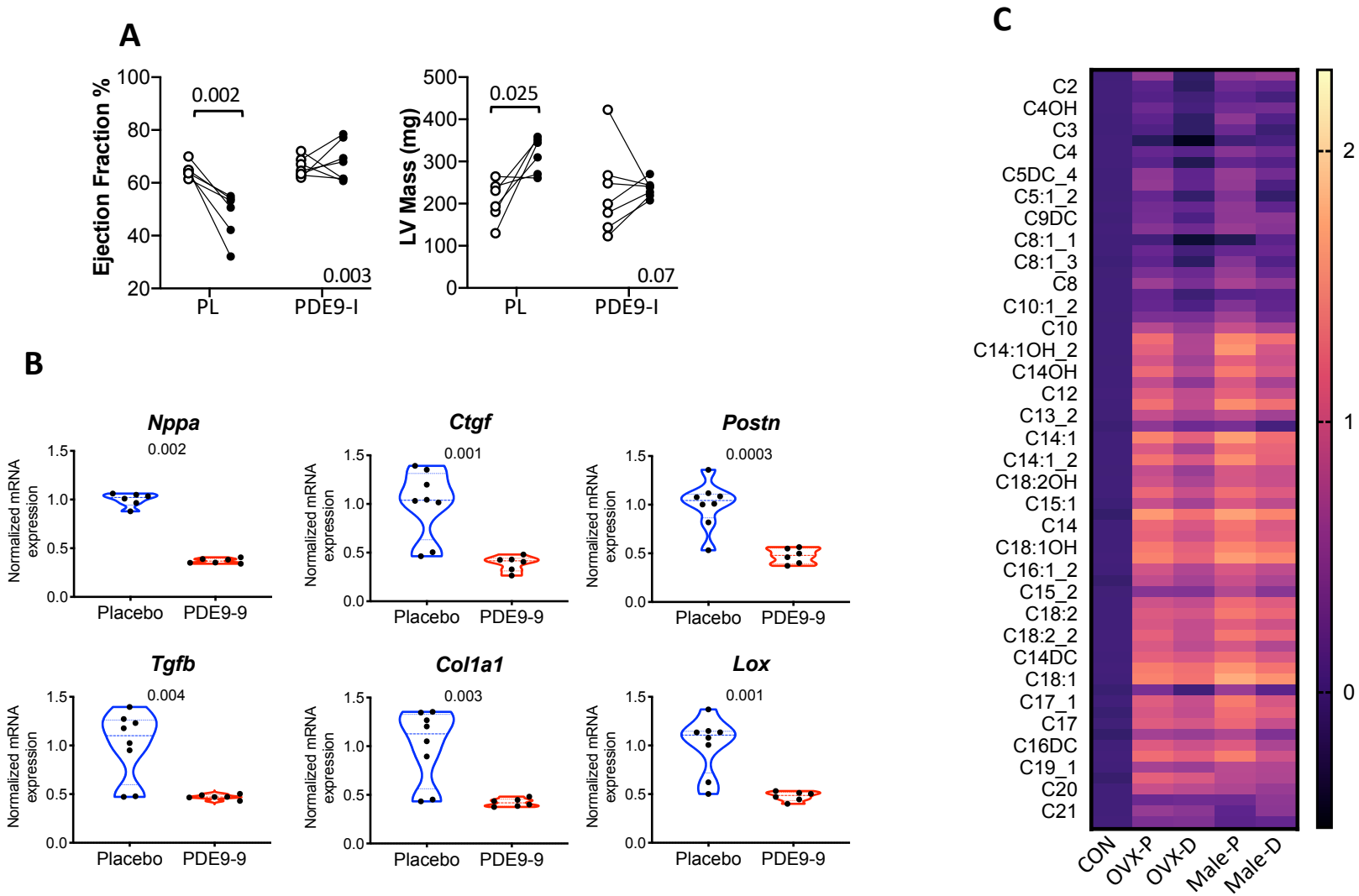

A
Ejection Fraction %
0.002
0.003
PL
PDE9-I
LV Mass (mg)
0.025
0.07
B
Nppa
0.002
Ctgf
0.001
Postn
0.0003
Tgfb
0.004
Col1a1
0.003
Lox
0.001
Normalized mRNA expression
Placebo
PDE9-9
C
C2
C4OH
C3
C4
C5DC_4
C5:1_2
C9DC
C8:1_1
C8:1_3
C8
C10:1_2
C10
C14:1OH_2
C14OH
C12
C13_2
C14:1
C14:1_2
C18:2OH
C15:1
C14
C18:1OH
C16:1_2
C15_2
C18:2
C18:2_2
C14DC
C18:1
C17_1
C17
C16DC
C19_1
C20
C21
CON
OVX-P
OVX-D
Male-P
Male-D

Figure S7

Supplemental Figure S7. PDE9-I improves male ob/CMS mouse cardiac function, pathological hypertrophic/fibrotic molecular signature, and fat metabolome A) Echocardiography for ejection fraction and LV mass in male ob/CMS mice treated with placebo or PDE9-I. (n=6,7/group, 2WANOVA P-value for interaction at lower right, upper P values for Tukey multiple comparisons test. **B)** Quantitative PCR analysis of mRNA for hypertrophic and pro-fibrotic signaling genes in myocardium of males at termination of the same protocol. Abbreviations as provided in Figure 2C legend. KW P-values displayed for each gene. **C)** Heat map (log transformed) for acyl-carnitine metabolites in myocardium of mice fed normal diet (CON) or OVX or Males with DIO/mTAC CMS treated with placebo (PL) or PDE9-I (n=5/group). Statistical analysis performed using 2-step Benjamini, Krieger, Yekutieli procedure for multiple Mann Whitney comparisons, 1% FDR. All analytes in both OVX and Males were significantly increased from non-obese control levels at adjusted Q value <0.002. 89% and 85% of all analytes were significantly reduced (Adjusted Q<0.05, most Q=0.01) by PDE9-I treatment versus placebo in OVX and Males, respectively. Decreases were distributed throughout the broad range of FA but most notable in long-chain FA.

