Supporting Information

Pharmacological Antagonism of RAGE Signaling Promotes Thermogenesis & Healthful Body Mass & Composition & Metabolism in Mice

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Supplementary Figures and Figure Legends

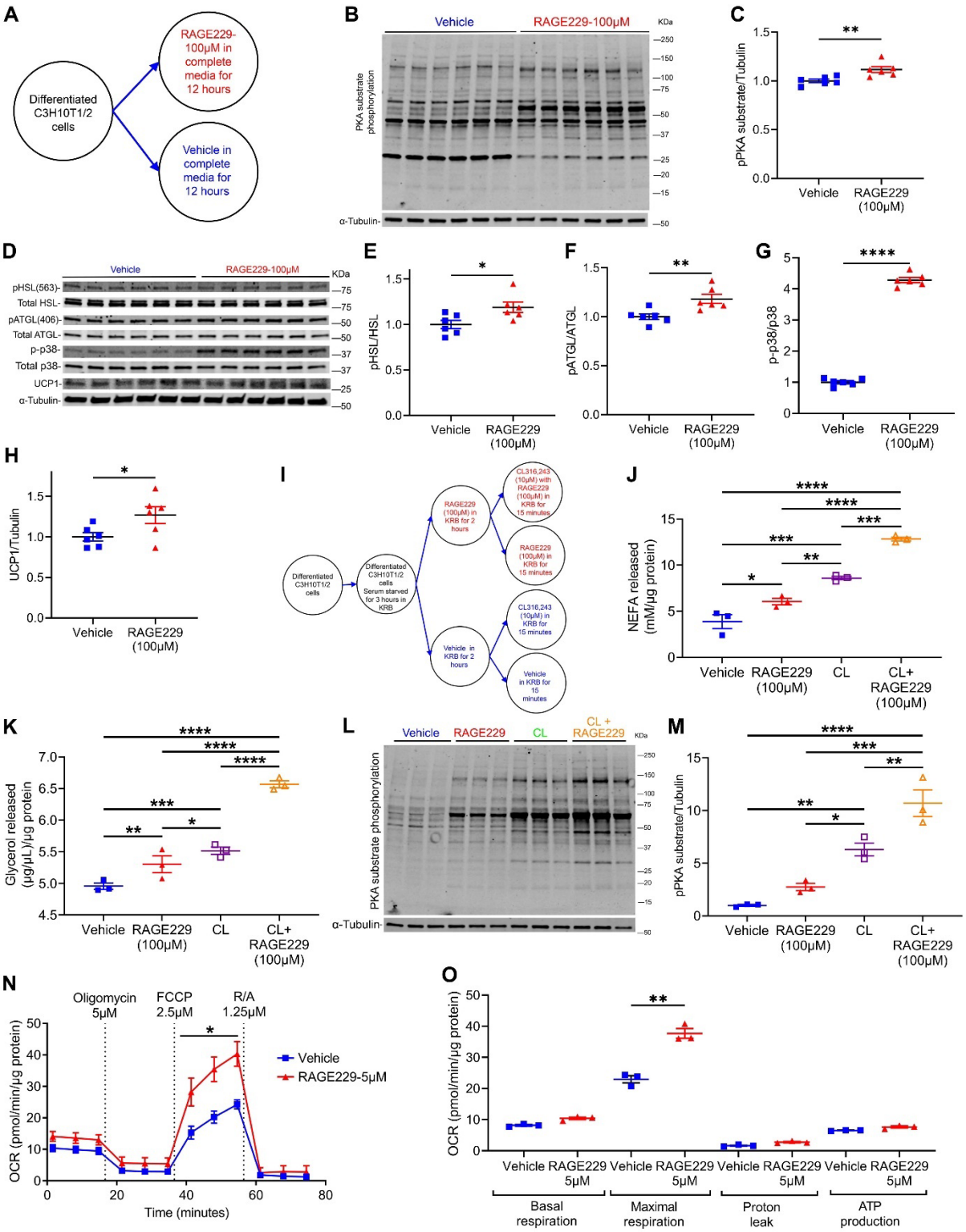


Figure S1. Treatment of mouse adipocyte-like C3H10T1/2 cells with RAGE229 increases thermogenic capacity, β3-adrenergic-stimulated lipolysis and mitochondrial function. (A) experimental schematic- differentiated adipocytes treated with RAGE229 (100 μM) or Vehicle

(DMSO) in complete media for 12 h (N=6 per treatment group, data presented from one of the two independent experiments, each performed with N=6 per group), (B-H) Western blot analysis of cell lysate probed for expression of PKA substrate phosphorylation/α-Tubulin, phosphorylated HSL-serine563/total HSL, phosphorylated ATGL-serine406/total ATGL, phosphorylated p38/total p38 MAP kinase and UCP1/α-Tubulin, (I) experimental schematic-differentiated adipocytes were serum-starved for 3 h in KRB with BSA before treatment with either RAGE229 (100μM) or vehicle for 2 h followed by CL316,243 (10μM) treatment for 15 mins (N=3 independent wells of the tissue culture plate per treatment group, data presented from one of the two independent experiments, each performed with N=3 independent wells per group), (J-K) NEFA and glycerol released into KRB after CL316,243 stimulation, (L-M) Western blot of cell lysate probed for expression of PKA substrate phosphorylation/α-Tubulin after CL316,243 stimulation, (N-O) Oxygen consumption rate (OCR) measured in adipocytes pre-treated with vehicle vs. RAGE229-5μM for 2 h (N=3 independent wells of the tissue culture plate per treatment group, data presented from one of the 3 independent experiments, each performed with N=3 independent wells per group). Data are presented as the Mean ± SEM. For group comparisons, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Wilcoxon rank sum test was used if normality was not established. Linear mixed-effect model was adopted for correlated/time-series data with post-hoc t-test for group comparisons at each time point. For J,K&M, 2-way ANOVA followed by multiple comparison without correction was used to compare groups. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

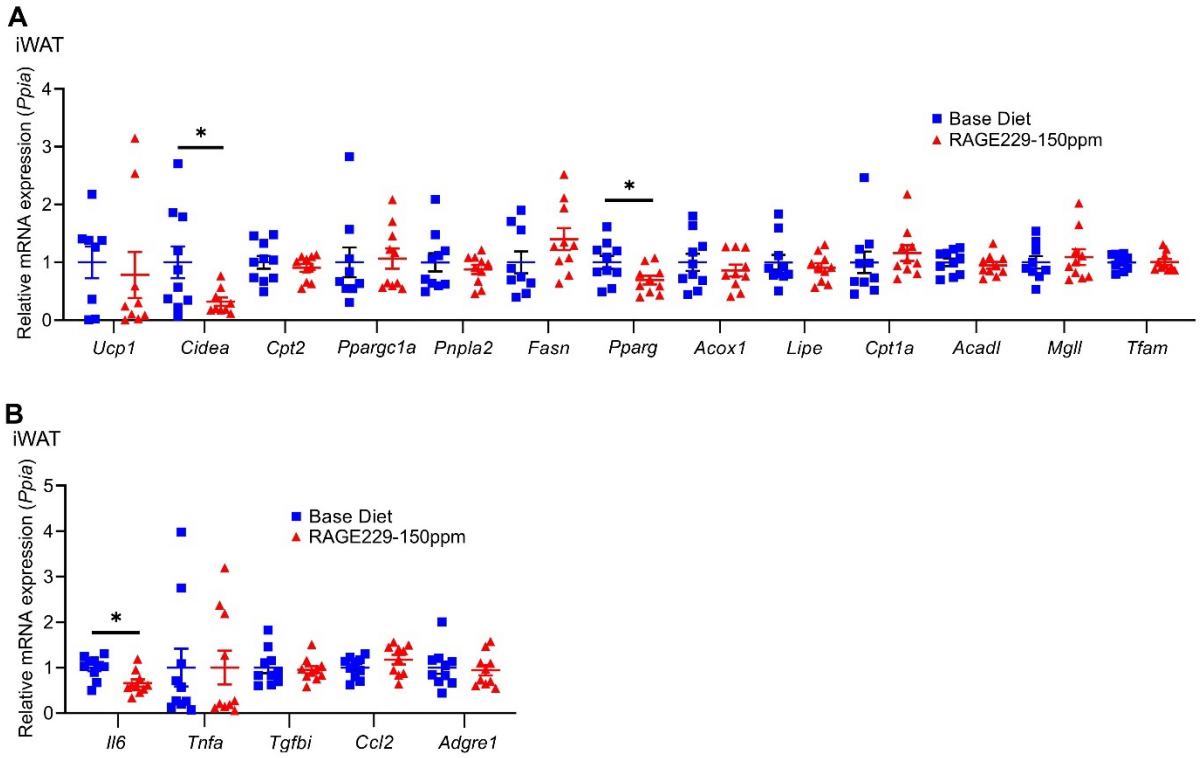


Figure S2. Pair-feeding lean male mice with RAGE229-150 ppm diet and effects on iWAT gene expression. (A) Relative expression of the indicated metabolic and thermogenic genes in iWAT (N=8-10 Base diet, N=9-10 RAGE229-150 ppm diet) (B) Relative expression of the indicated inflammation genes in iWAT (N=10 per group). The iWAT was collected from randomly fed mice after three d pair-feeding. Data are presented as the Mean ± SEM. For group comparison, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Wilcoxon rank sum test was used if normality was not established. *P<0.05.

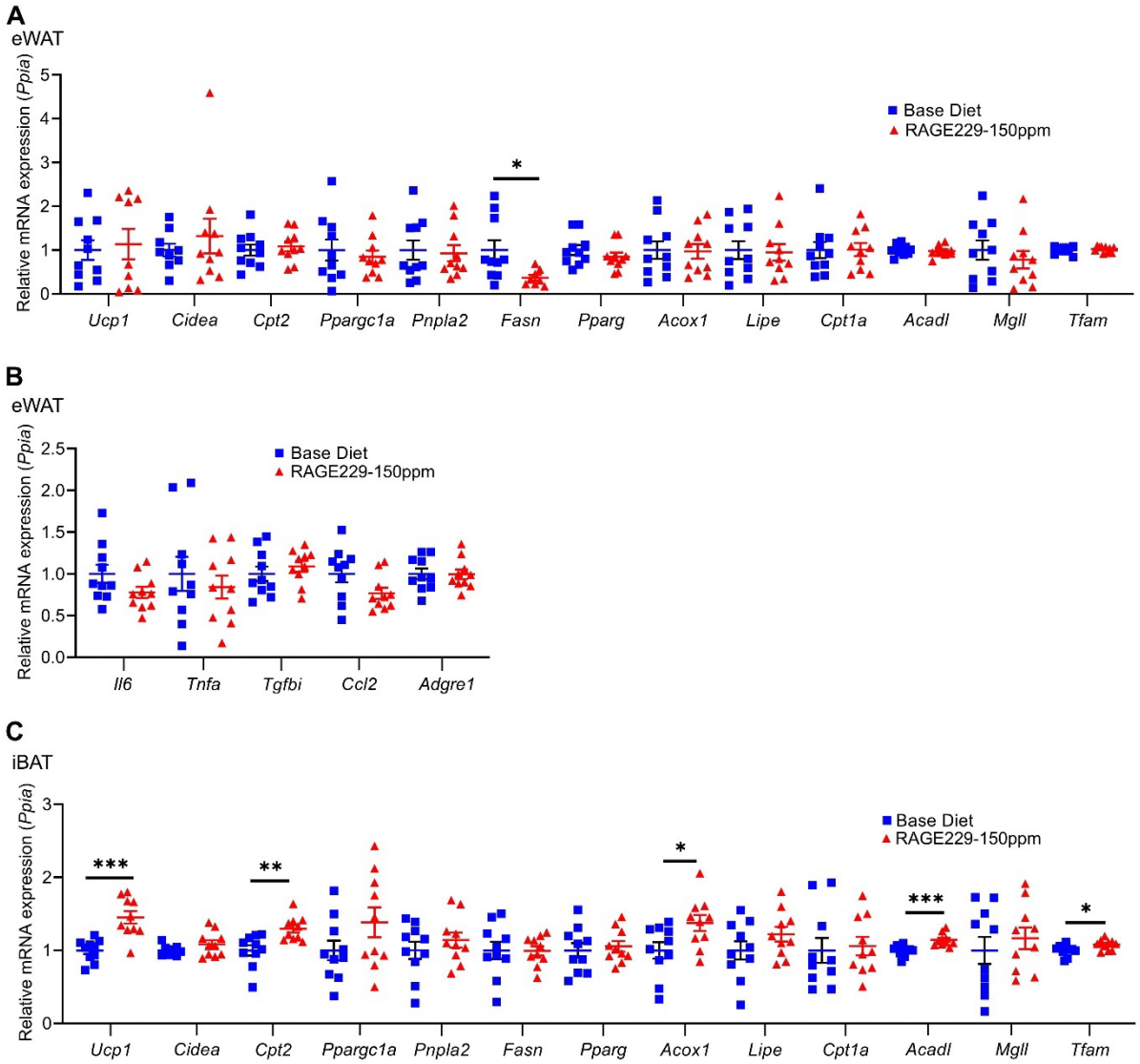
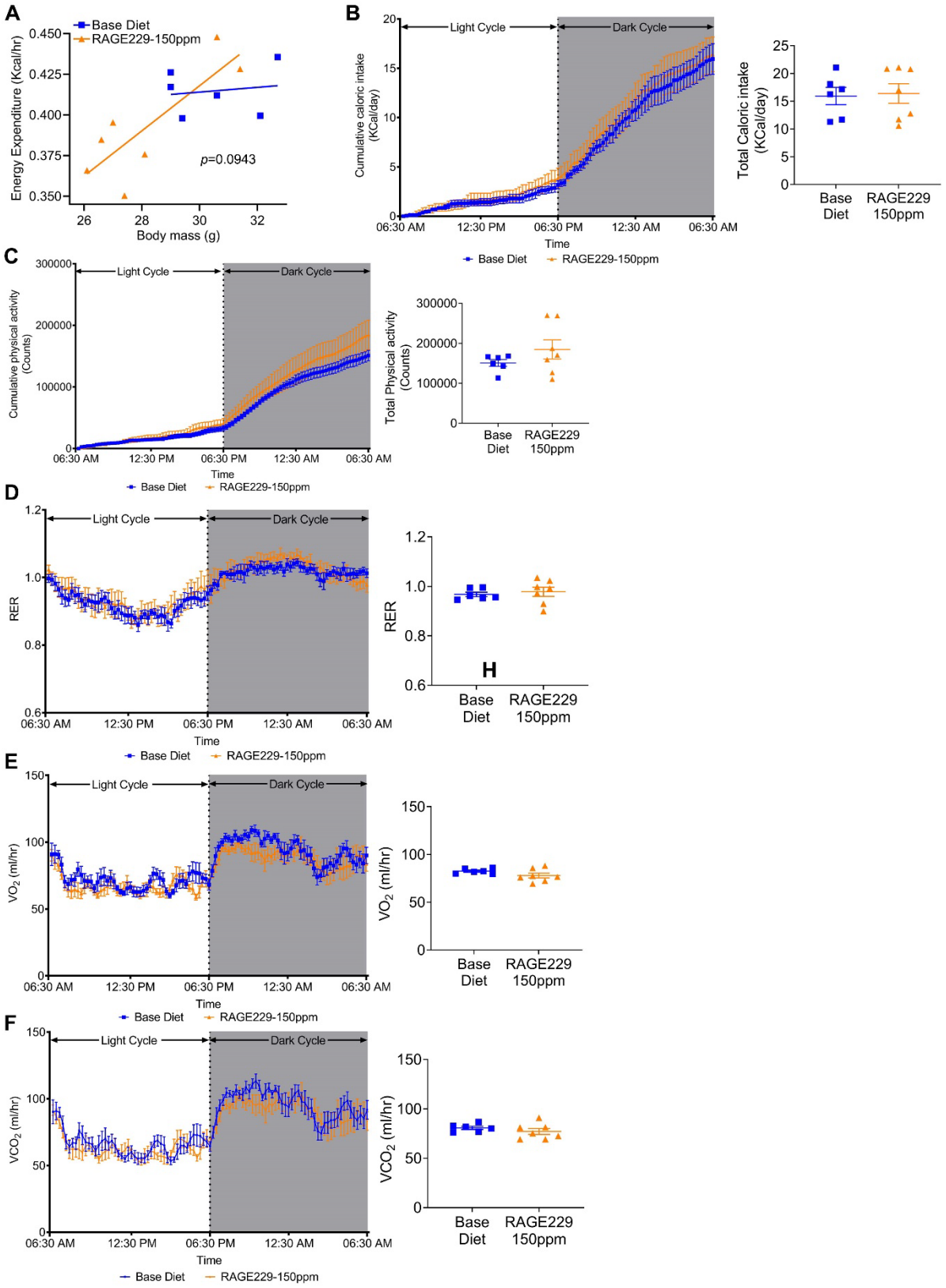


Figure S3. Pair-feeding lean male mice with RAGE229-150 ppm diet results in higher PKA-dependent lipolytic and thermogenic markers in eWAT and iBAT depots compared with Base diet. (A) Relative expression of the indicated metabolic and thermogenic genes in eWAT (N=9-10 Base diet, N=8-10 RAGE229-150 ppm diet), (B) Relative expression of the indicated inflammation genes in eWAT, (N=10 per group), (C) relative expression of the indicated metabolic and thermogenic genes in iBAT (N=8-10 Base diet, N=10 RAGE229-150 ppm diet). Tissues were collected from randomly fed mice after three d of pair-feeding. Data are presented as the Mean ± SEM. For group comparison, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Wilcoxon rank sum test was used if normality was not established. *P<0.05, **P<0.01, ***P<0.001.


A
Base Diet
RAGE229-150ppm
Energy Expenditure (Kcal/hr)
p=0.0943
Body mass (g)
B
Light Cycle
Dark Cycle
Cumulative caloric intake (KCal/day)
Time
Base Diet
RAGE229-150ppm
Total Caloric intake (KCal/day)
Base Diet
RAGE229 150ppm
C
Cumulative physical activity (Counts)
Total Physical activity (Counts)
D
RER
H
E
VO2 (ml/hr)
F
VCO2 (ml/hr)
06:30 AM
12:30 PM
06:30 PM
12:30 AM

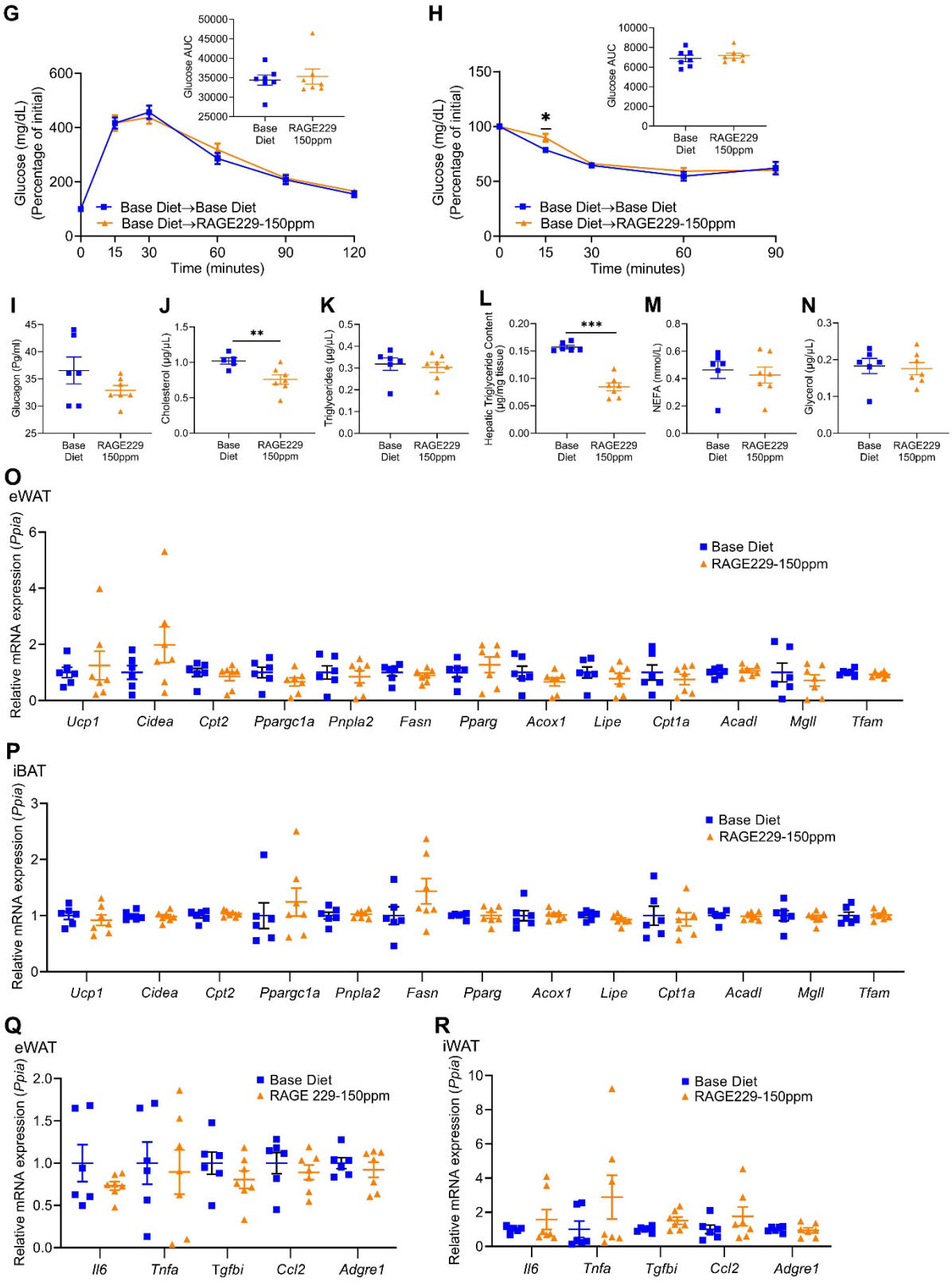


Figure S4. Administration of RAGE229-150 ppm diet to lean male mice causes loss of body and fat mass and improves metabolic health. (A) Average EE per h vs. body mass data subjected to analysis of covariance (ANCOVA); see Table S1 for additional analyses, (B-F) caloric intake over 24 h and total caloric intake per d; physical activity over 24 h and total