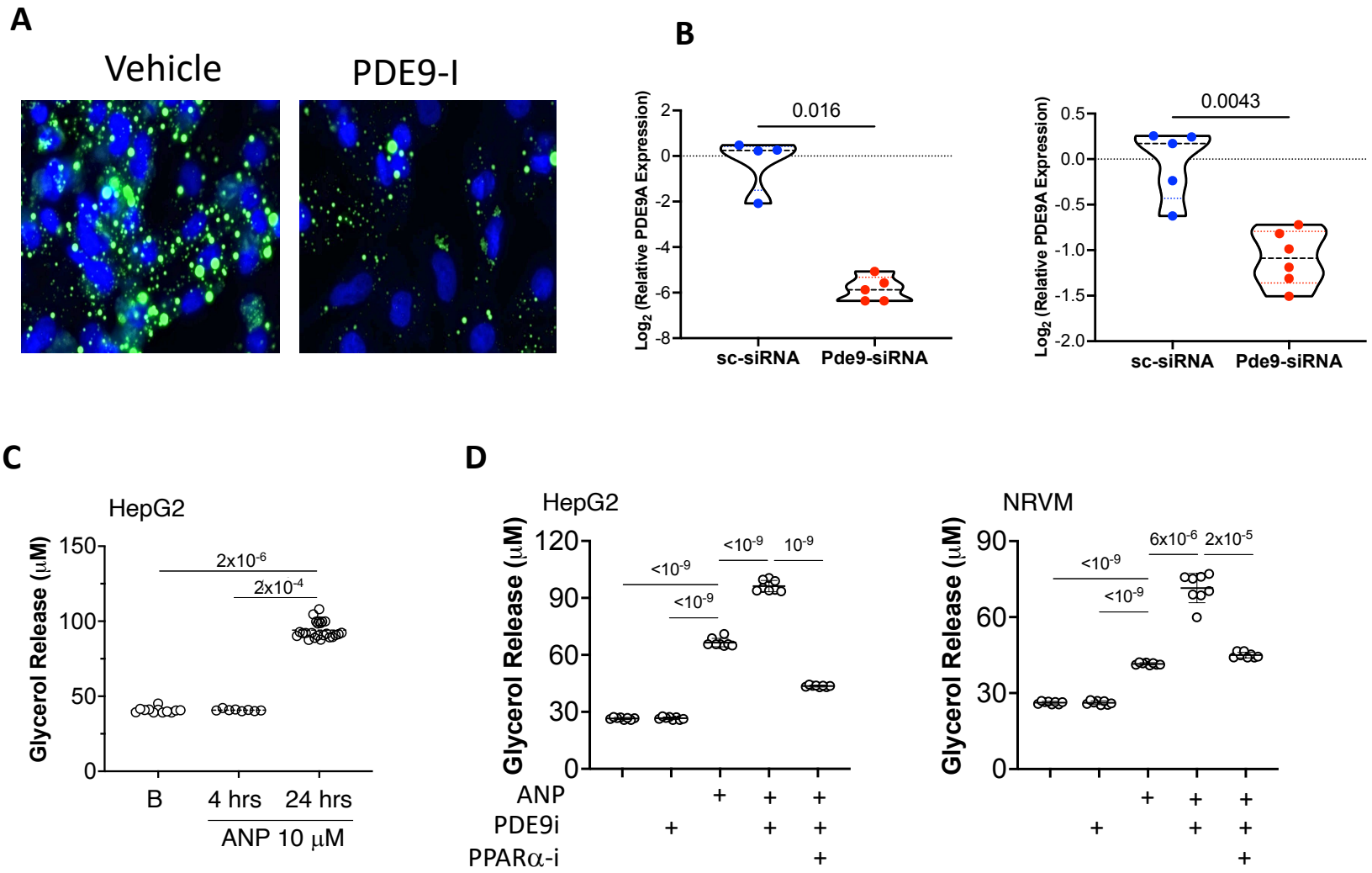
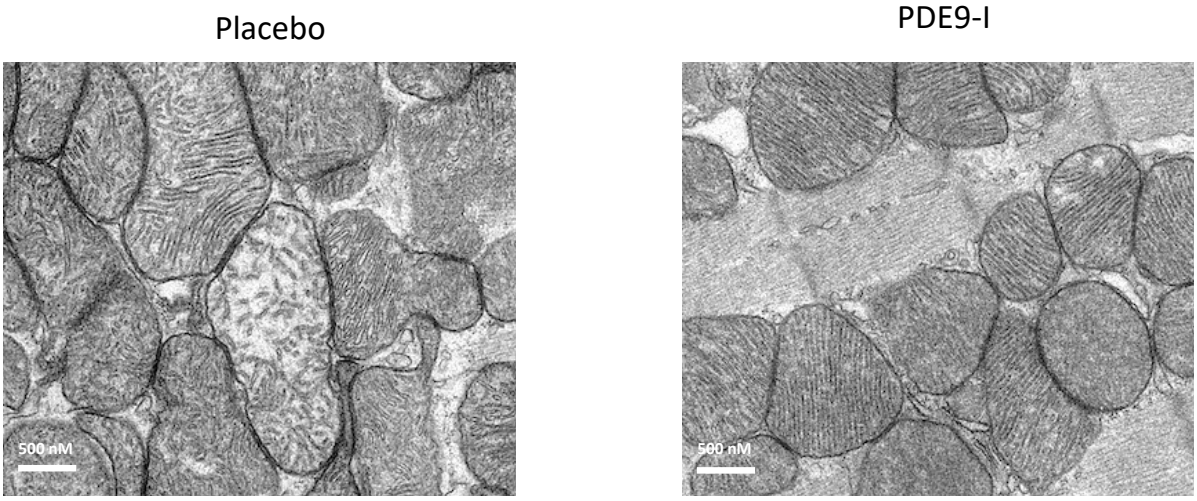

A
Vehicle
PDE9-I
B
Log2 (Relative PDE9A Expression)
0.016
sc-siRNA
Pde9-siRNA
0.0043
C
HepG2
Glycerol Release (μM)
2x10-6
2x10-4
B
4 hrs
24 hrs
ANP 10 μM
D
HepG2
NRVM
<10-9
10-9
6x10-6
2x10-5
ANP
PDE9i
PPARα-i