physical activity per d; RER, VO_2 consumption and VCO_2 production measured over 24 h and average per d measured at 10 weeks post-diet switch (N=6 Base diet, N=7 RAGE229-150 ppm diet), (G) post-overnight fasting glucose tolerance test shown as percent change from baseline blood glucose at 6 weeks post-diet switch (N=7 per group), (H) post-6 h fasting insulin tolerance test shown as percent change from baseline blood glucose at 7 weeks post-diet switch (N=7 per group), (I-N) post-overnight fasting plasma glucagon, cholesterol, triglycerides, hepatic triglycerides, plasma NEFA and plasma glycerol measured at 12 weeks post-diet switch (N=5-6 Base diet, N=7 RAGE229-150 ppm diet), (O-P) relative expression of the indicated metabolic and thermogenic genes in eWAT and iBAT (N=6 Base diet, N=7 RAGE229-150 ppm diet) at 6 weeks post-diet switch, (Q-R) relative expression of the indicated inflammation genes in eWAT and iWAT (N=6 Base diet, N=7 RAGE229-150 ppm diet) at 6 weeks post-diet switch. Tissues for gene expression analysis were collected from randomly-fed mice. Data are presented as the Mean ± SEM. Linear mixed-effect model was adopted for correlated/time-series data with post-hoc t-test for group comparisons at each time point. For group comparison, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Kruskal Wallis test/Wilcoxon rank sum test was used if normality was not established. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

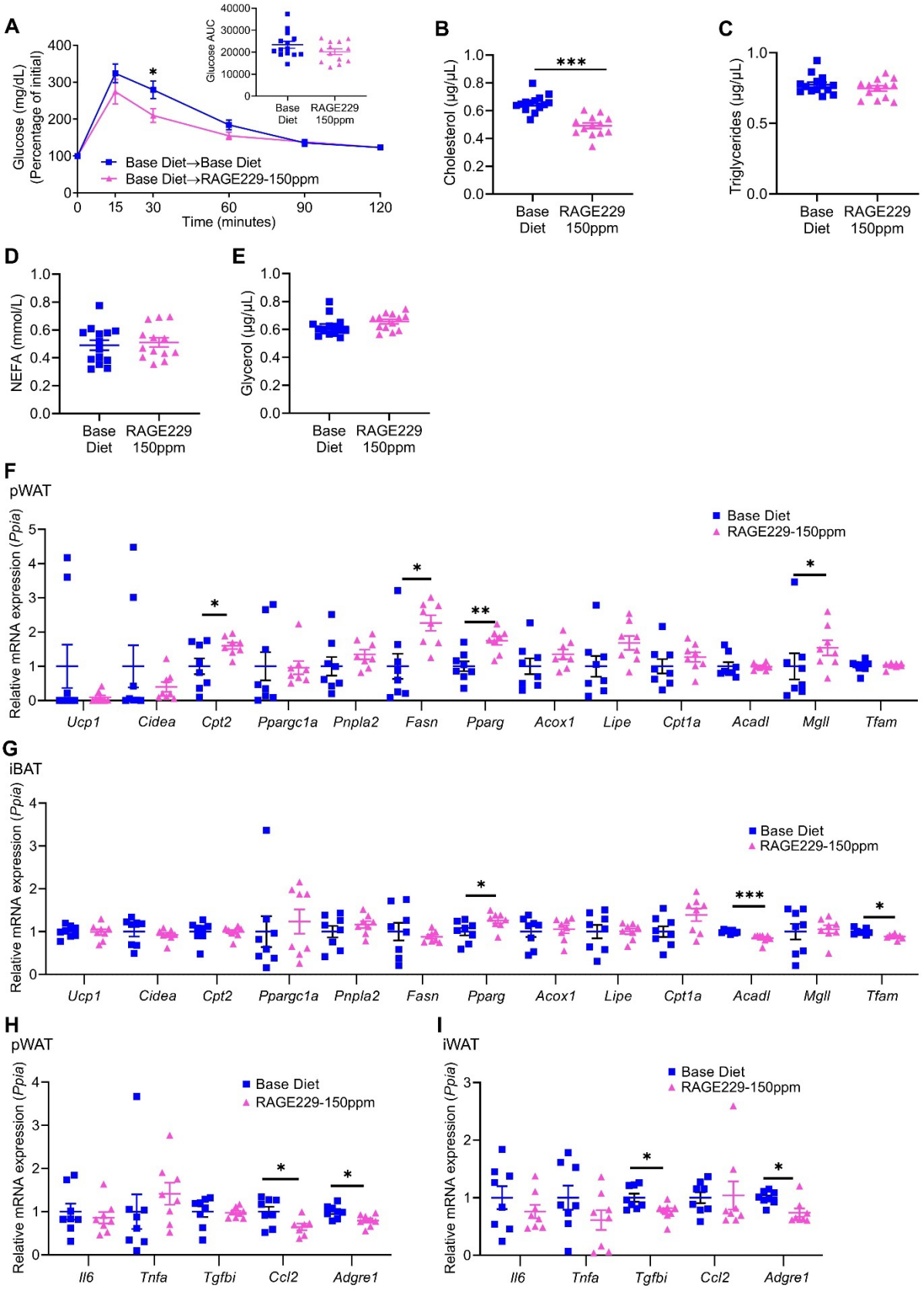
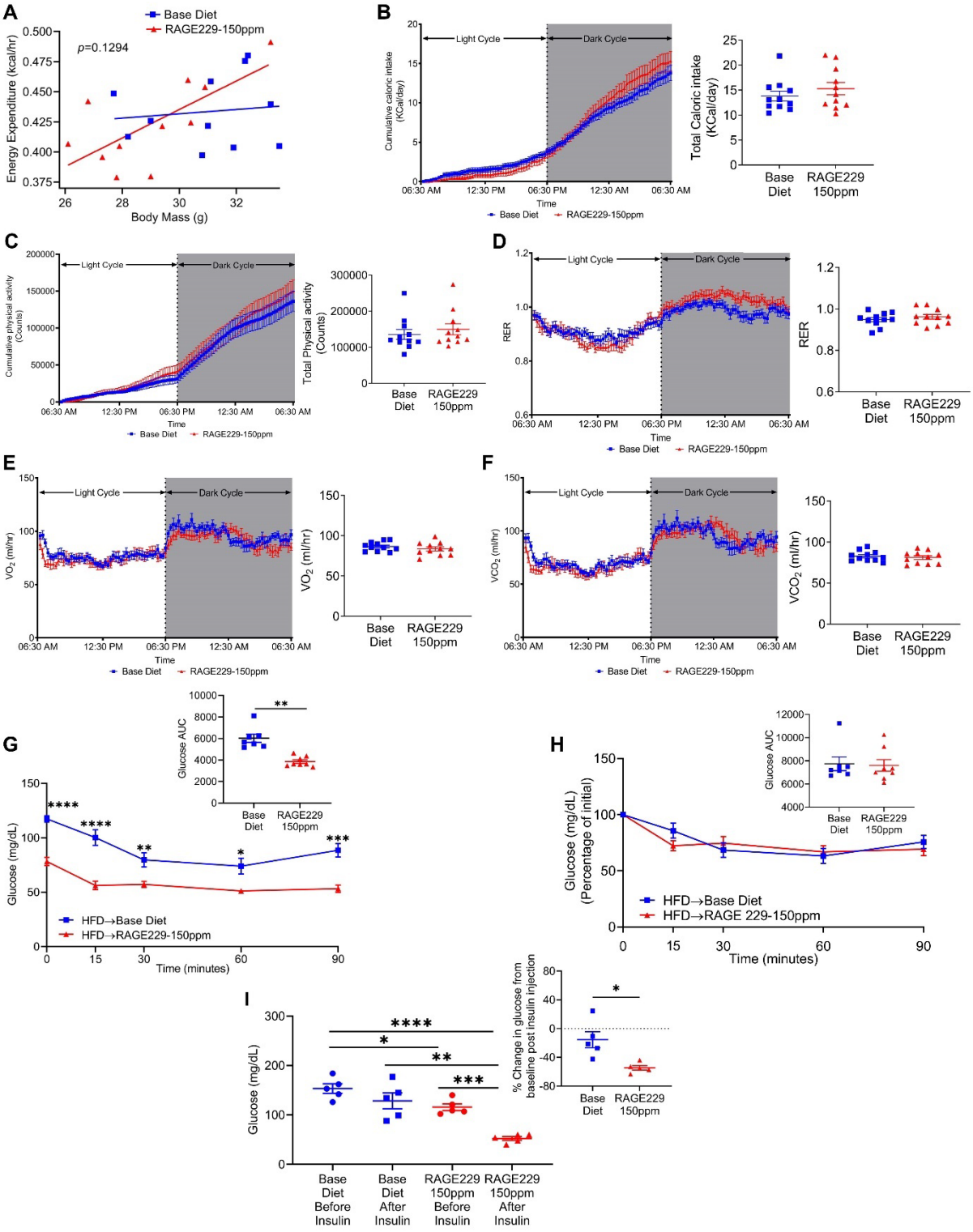
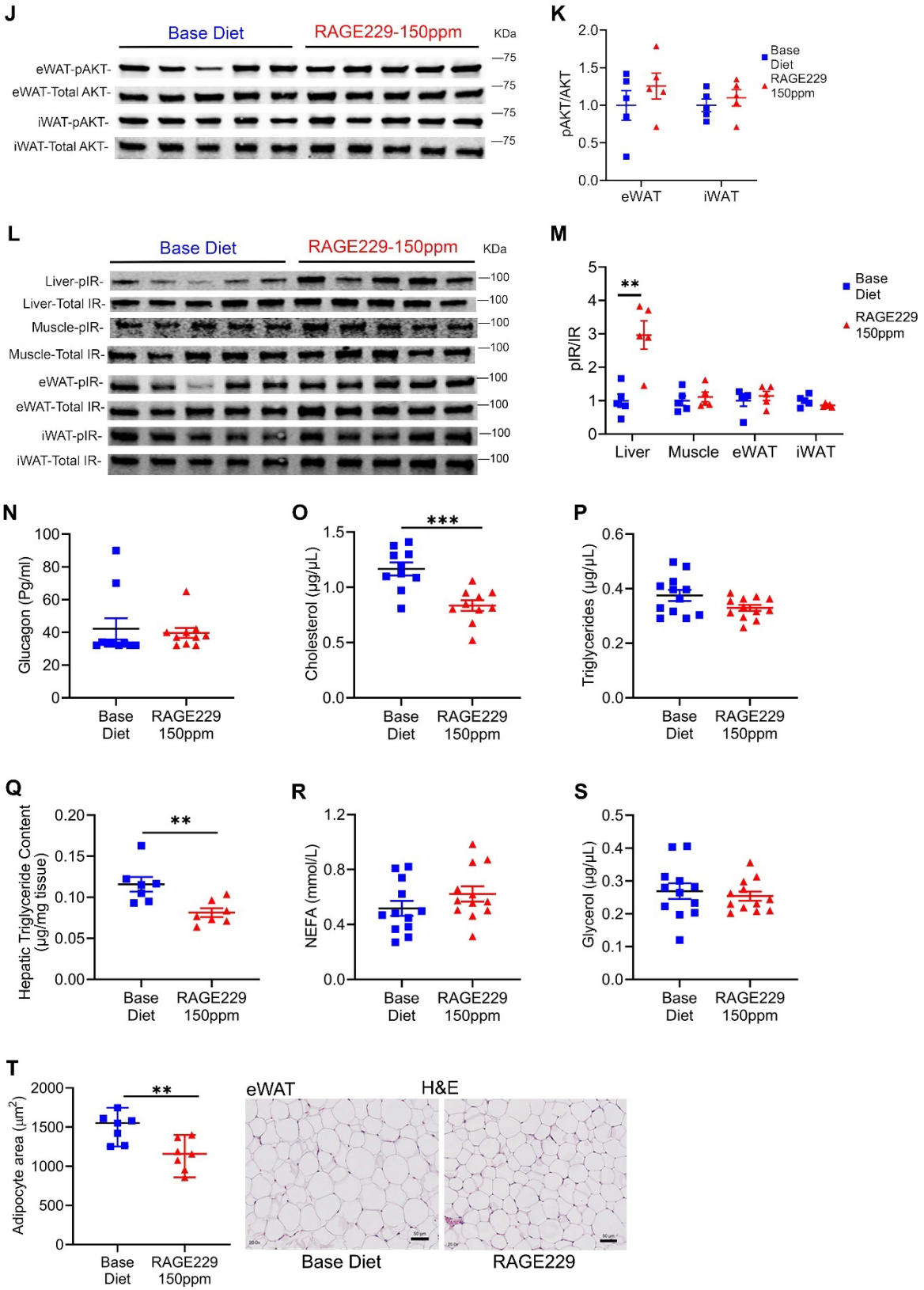


Figure S5. The effects of RAGE229-150 ppm diet on body and fat mass and metabolic benefits are sex-independent: studies in female mice. (A) post-6 h fasting glucose tolerance test shown as percent change from baseline blood glucose at 6 weeks post-diet switch (N=14 Base diet, N=13 RAGE229-150 ppm diet), (B-E) Post-6 h fasting plasma cholesterol,

triglycerides, plasma NEFA and plasma glycerol at 8 weeks post-diet switch (N=14 Base diet, N=13 RAGE229-150 ppm diet), (F-G) relative expression of indicated metabolic and thermogenic genes in pWAT and iBAT (N=8 per group) at 8 weeks post-diet switch, (H-I) relative expression of the indicated inflammation genes in pWAT and iWAT (N=8 Base diet, N=7-8 RAGE229-150 ppm diet) at 8 weeks post-diet switch. Data are presented as the Mean ± SEM. Linear mixed-effect model was adopted for correlated/time-series data with post-hoc t-test for group comparisons at each time point. For group comparison, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Kruskal Wallis test/Wilcoxon rank sum test was used if normality was not established. $*P<0.05$ $**P<0.01$, $***P<0.001$.


A
Base Diet
RAGE229-150ppm
p=0.1294
Energy Expenditure (kcal/hr)
Body Mass (g)
B
Light Cycle
Dark Cycle
Cumulative caloric intake (KCal/day)
Time
Total Caloric intake (KCal/day)
Base Diet
RAGE229 150ppm
C
Cumulative physical activity (Counts)
Total Physical activity (Counts)
D
RER
E
VO2 (ml/hr)
F
VCO2 (ml/hr)
G
Glucose AUC
Glucose (mg/dL)
HFD→Base Diet
HFD→RAGE229-150ppm
Time (minutes)
H
Glucose (Percentage of initial)
HFD→RAGE 229-150ppm
I
Base Diet Before Insulin
Base Diet After Insulin
RAGE229 150ppm Before Insulin
RAGE229 150ppm After Insulin
% Change in glucose from baseline post insulin injection


J
Base Diet
RAGE229-150ppm
KDa
eWAT-pAKT-
eWAT-Total AKT-
iWAT-pAKT-
iWAT-Total AKT-
75
K
pAKT/AKT
Base Diet
RAGE229 150ppm
eWAT
iWAT
L
Liver-pIR-
Liver-Total IR-
Muscle-pIR-
Muscle-Total IR-
eWAT-pIR-
eWAT-Total IR-
iWAT-pIR-
iWAT-Total IR-
100
M
pIR/IR
**
Liver
Muscle
eWAT
iWAT
N
Glucagon (Pg/ml)
O
Cholesterol (µg/µL)
***
P
Triglycerides (µg/µL)
Q
Hepatic Triglyceride Content (µg/mg tissue)
R
NEFA (mmol/L)
S
Glycerol (µg/µL)
T
Adipocyte area (µm²)
eWAT
H&E
Base Diet
RAGE229

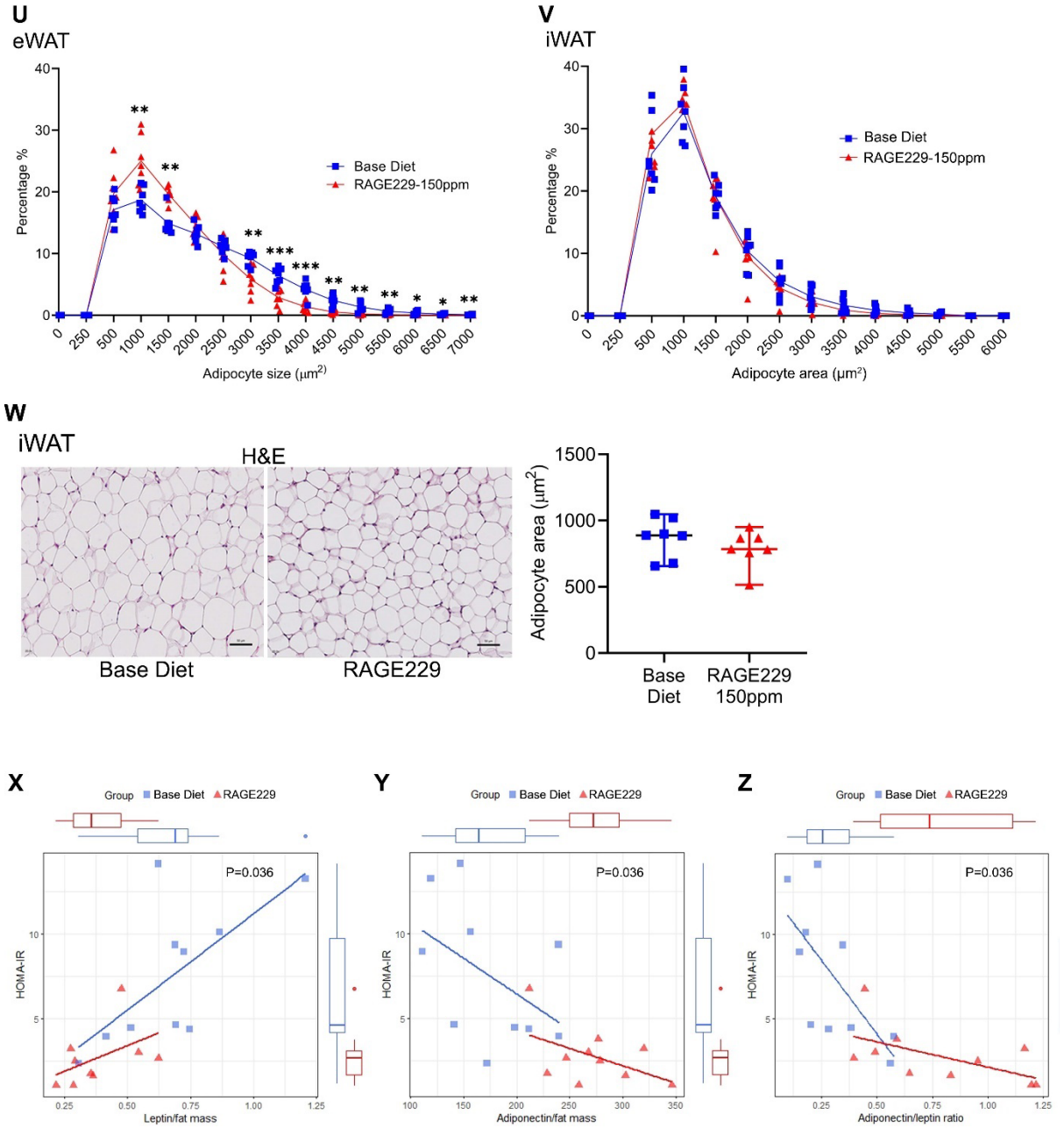
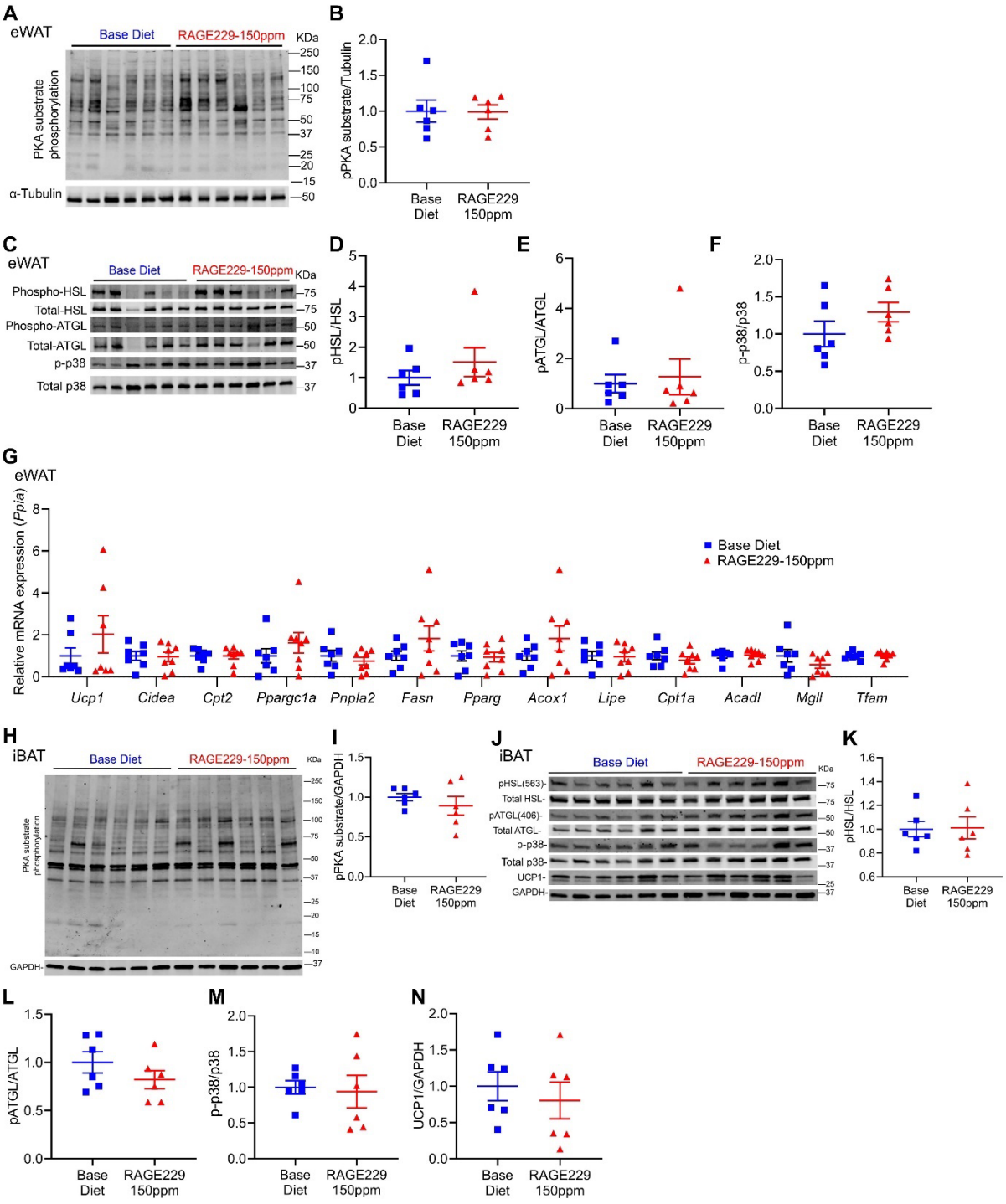