Figure S8

Supplemental Figure S8. PDE9-I stimulates lipolysis in cardiomyocytes and heptatic HepG2 cells. **A)** Cardiomyocytes were loaded with 5 μM BODIPY 493/503 to fluorescently label lipid macro droplets (green), nuclei are stained with DAPI (blue); (x3 replicates). Cells were fed a fatty acid mixture described in Methods and then incubated with vehicle or PDE9-I (PF-7493) for 24 hours prior to imaging. **B)** Result of PDE9a targeted siRNA versus scrambled (Scr) control in reducing PDE9a gene expression in human cultured adipocytes (left) and NRVM (right). **C)** Glycerol release at baseline (B, n=8), and after 4 hrs (n=8) or 24 hrs (n=24) ANP exposure in adipocytes. Analysis by KW test, P values DMCT shown. **D)** Lipolysis assessed by stimulated glycerol release in HepG2 cells or cardiomyocytes (NRVM) treated with ANP for 24 hours ±PDE9-I. PDE9-I (PF-7493, 5 μM) stimulated lipolysis in the presence of ANP in both cell types, but this was blunted by concomitant PPARα inhibition. N=8/group; Welch ANOVA with Dunnet's T3 multiple comparisons test results displayed.



Supplemental Figure S9. Impact of PDE9-I on mitochondrial volume density and myocardial lipid accumulation. Example EM images of myocardium from male DIO/mTAC mice treated with either therapy. Upper shows typical enlarged mitochondria with reduced density observed in the placebo group. (Replicated x 4 for each group).

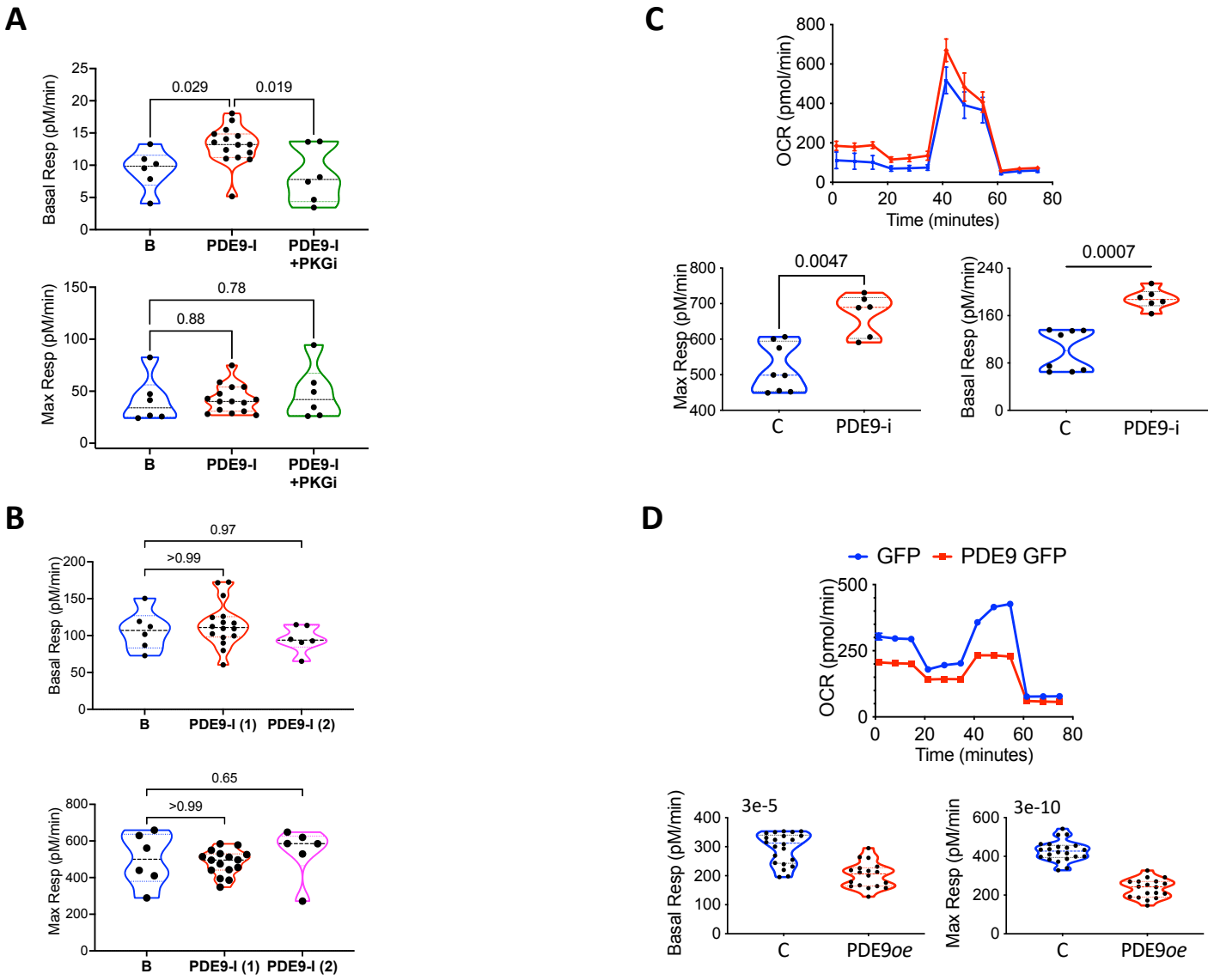

A
Basal Resp (pM/min)
0.029
0.019
B
PDE9-I
PDE9-I +PKGi
Max Resp (pM/min)
0.78
0.88
B
0.97
>0.99
PDE9-I (1)
PDE9-I (2)
0.65
>0.99
C
OCR (pmol/min)
Time (minutes)
Max Resp (pM/min
0.0047
C
PDE9-i
Basal Resp (pM/min)
0.0007
D
GFP
PDE9 GFP
3e-5
PDE9oe
3e-10