Figure S6. Administration of RAGE229-150 ppm diet enhances loss of body mass and adiposity and improves metabolic health in male mice with established obesity undergoing weight loss. (A) average EE per h vs. body mass data subjected to analysis of covariance (ANCOVA); see Table S3 for additional analyses, (B-F) cumulative caloric intake over 24 h and total caloric intake per d; cumulative physical activity over 24 h and total physical activity per d; RER; , VO_2 consumption and VCO_2 production measured over 24 h and average per d measured at 10 weeks post-diet switch in mice switched from HFD to Base diet (N=11) or RAGE229-150 ppm diet (N=11), (G) Post-6 h fasting insulin tolerance test in mice with obesity at 7 weeks post-diet switch to either Base diet (N=7) or RAGE229-150 ppm diet (N=8), (H) post-6 h fasting insulin tolerance test shown as percent change from baseline blood glucose in mice with obesity at 6 weeks post-diet switch to either Base diet (N=7) or RAGE229-150 ppm diet (N=8) (I) Plasma concentrations of glucose are reported pre- and 15 mins post-IP

injection of insulin in base diet- fed mice and in mice fed RAGE229-150 ppm diet; inset shows the % change in plasma concentrations of glucose in both groups of mice. N=5 per group. (J-K) Western blot of eWAT and iWAT collected after 15 min of insulin bolus and then probed for expression of phosphorylated AKT-serine473/total AKT (N=5 per group), (L-M) Western blot of liver, muscle, eWAT and iWAT collected after 15 min of insulin bolus and then probed for expression of phosphorylated IR beta-Tyrosine 1150/1151/total IR beta (N=5 per group), (N-S) post-overnight fasting plasma glucagon, cholesterol (N=10 per group), triglycerides (N=12 per group), hepatic triglycerides (N=7 per group), plasma NEFA and plasma glycerol (N=12 per group) at 12 weeks post-diet switch, (T) adipocyte area of eWAT presented as median (N=7 per group) and 95% confidence intervals with representative images (10x magnification, scale bar-50 μM) measured at 12 weeks post-diet switch (U-V) adipocyte area distribution curves of eWAT and iWAT, respectively (N=7 per group), (W) adipocyte area of iWAT presented as median (N=7 per group) and 95% confidence intervals with representative images (10x magnification, scale bar-50μM) measured at 12 weeks post-diet switch. (X-Y-Z) Scatter plot with marginal histograms of HOMA-IR vs. leptin/fat mass (X) Adiponectin/fat mass (Y) and Adiponectin/leptin ratio (Z). Data are presented as the Mean ± SEM (other than adipocyte size). Linear mixed-effect model was adopted for correlated/time-series data with post-hoc t-test for group comparisons at each time point. For group comparison, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Kruskal Wallis test/Wilcoxon rank sum test was used if normality was not established. In X-Z, *P*-value indicates FDR-adjusted *P*-value for the mediation effect of RAGE229-150 ppm diet on HOMA-IR through Leptin/fat mass (X), Adiponectin/fat mass (Y) and Adiponectin/Leptin ratio (Z). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.001.


A
eWAT
Base Diet
RAGE229-150ppm
KDa
PKA substrate phosphorylation
α-Tubulin
B
pPKA substrate/Tubulin
Base Diet
RAGE229 150ppm
C
eWAT
Phospho-HSL
Total-HSL
Phospho-ATGL
Total-ATGL
p-p38
Total p38
D
pHSL/HSL
E
pATGL/ATGL
F
p-p38/p38
G
eWAT
Relative mRNA expression (Ppia)
Base Diet
RAGE229-150ppm
Ucp1
Cidea
Cpt2
Ppargc1a
Pnpla2
Fasn
Pparg
Acox1
Lipe
Cpt1a
Acadl
Mgll
Tfam
H
iBAT
GAPDH
I
pPKA substrate/GAPDH
J
iBAT
pHSL(563)-
Total HSL-
pATGL(406)-
Total ATGL-
p-p38-
Total p38-
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p-p38/p38
N
UCP1/GAPDH

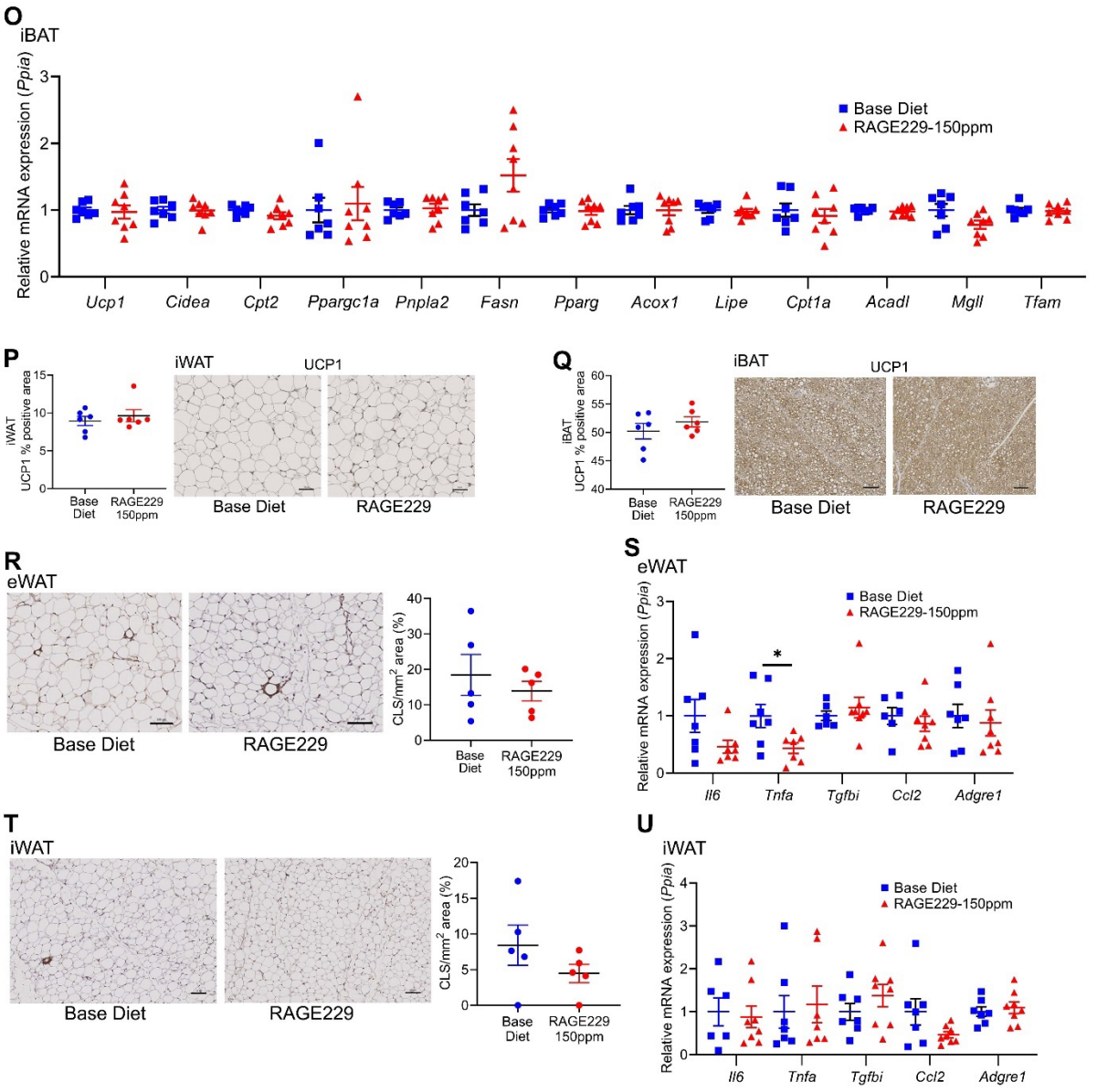

Figure S7. Effect of administration of RAGE229-150 ppm diet on molecular markers of lipolysis, metabolism, thermogenesis and inflammation in adipose tissue depots of male mice with established obesity undergoing weight loss. (A-F) Western blot of eWAT lysate probed for expression of PKA substrate phosphorylation/α-Tubulin, phosphorylated HSL-serine563/total HSL, phosphorylated ATGL-serine406/total ATGL and phosphorylated p38/total p38 MAP kinase, at 6 weeks post-diet switch (N=6 per group), (G) relative expression of the indicated metabolic and thermogenic genes in eWAT at 6 weeks post-diet switch (N=7 Base diet, 7-8 RAGE229-150 ppm diet) (H-N) Western blot analysis of iBAT lysate for expression of PKA substrate phosphorylation/GAPDH, phosphorylated HSL-serine 563/total HSL, phosphorylated ATGL-serine406/total ATGL, phosphorylated p38/total p38 MAP kinase, and UCP1/GAPDH at 6 weeks post-diet switch (N=6 per group), (O) relative expression of the indicated metabolic and thermogenic genes in iBAT at 6 weeks post-diet switch (N=7 Base diet, N=8 RAGE229-150 ppm diet), (P-Q) UCP1 staining positive area (%) (N=6 per group with 5-6 images per sample) with representative images shown (10x

magnification, scale bar-50 μM) in iWAT and iBAT at 12 weeks post-diet switch, (R) crown-like structures (CLS) % per mm^2 area in eWAT with representative images shown (10x magnification, scale bar-100 μM, N=5 per group) at 12 weeks post-diet switch, (S) relative expression of the indicated inflammation genes in eWAT (N=6-7 Base diet, 6-8 RAGE229-150 ppm diet) at 6 weeks post-diet switch, (T) CLS % per mm^2 area in iWAT with representative images shown (10x magnification, scale bar-100 μM, N=5 per group) at 12 weeks post-diet switch, (U) relative expression of indicated inflammation genes in iWAT (N=6-7 Base diet, 7-8 RAGE229-150 ppm diet) at 6 weeks post-diet switch. Tissues for gene expression and Western blot analysis were collected from randomly-fed mice. Data are presented as the Mean ± SEM. For group comparison, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Kruskal Wallis test/Wilcoxon rank sum test was used if normality was not established. $*P<0.05$.

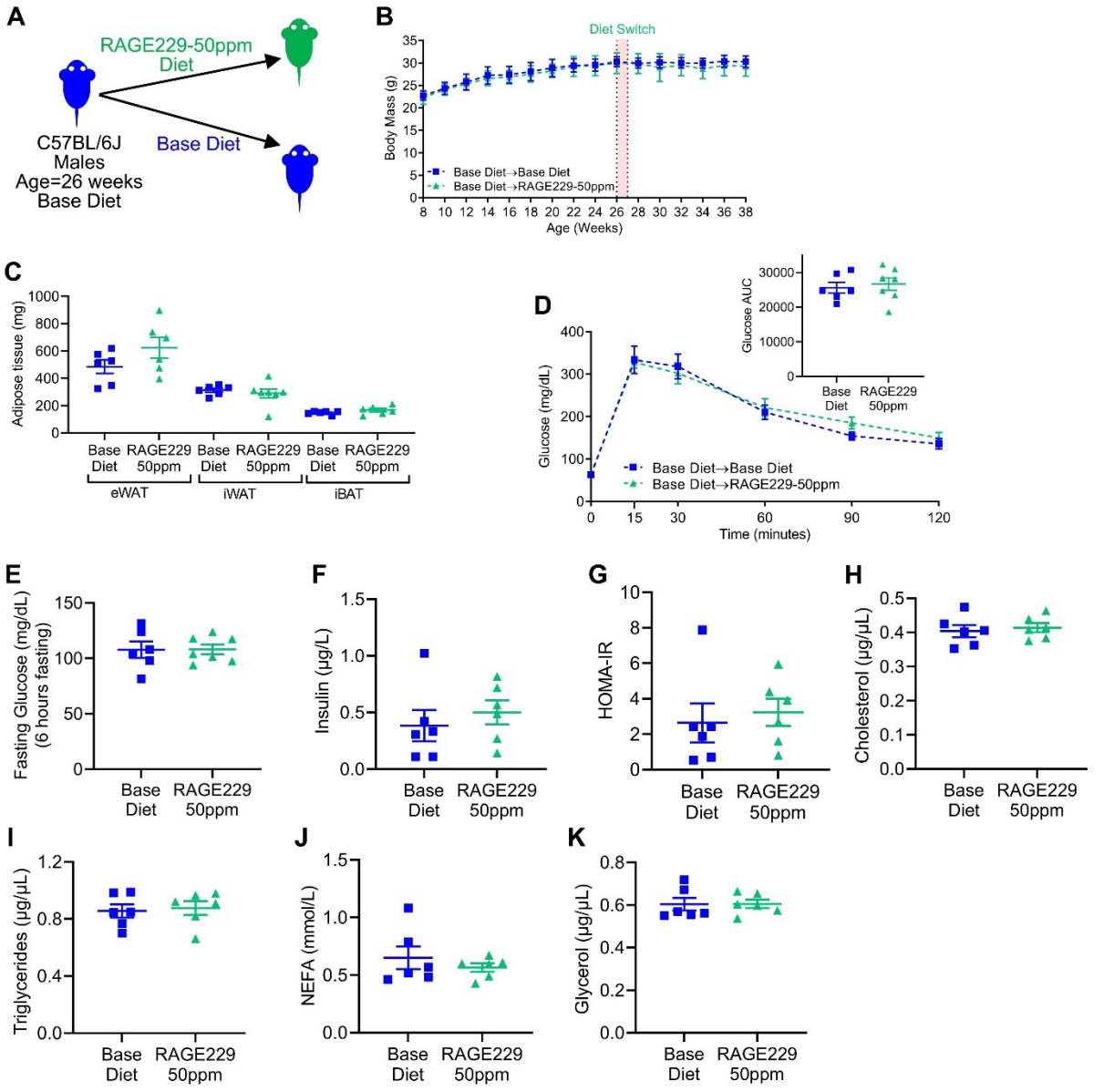

Figure S8. The effects of RAGE229 in lean male mice are dose-dependent; studies testing RAGE229 50 ppm diet. (A) Experimental schematic, (B) Body mass measured over the indicated time course in mice fed Base diet until age 26 weeks; mice then either remained on Base diet (N=6) or were switched to RAGE229-50 ppm diet (N=7), (C) adipose tissue depot mass at 12 weeks post-diet switch (N=6 Base diet, N=6-7 RAGE229-150 ppm diet), (D) Post-overnight fasting glucose tolerance test at 10 weeks post-diet switch (N=6 Base diet, N=7 RAGE229-150 ppm diet), (E-K) Post-6 h fasting plasma glucose (N=6 Base diet, N=7 RAGE229-150 ppm diet), insulin, HOMA-IR, plasma cholesterol, triglycerides, NEFA and glycerol (N=6 per group) at 12 weeks post-diet switch. Data are presented as the Mean ± SEM. For analysis of absolute body mass (B) over the indicated time period, piecewise linear mixed effect model was fitted with node set at 26 weeks and difference was tested between the body mass of the weight of the Base diet and RAGE229- 150 ppm diet groups after the diet switch. Linear mixed-effect model was adopted for correlated/time-series data (B,D) with post-hoc t-test for group comparisons at each time point. For group comparison, the Shapiro-Wilk

normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Kruskal Wallis test/Wilcoxon rank sum test was used if normality was not established.

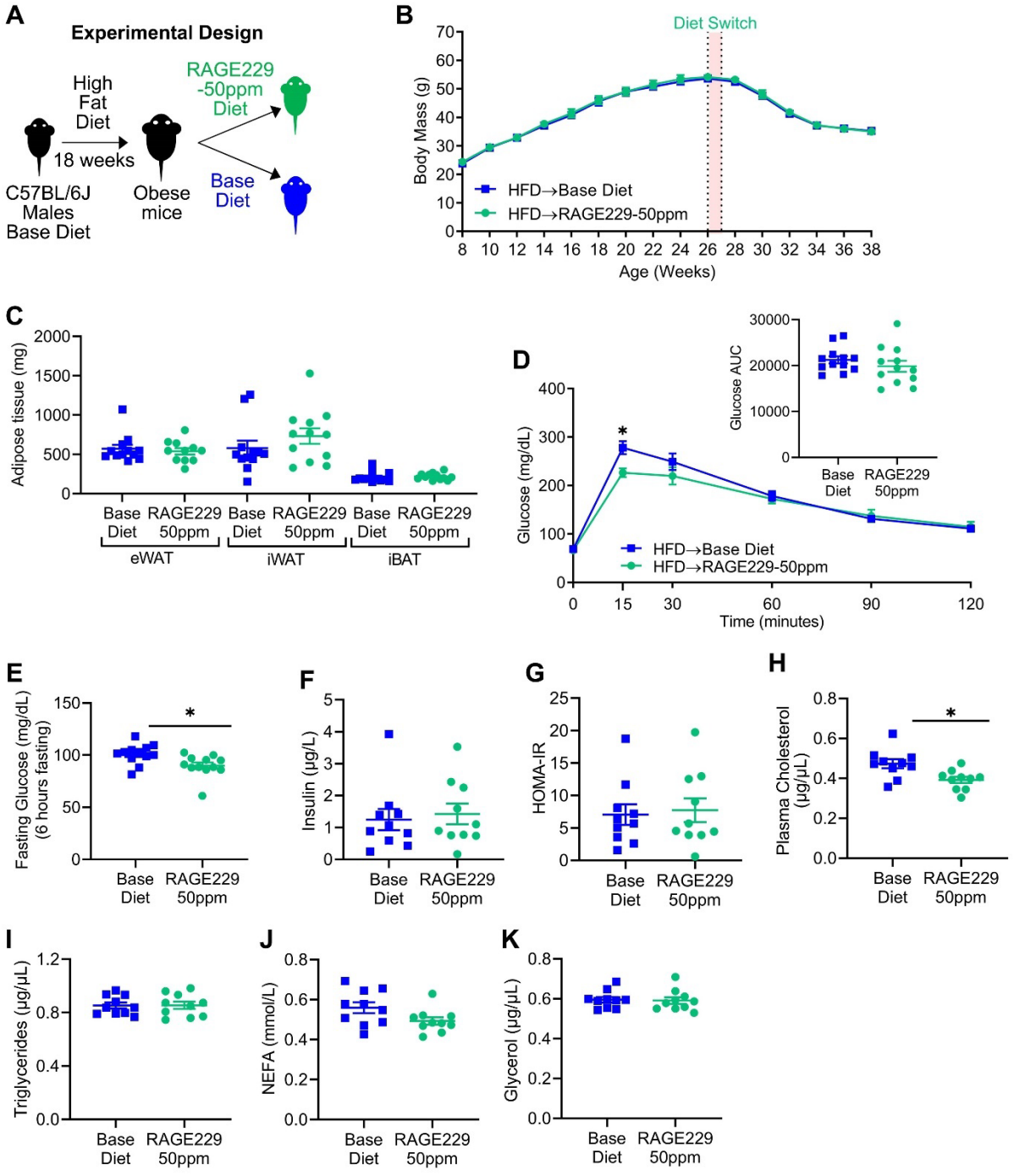

Figure S9. The effects of RAGE229 in mice with obesity are dose-dependent: studies testing RAGE229 50 ppm diet. (A) Experimental schematic, (B) Body mass measured over the indicated time course in male mice fed HFD; between age 8 and 26 weeks to establish obesity; mice were then switched to Base diet (N=12) or RAGE229-50 ppm diet (N=12), (C) adipose tissue depot mass at 12 weeks post-diet switch, (D) Post-overnight fasting glucose tolerance test performed at 10 weeks post-diet switch (N=12 per group), (E-K) Post-6 h fasting plasma glucose (N=12 per group), insulin, HOMA-IR, plasma cholesterol, triglycerides, NEFA and glycerol (N=10 per group) at 12 weeks post-diet switch. Data are presented as the Mean ± SEM. For analysis of absolute body mass (B) over the indicated time period, piecewise linear mixed effect model was fitted with node set at 26 weeks and difference was tested between the

body mass of the weight of the Base diet and RAGE229- 150 ppm diet groups after the diet switch. Linear mixed-effect model was adopted for correlated/time-series data (B,D) with post-hoc t-test for group comparisons at each time point. For group comparison, the Shapiro-Wilk normality test was conducted first for each group with pre-specified significance level of 0.1, and if passed, the t-test was implemented while non-parametric Kruskal Wallis test/Wilcoxon rank sum test was used if normality was not established. $*P<0.05$.