Figure S10

Supplemental Figure S10. Acute PDE9-I has minimal impact on mitochondrial basal or maximal respiration, and chronic PDE9-I (24 hrs) stimulates while PDE9a overexpression depresses both in cardiomyocytes. **A)** Basal and maximal O_2 consumption in human adipocytes at baseline (B, n=6) and after 1-hr PDE9-I exposure without (n=14) or with (n=6) concomitant PKG inhibition (PKG-I, DT3, 1 μM). Data analyzed by KW test, with DMCT P values displayed. **B)** Similar experiment and analysis in cardiomyocytes, with two different PDE9-I: (1) PF-7943, (2) BAY 73-6691, both 5 μM for 1 hour. **C)** Mitochondrial respiration in cardiomyocytes exposed to 24 hours PDE9-I (PF-7943, 5 μM). Upper panel shows time course of protocol (c.f. Figure 5d for adipocytes), mean ±SD, n=8 control, n=6 PDE9-I. Lower panels show summary data for basal and maximal O_2 consumption, same sample size, KW test P values displayed. **D)** Oxygen consumption in myocytes pre-infected with AdV expressing *Pde9a-Gfp* or *Gfp* for 48 hrs (n=18, 24, 22 respectively; KW, DMCT p-values displayed)

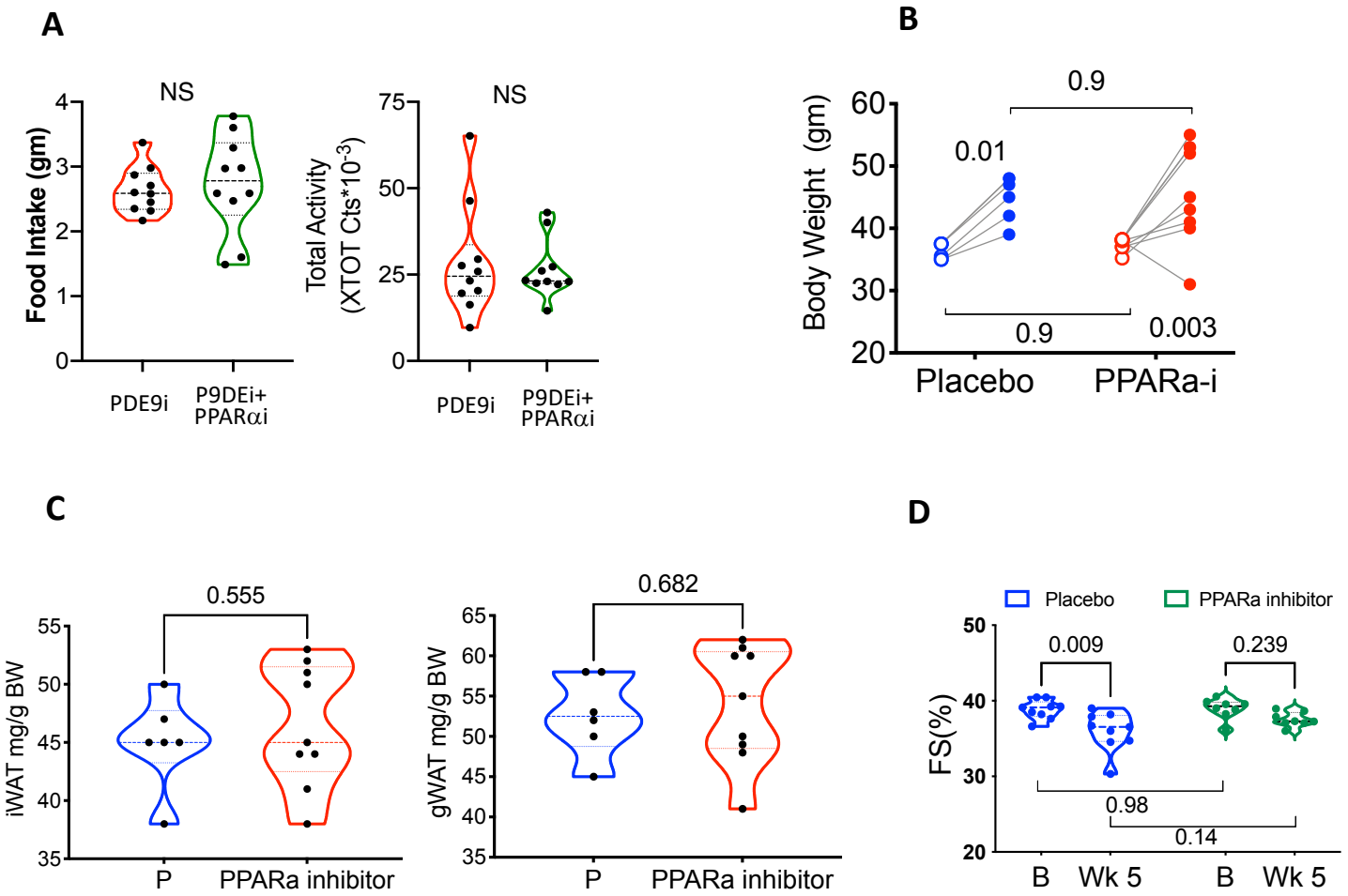

A
NS
Food Intake (gm)
4
3
2
1
0
PDE9i
P9DEi+ PPARαi
NS
Total Activity (XTOT Cts*10-3)
75
50
25
0
PDE9i
P9DEi+ PPARαi
B
0.9
0.01
Body Weight (gm)
60
50
40
30
20
0.9
0.003
Placebo
PPARa-i
C
0.555
iWAT mg/g BW
55
50
45
40
35
P
PPARa inhibitor
0.682
gWAT mg/g BW
65
60
55
50
45
40
35
P
PPARa inhibitor
D
Placebo
PPARa inhibitor
FS(%)
50
40
30
20
0.009
0.239
0.98
0.14
B
Wk 5
B
Wk 5

Figure S11

Supplemental Figure S11. Effect of PPARa inhibition on food intake or activity in OVX DIO/mTAC mice; and on weight, fat mass, and left ventricular function in OVX DIO mice. **A)** Food intake and activity OVX obese/CMS mice treated with either PDE9-I alone or in combination with PPARα-I had no significant differences in daily activity or food intake. **B)** Total body weight of female OVX DIO mice without mTAC treated with placebo of PPARα inhibitor (GW 6471). PPARα inhibition did not significantly change the magnitude of weight gain found in both groups (P=0.96 for interaction term) over the 6-week period. 2WANOVA, N=5 placebo; 9 PPARα–I, Sidak's multiple comparison test P values displayed for various pairs. **C)** Fat mass of iWAT and gWAT in same study testing effect of PPARα-I alone. There was no significant difference in mass between groups. N=6 placebo, 9 PPARα-I; results of MW test shown. **D)** LV percent fractional shortening (%FS) data from same experiment. There was a slight though significant decline in %FS in the placebo but not PPARα-I group. Same statistical analysis used as in panel b, and P values from Sidak's MCT shown.

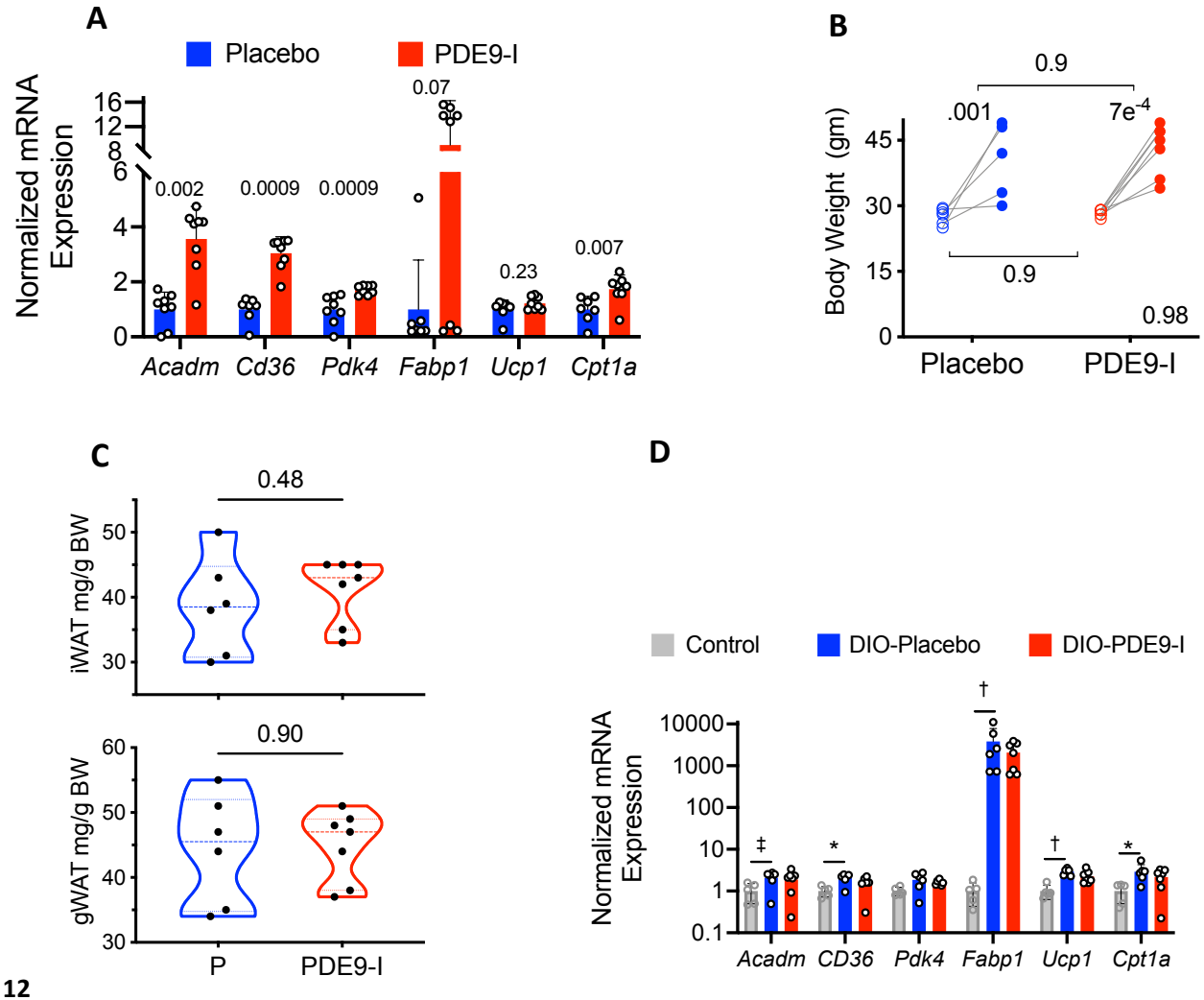

A
Placebo
PDE9-I
Normalized mRNA Expression
16
12
8
6
4
2
0
0.002
0.0009
0.0009
0.07
0.23
0.007
Acadm
Cd36
Pdk4
Fabp1
Ucp1
Cpt1a
B
0.9
.001
7e-4
0.9
0.98
Body Weight (gm)
45
30
15
0
Placebo
PDE9-I
C
0.48
iWAT mg/g BW
50
40
30
0.90
gWAT mg/g BW
60
50
40
30
P
PDE9-I
D
Control
DIO-Placebo
DIO-PDE9-I
Normalized mRNA Expression
10000
1000
100
10
1
0.1
‡
*
†
†
*
Acadm
CD36
Pdk4
Fabp1
Ucp1
Cpt1a

Figure 12

Supplemental Figure S12. Effect of PDE9I on PPARa-associated mRNA abundance in BAT from male mice with DIO but without mTAC; and on weight, fat mass, and BAT expression of similar genes in non-OVX female mice with DIO and no mTAC. A) Expression shown relative to placebo treated group (N=8/group, analysis by 2S-BKY-MW, Q values displayed. **B)** Body weight before and after 6-week treatment with placebo or PDE9-I in non-OVX DIO mice (no mTAC) shows similar weight gain. 2WANOVA, SMCT P values displayed. **C)** Weight of gWAT and iWAT are not significantly different between same treatment groups. KW P values shown. **D)** Expression of PPARa-associated genes in non-OVX DIO without mTAC with placebo (n=6) versus PDE9-I (n=7), and non-obese controls (n=5). Data plot on log scale. While most of the genes have increased expression with DIO, there is no further change from PDE9-I. Statistical analysis as in panel **A**, Q values for control vs DIO comparison are displayed. For placebo vs PDE9-I all Q values were >0.41.