Supplementary Tables

Table S1. Energy Expenditure (EE): ANCOVA* (Related to Figure S4A)

	Estimate	Pr(>|t|)
(Intercept)	0.3704658	0.0507027
RAGE229-150 ppm Diet vs. Base Diet (EE)	-0.3691789	0.0949001
Body mass	0.0014538	0.7934249
RAGE229-150 ppm Diet vs Base Diet: Body mass	0.0124373	0.0943328

Multiple R^2 *P* adj= 0.5371
*=Analysis of Covariance

Table S2. Glucose decay constant (Linear regression results)* (Related to Figure 4H)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.460	0.052	86.433	0.000
Time	-0.006	0.001	-5.422	0.000
dietRAGE229-150ppm	-0.158	0.073	-2.169	0.034
time:dietRAGE229-150ppm	0.000	0.001	-0.289	0.773

Table S3. Energy Expenditure (EE): ANCOVA* (Related to Figure S6A)

	Estimate	Pr(>|t|)
(Intercept)	0.3779278	0.0167635
RAGE229-150 ppm Diet vs. Base Diet (EE)	-0.2976156	0.1356121
Body mass	0.0017952	0.7018118
RAGE229-150 ppm Diet vs. Base Diet: Body mass	0.0100398	0.1294031

Multiple R^2 *P* adj= 0.2029
*=Analysis of Covariance

Table S4. Glucose decay constant (Linear regression results)*(Related to Figure S6G-H)

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.623	0.053	87.278	0.000
time	-0.003	0.001	-3.183	0.002
dietRAGE229-150ppm	-0.432	0.073	-5.960	0.000
time:dietRAGE229-150ppm	0.000	0.001	-0.046	0.963

Table S5: Mediation Analysis (Male Mice with Obesity) (Related to Figure S6X-Y-Z)

Y	X	Group. Y	pval1	pval1_ fdr	Group. X	pval2	pval2_ fdr	X.Y	pval3	pval3_ fdr	Group. X Y	pval4	pval4_ fdr
HOMA-IR	Weight loss	-4.043	0.022	0.0220	-1.289	0.000	0.000	0.138	0.875	0.8750	-0.178	0.876	0.876
HOMA-IR	Fat mass (g)	-7.736	0.000	0.0000	-1.376	0.000	0.000	-2.555	0.006	0.0120	3.515	0.015	**0.030**
HOMA-IR	Leptin/fat mass	-1.879	0.149	0.3945	-1.182	0.001	0.001	2.492	0.000	0.0000	**-2.945**	0.012	**0.036**
HOMA-IR	Adiponectin /fat mass	-1.537	0.413	0.4130	1.498	0.000	0.000	-2.195	0.023	0.0230	**-3.287**	0.036	**0.036**
HOMA-IR	Adiponectin /leptin ratio	-1.875	0.263	0.3945	1.384	0.000	0.000	-2.131	0.013	0.0195	**-2.949**	0.030	**0.036**
HOMA-IR	Cholesterol (µg/µl)	-2.915	0.113	0.2260	-1.395	0.000	0.000	1.368	0.146	0.1510	-1.909	0.166	0.181
HOMA-IR	TAG (µg/mg)	-2.211	0.396	0.3960	-1.429	0.000	0.000	1.865	0.151	0.1510	-2.666	0.181	0.181

FDR=false discovery rate

p val1(Group Y) reflects if there is a significant, direct effect of treatment group (RAGE229 diet) on the outcome (Y) (HOMA-IR)
p val 2 (Group X) reflects if there is a significant effect of Group(RAGE229 diet) on variable X (eg. weight loss, etc)
p val 3 (X-Y) reflects the significance of the correlation between variable X and outcome Y
p val 4 reflects if there is a significant, indirect effect of treatment group (RAGE229 diet) on the outcome mediated by X (eg., weight loss.)

*Note that in order to support conclusions regarding mediation of RAGE229 through X on Y, the **"Group X Y"** must be a negative value, as is the case for leptin/fat mass; adiponectin/fat mass; and adiponectin/leptin ratio. Hence, these data suggest that RAGE229 lowers HOMA-IR by (1) decreasing leptin/fat mass, (2) increasing adiponectin/fat mass and (3) increasing adiponectin/leptin ratio.*

Table S6. List of Taqman Primers

Primer	Assay number	Catalogue number
Acox1	Mm01246834_m1	4331182
Acadl	Mm00599660_m1	4331182
Adgre1	Mm00802529_m1	4331182
Ccl2	Mm00441242_m1	4331182
Cidea	Mm00432554_m1	4331182
Cpt1a	Mm00550438_m1	4331182
Cpt2	Mm00487205_m1	4331182
Fasn	Mm00662319_m1	4331182
Il6	Mm00446190_m1	4331182
Lipe	Mm00495359_m1	4331182
Mgll	Mm00449274_m1	4331182
Pnpla2	Mm00503040_m1	4331182
Pparg	Mm00440940_m1	4331182
Ppargc1	Mm01208835_m1	4331182
Ppia	Mm02342430_g1	4331182
Tfam	Mm00447485_m1	4331182
Tgfbi	Mm01337605_m1	4331182
Tnfa	Mm00443258_m1	4331182
Ucp1	Mm01244861_m1	4331182

Table S7. List of Antibodies

Antibody	**Source**	**Catalogue number**
Phospho-HSL-Serine-563	Novus biological (in vivo studies)	NBP3-05457
Phospho-HSL-Serine-563	Cell Signaling (in vitro studies)	4139
Total HSL	Cell Signaling	4107
Phospho-ATGL-serine-406	ABCAM	ab135093
Total ATGL	Cell Signaling	2439
UCP1(D9D6X)	Cell Signaling (for mice BAT)	14670
UCP1	ABCAM (cultured C3H10T1/2 cells)	10983
UCP1	ABCAM (immunostaining)	23841
F4/80	ABCAM	6640
α-Tubulin	Cell Signaling	3873
GAPDH	Santa Cruz	sc-32233
Phospho-P38 MAPK	Cell Signaling	9211
Total-P38 MAPK	Cell Signaling	9212
Phospho-PKA Substrate (RRXS*/T*) (100G7E)	Cell Signaling	9624
Phospho-AKT (Ser473)	Cell Signaling	9271
Total AKT	Cell Signaling	9272
Phospho-Insulin receptor beta (Tyrosine 1150/1151)	Cell Signaling	3024
Insulin Receptor beta	Cell Signaling	3025

Methods

See the main document for the description of the some of the methods employed for the work reported in this manuscript.

Pair-feeding studies

At the indicated time points, mice were weight-matched and randomized into three groups; an ad-libitum RAGE229 diet-fed "guide" group, and pair-fed base diet vs. RAGE229-150 ppm diet-fed groups. Mice were single housed for 4 days to adapt to single housing environment and then acclimatized to new diet over 6 days. After acclimation, the guide group had free access to and was fed the RAGE229 diet *ad libitum*. The average caloric intake over a 24-hour period of RAGE229-fed guide group was measured and the following day, equivalent numbers of these calories were fed to the pair-fed-RAGE229 diet and the pair-fed-base diet groups. Body weight and food intake were measured daily. To avoid pair-fed mice being left without food, especially during the dark period, one third part of the stipulated amount of diet was given at the beginning of the light cycle and two third parts of diet were given towards the end of the light cycle. Where indicated, rectal temperature was measured daily in awake mice for the indicated period of days during the first hour into the light cycle to avoid diurnal variation, using a rectal probe attached to a digital thermometer (ThermoWorks, Alpine UT). At the indicated time point of pair-feeding, the pair-fed groups were euthanized in a randomly-fed state and plasma and tissues were collected and snap-frozen until further use.

Glucose and insulin tolerance tests

Following the indicated fasting period, fasting concentrations of blood glucose were measured using handheld glucometer (Freestyle Freedom Line, Abbott Diabetes Care Inc, Alameda CA) from blood collected from the tail (t=0). Mice were given intraperitoneal (IP) injections of glucose (1g/kg body weight) or insulin (0.85 IU/kg body weight) (Humulin R- Lilly 0002-8215-01) and blood glucose was measured at 15, 30, 60, 90 and 120 min following the glucose administration or at 15, 30, 60 and 90 min following insulin administration. The glucose decay constant (Kg) post-insulin injection (insulin tolerance test) was calculated as the negative slope of the linear regression equation between serum glucose (after natural log transformation) and time (1) .

In vivo insulin signalling study

The in vivo insulin signaling study was performed as follows(2): Following a 6 hour fast, fasting concentrations of blood glucose were measured. Mice were then given an IP injection of insulin (100mU in 300µL of 5% glucose) (Humulin R- Lilly 0002-8215-01) and 15 min later, blood glucose was measured. Mice were then immediately euthanized and the liver, quadriceps muscle, eWAT and iWAT were collected, snap-frozen and stored at -80°C until further analyses.

Dual Energy X-Ray (DEXA) Absorptiometry

At the indicated time points, mice were weighed, briefly anesthetized via inhalation of isoflurane and placed on a scanning area of a precalibrated Lunar PIXImus DEXA instrument (PIXImus, WI-USA). At least 3 scans per mouse were performed and the average lean and fat mass were computed and recorded.