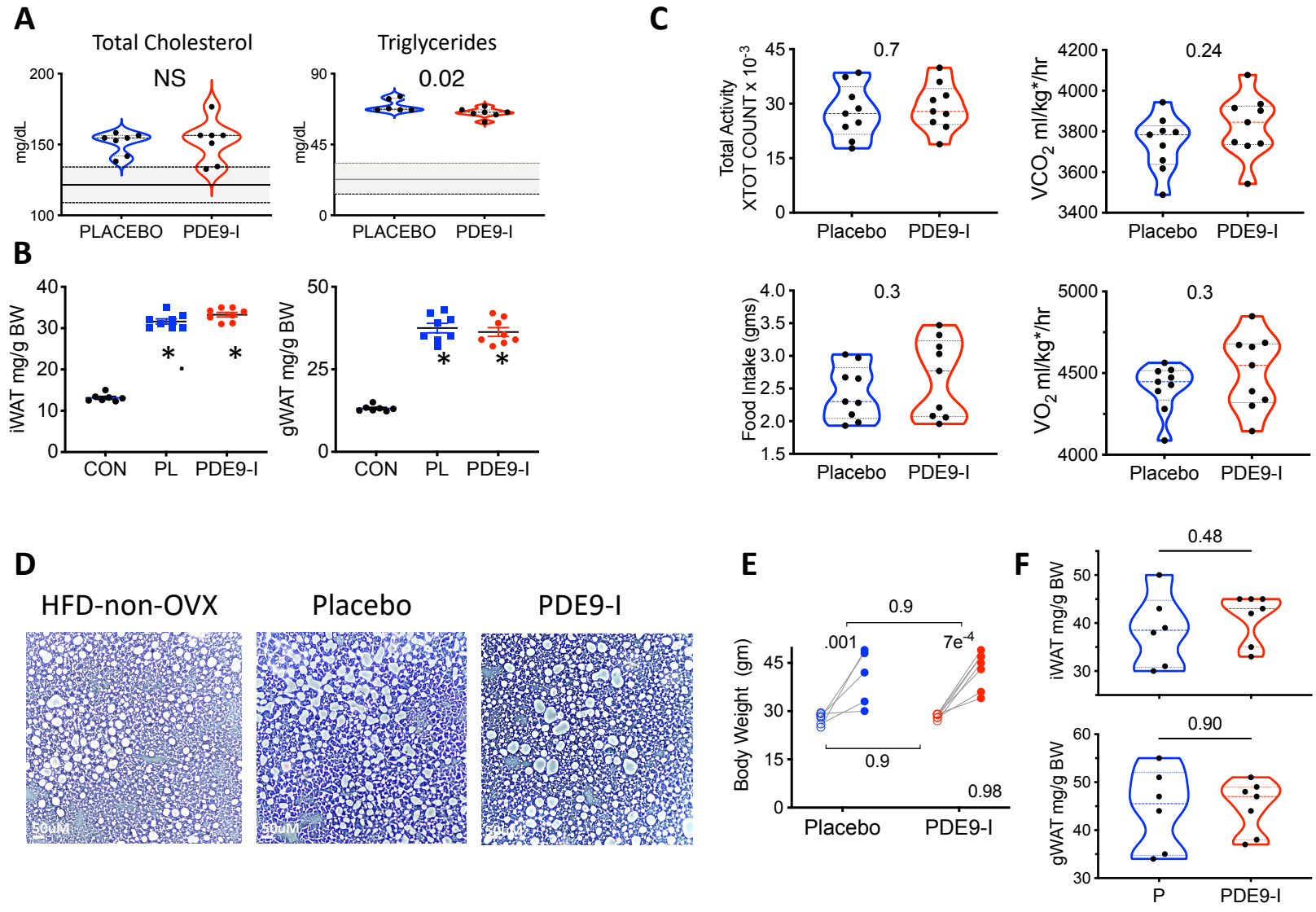

A
Total Cholesterol
NS
mg/dL
200
150
100
PLACEBO
PDE9-I
Triglycerides
0.02
90
45
0
B
iWAT mg/g BW
40
30
20
10
0
CON
PL
PDE9-I
gWAT mg/g BW
50
25
0
C
Total Activity XTOT COUNT x 10-3
0.7
45
30
15
Placebo
PDE9-I
VCO2 ml/kg*/hr
0.24
4200
4000
3800
3600
3400
Food Intake (gms)
0.3
4.0
3.5
3.0
2.5
2.0
1.5
D
HFD-non-OVX
Placebo
PDE9-I
50uM
E
Body Weight (gm)
0.9
.001
7e-4
0.9
0.98
F
iWAT mg/g BW
0.48
gWAT mg/g BW
0.90
P

Figure S13

Supplemental Figure S13. Non-OVX females display no significant changes in serum lipids, iWAT or gWAT weight, food intake, activity, whole body metabolism, or hepatic lipid accumulation in response to PDE9-I. A) Non-OVX mice with DIO/mTAC model treated with placebo or PDE9-I for 8 weeks show similar plasma lipids levels. N=7/group; MW P-values shown. **B)** Both iWAT or gWAT mass increase similarly in placebo (PL, n=8) and PDE9-I (n=8) treated non-OVX over lean-controls (n=7). **C)** Activity, food intake, VO_2, and VCO_2 (latter normalized to lean + 0.2x fat mass) are similar between placebo and PDE9-I treatment (n=9/group), MW test. **D) L**ipid accumulation in the liver is similar in non-OVX in either group. Replicated n=6/group. **e)** DIO obesity without mTAC in non-OVX exhibits similar body weight changes and iWAT and gWAT mass in placebo (P) and PDE9-I groups. For weight analysis, 2WANOVA, P value to lower right is interaction term, others values for paired comparisons by SMCT.

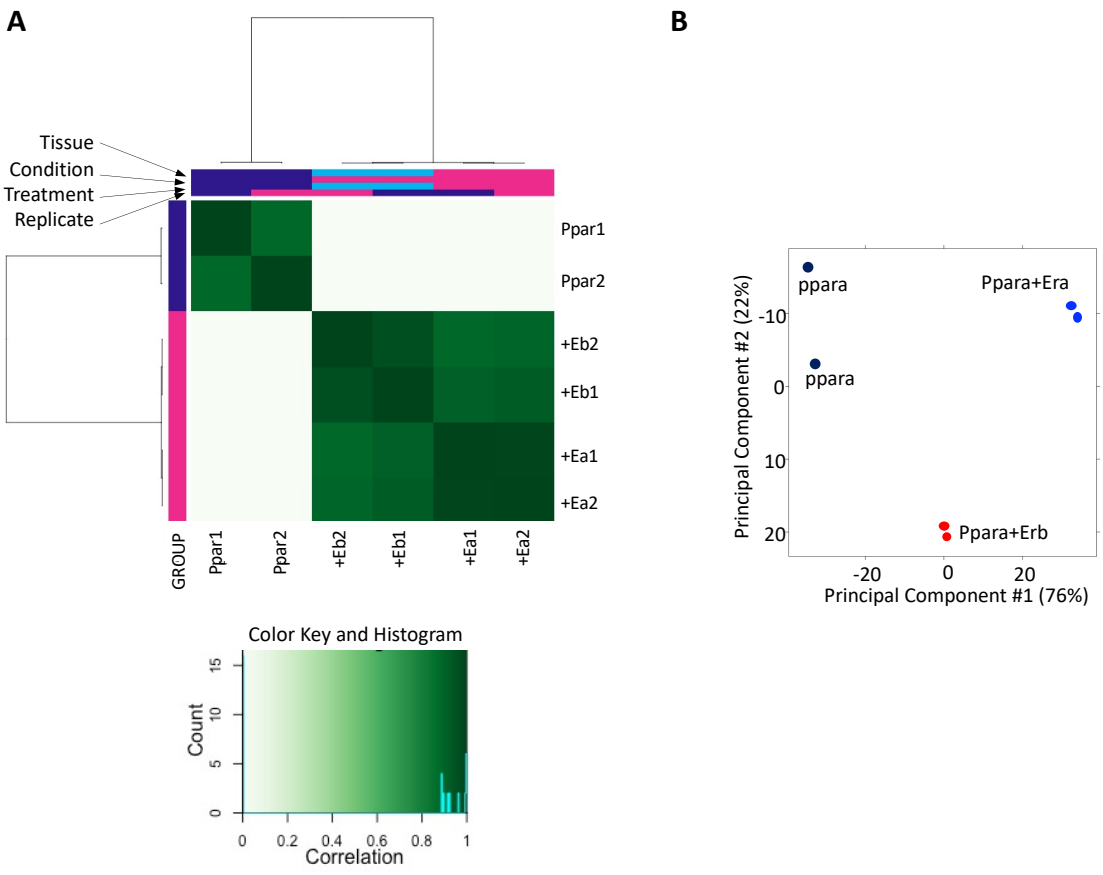
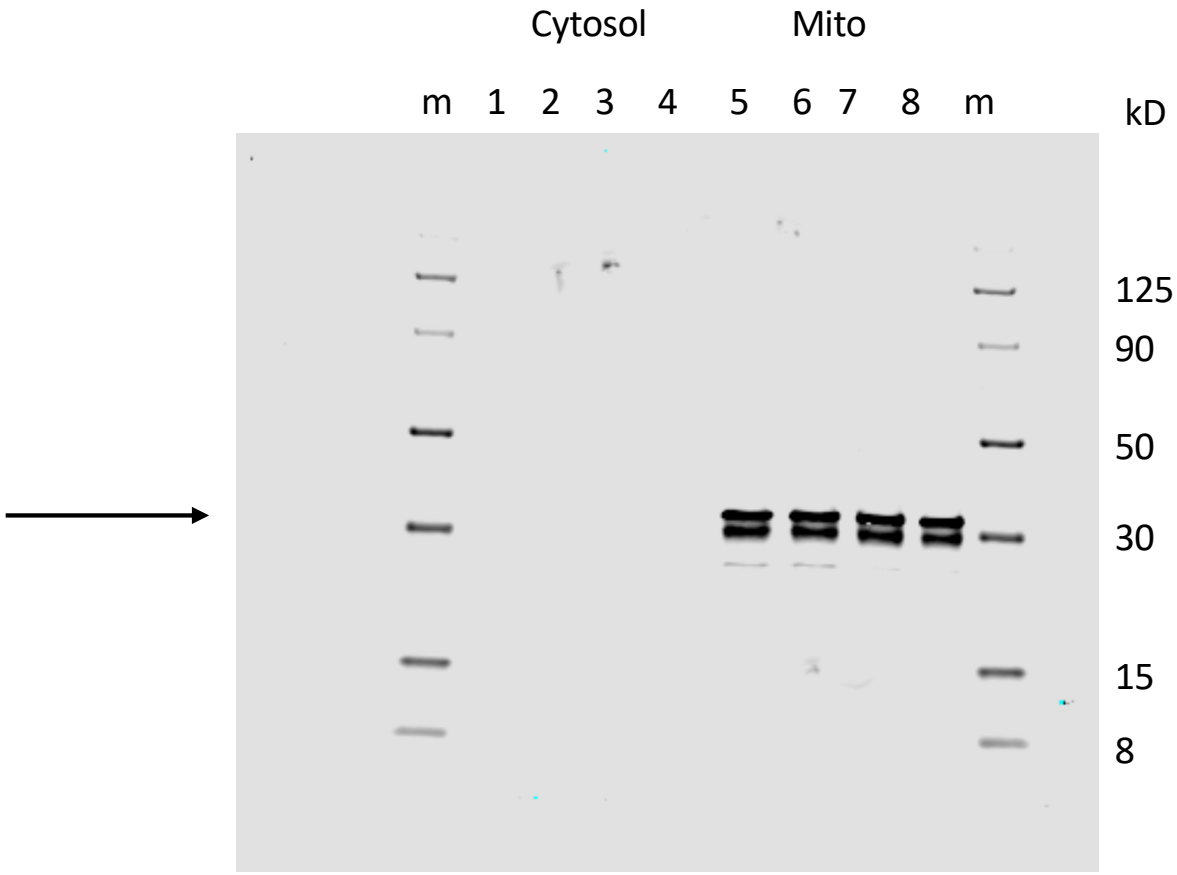


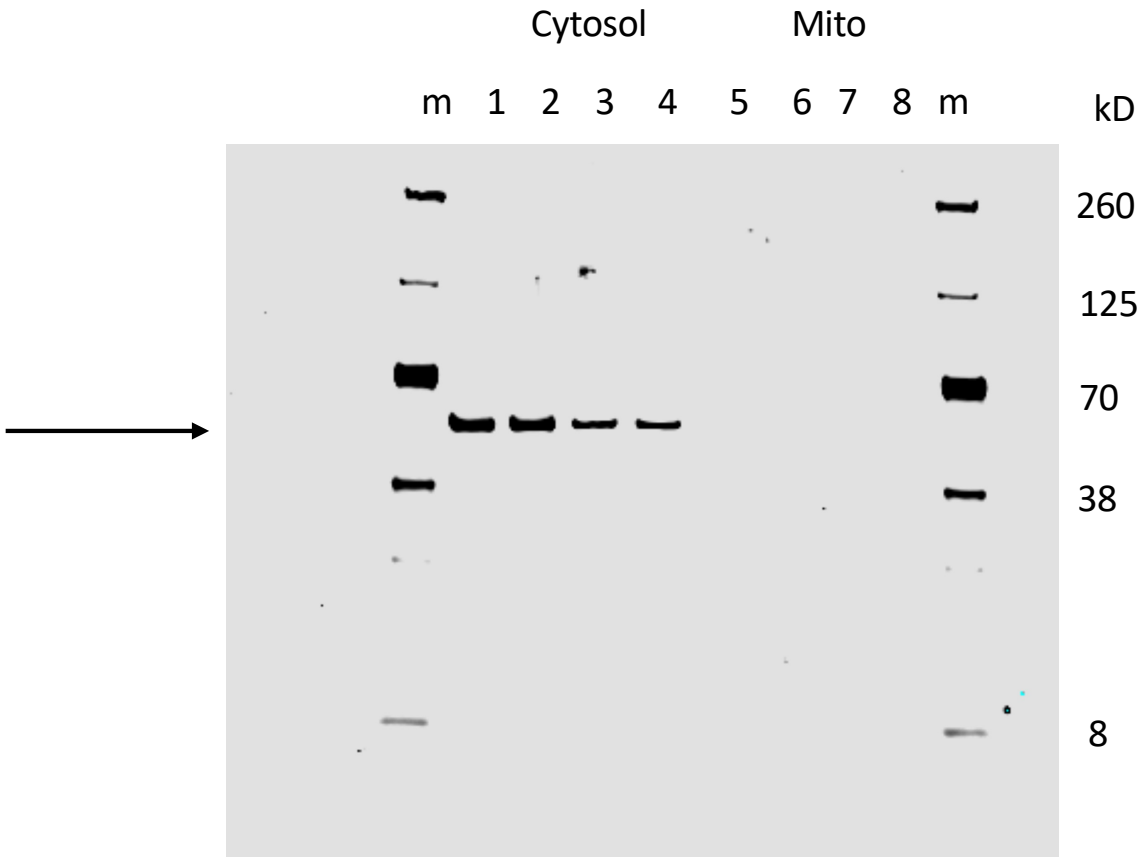
Figure S14

Supplemental Figure S14. A) Non-negative matrix factorization analysis of genes with PPARa chromatin binding identified by ChIPSeq displays strong internal correlation of each experimental run within each experimental group (PPARα alone, PPARα+ETα, PPARα+ERβ). The latter cluster more closely together but quite differently from PPARα alone. **B)** PCA analysis of same gene sets also shows consistency between experiments and marked separation between the three groups.

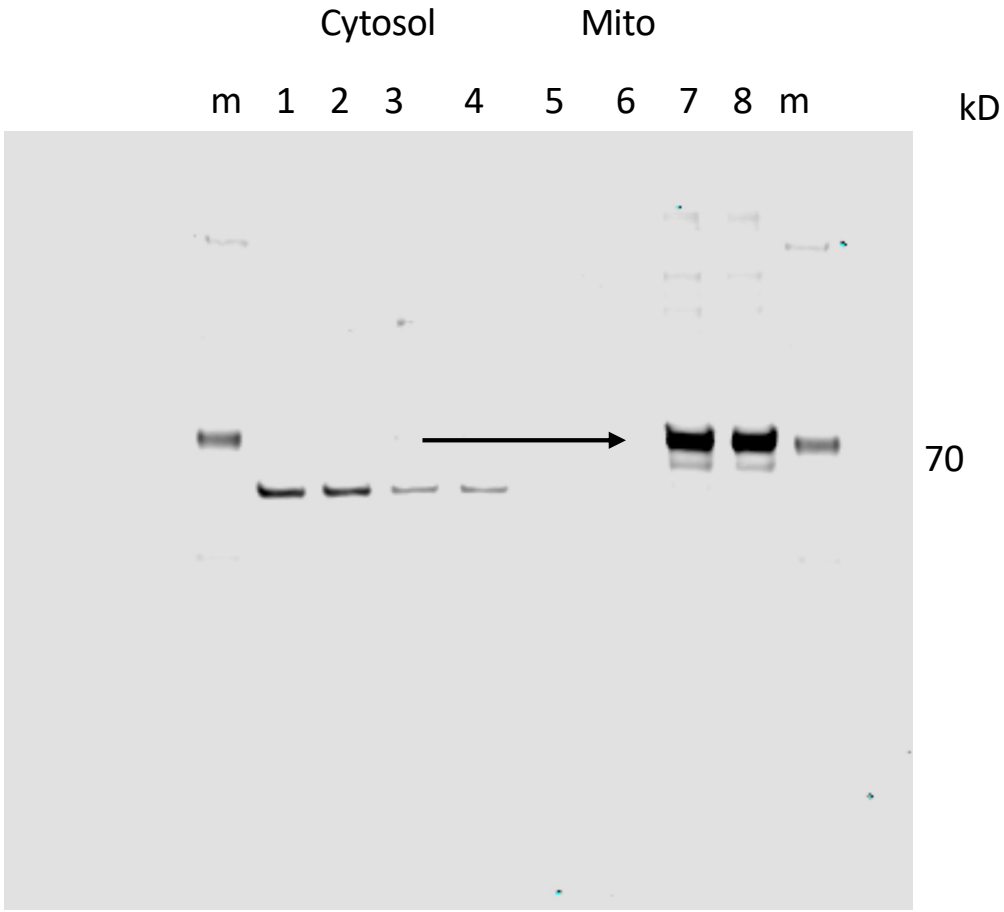
VDAC – Raw Gels



Alpha Tubulin – Raw Gels



FLAG – Raw Gel



Total Protein Stain – Raw Gels