Blood and Tissue collection

Following an indicated period of fasting or in a random (fed) state, mice were given an IP injection of ketamine (80mg/kg) and xylazine (10 mg/kg). Once the depth of anesthesia was confirmed, blood was collected through cardiac puncture and plasma was prepared using EDTA as anticoagulant and centrifugation at 2000× g for 10 min was performed; plasma was stored at -80°C until further use. White adipose tissue (WAT) as follows: epididymal (eWAT) in male mice or perigonadal (pWAT) in female mice; subcutaneous (inguinal) WAT (iWAT), and brown adipose tissue (BAT) as follows: interscapular brown adipose tissue (iBAT) depots and liver were collected. Tissues were snap-frozen and stored at -80°C until further analyses. Sections of the adipose depots were fixed in 4% paraformaldehyde for further experiments.

Plasma and Liver analysis

Plasma was analysed for the concentrations of plasma insulin using Mercodia mouse insulin ELISA kit (10-1247); concentrations of leptin, adiponectin and glucagon were measured using mouse leptin quantikine ELISA kit (M0B00B); adiponectin quantikine ELISA kit (MRP300); and glucagon quantikine ELISA kit (DGCG0), respectively (R&D systems). Plasma concentrations of glycerol were measured using glycerol reagent (Sigma, F6428); NEFA was measured using colorimetric NEFA-HR (2) kit (WAKO); triglyceride concentrations were determined using triglyceride determination kit (Sigma-TR0100); and total cholesterol was determined using Infinity cholesterol reagent (Thermofisher-13421). The homeostatic model assessment of insulin resistance (HOMA-IR) was determined by using the following formula:

fasting plasma insulin (mU/L) × fasting plasma glucose (mmol/L)/22.5 (3). Hepatic triglyceride content was determined by extracting triglycerides using the Folch extraction technique (4) by homogenizing the tissue in chloroform : methanol (2:1) mixture and then incubating overnight while shaking. This was followed by addition of 0.1N sodium chloride, vortexing and centrifugation, which separated the aqueous and organic phase. The organic phase was collected, evaporated under nitrogen gas to complete dryness and then 3M alcoholic potassium hydroxide was added followed by heating the samples at 70ºC for 60 mins. The resultant extract was used to measure triglyceride content using triglyceride determination kit (Sigma-TR0100) and normalized to tissue weight.

RNA isolation and mRNA expression

Total RNA from adipose tissue depots was extracted by homogenizing tissue samples in Qiazol (Qiagen-79306) and using RNeasy kit (Qiagen-74004). The RNA content was measured using Nano-drop spectrophotometer (Thermofisher ND-100). RNA was reverse transcribed to generate cDNA using iScript™ cDNA Synthesis Kit (Biorad-1708891). Quantitative PCR (qPCR) was performed using the TaqMan method (50°C for 2 mins, 95°C for 10 mins, and 40 cycles of 95°C for 15 s and 60°C for 1 min) with premade primer sets (Applied Biosystems, **Table S6**) using a 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The relative transcripts abundance was normalized to the expression of housekeeping genes (indicated in figure legends) using the ΔΔCt method.

In vitro β-adrenergic stimulated lipolysis

Differentiated murine adipocytes were serum starved for 3 h in Krebs-Ringer bicarbonate (KRB) buffer with 2% fatty acid free bovine serum albumin (BSA) (Roche 3117057001). After three h, cells were treated with RAGE229 (100 µm) or equal volumes of vehicle (DMSO) for 2 h before stimulation with the β3-adrenergic agonist-CL316,243 (10 µM) (Sigma-C5976) for 15 mins (5). After stimulation, media was collected and glycerol and NEFA were measured using free glycerol reagent (Sigma Aldrich-F6428) and colorimetric NEFA assay (WAKO-99934691), according to the manufacturer's instructions. Cells were washed with PBS and lysed to extract proteins.

Differentiated human adipocytes were serum starved for 3 h in adipocyte starvation media (Cell Applications- 811S-250). After three h, cells were treated with RAGE229 (100 µm) or vehicle (DMSO) in KRB buffer with 4% fatty acid free BSA for 2 h before stimulating with isoproterenol (0.25 µM) for 2 h (6). After stimulation, media was collected; glycerol was

measured using free glycerol reagent (Sigma Aldrich). Cells were washed with PBS and lysed to extract proteins.

Mitochondrial stress test

C3H10T1/2 cells were seeded in 8 well Seahorse plates at density of 5,000 cells per well and differentiated as mentioned above. Human subcutaneous adipocytes were seeded in 24 well Seahorse plates at a density of 15,000 cells per well and differentiated as noted above. Differentiated adipocyte were treated with RAGE229-5 μM in growth media for 2 h. Cells were then washed and incubated with XF seahorse media for 1 h in a non-CO_2 incubator. The oxygen consumption rate (OCR) was determined with Seahorse XF HS Mini Analyzer (Agilent. Santa Clara CA) for mouse cells and an XF24 Extracellular Flux Analyzer for human cells (Seahorse Bioscience, Proteigene, Saint Marcel, France). Uncoupled and maximum OCR were determined with oligomycin (5 μM (Sigma-75351)) and FCCP (2.5 μM (Sigma-C2920)). Rotenone (1.25 μM, (Sigma-R8875)) and antimycin A (1.25 μM (Sigma-A8674)) were used to inhibit complex I- and III-dependent respiration, respectively. OCR was normalized to the protein concentration of the respective well.

Mitochondrial DNA content

Genomic DNA was isolated using QIAamp DNA mini kit (Qiagen, 51304) from the differentiated adipocytes pre-treated for 2 h with RAGE229-5 μM or vehicle in growth media. From 40 μg of genomic DNA, the gene expressions of mitochondrial marker gene cytochrome c oxidase subunit I (CO1) and nuclear marker gene NADH:Ubiquinone Oxidoreductase Core Subunit V1 (NDUFV1) was measured with SYBR green (Biorad 1708880) using 7500 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific). The CO1 primers were: (forward primer) 5'-TGC TAG CCG CAG GCA TTA C-3' and (reverse primer) 5'-GGG TGC CCA AAG AAT CAG AAC-3'. The NDUFV1 primers were: (forward primer) 5'-CTT CCC CAC TGG CCT CAA G-3' and (reverse primer) 5'-CCA AAA CCC AGT GAT CCA GC-3'. The ratio of CO1 to NDUFV1 expression was calculated (7).

Protein extraction and Western blot analysis

Tissues or differentiated cells were homogenized with 1X-RIPA (Cell Signaling, 9806) supplemented with complete protease (ThermoFisher-A32963) and phosphatase (ThermoFisher-A32957) inhibitors and homogenate was kept on ice for one h followed by centrifugation at 14,000 rpm for 15 mins. The supernatant was collected; protein content was measured using BCA kit (Thermo fisher scientific- 23225). Reducing agent (NuPAGE-

NP0009) and loading dye (NuPAGE-NP0007) were added to the sample after diluting the samples to match the protein concentration with RIPA buffer. The protein was resolved on 4-20% TGX gels (BioRad) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked for one h at room temperature with blocking buffer (Odyssey LI-COR, Lincoln, NE) and then incubated with primary antibody (1:1000 dilution) (**Table S7**) overnight at 4°C. Membranes were washed (3 x 5 mins) with 1X- Tris-buffered saline with 0.1% Tween (TBST), incubated for one h at room temperature with secondary antibody (IRDye 680RD goat anti–mouse IgG (LI-COR-925-68070) and/or IRDye 800CW goat anti–rabbit IgG (LI-COR-925-32211)) diluted (1:20000) in TBST with 0.5% sodium dodecyl sulfate. Membranes were washed with TBST (3 x 5 mins) and scanned using the LI-COR Odyssey Classic (LI-COR, Lincoln, NE) and quantified with Image Studio Lite software (LI-COR, Lincoln, NE) based on direct fluorescence measurement. Membranes probed for phosphorylated proteins were stripped with 0.2N sodium hydroxide for 20 minutes at 37ºC, washed with TBST (3 x 5 mins), blocked with blocking buffer for one h and then probed for respective total protein.

Histology and immunohistochemistry

Adipose tissue (eWAT, iWAT and iBAT) samples were fixed in 4% paraformaldehyde solution and 24 h later washed three times with PBS. Tissues were embedded in paraffin and 5 μm thick sections were collected onto histology slides. For eWAT and iWAT measurement of adipocyte area, sections were stained with Mayer's hematoxylin and eosin (H&E) stain (8). For F4/80 antigen staining of eWAT and iWAT, and UCP1 antigen staining of iBAT and iWAT, paraffin-fixed sections were deparaffinized in xylene, and hydrated by graded washes of ethanol to water. Following this, antigen retrieval was performed by steaming the section in epitope retrieval solution (IHC-Teck, IW-1100), for 60 mins. Slides were permeabilized using 0.25% Triton x-100, incubated with hydrogen peroxide to inhibit endogenous peroxidase activity, followed by blocking with 5% normal rabbit serum (NRS) for 30 mins followed by Avidin and biotin for 15 mins each (Avidin/Biotin Blocking Kit, Vector Laboratories SP-2001). Slides were incubated with primary antibody (1:100) (Table S4) at 4ºC overnight. The sections were then washed and incubated with respective secondary antibody, developed with diaminobenzidine (DAB) and then counter- stained with hematoxylin.

Image analysis and quantification

Both H&E-stained and antibody-stained whole slides were scanned (Leica SCN400 scanner). For adipocyte area, whole sections were analysed for adipocyte area using Visiopharm image

analysis software (Visiopharm, Horsholm, DK). Tissue was first detected and outlined using tissue detect function, which restricted the subsequent analysis within the outlined area only. To avoid including the damaged parts of the tissue, erase function was used manually to further limit the tissue outlining. An algorithm was then designed which first used simple thresholding to classify pixels with intensities above 200 as adipose, and those above 15,000 (for iWAT) or 25,000 (for eWAT) as irregular (not adipose). In the post processing steps, adipocytes were labelled as adipose and the irregular area was labelled as clear; the algorithm eliminated adipocytes from analysis if the area was outside defined limits (250–6000 μm^2 for iWAT and 250–7000 μm^2 for eWAT) and also if the shape (form) factor is over 0.1 (for iWAT) or 0.2 (for eWAT); the adipocyte labels were then counted and the area was given as the output in comma-separated values (CSV) file. The median of adipocyte area of each sample was used for graphical presentation and analysis. To determine the percent distribution of adipocyte area in each group, adipocytes from each section were categorized into different classes and percentage of each class was calculated based on total number of adipocytes in each section.

For UCP1-stained sections, using the Slidepath Digital Imaging Hub software (Leica Biosystems), 5-6 randomly-selected section images per mouse were captured at 10x magnification. Using Fiji distribution of ImageJ, images were deconvoluted to separate the DAB Images from the hematoxylin counter-stain using the Fiji plug-in Color De-convolution 1.7 (vector-H DAB). The resulting DAB images were subjected to automated color threshold using the Otsu method (9-11) followed by quantification of UCP1 positive area. Average of 5-6 images per animal were used for statistical analysis. For F4/80-stained images, using OMERO (Glencoe software), section size was determined and whole section was scanned manually to avoid bias, for number of crown-like structure (CLS) and presented as percentage of number of CLS per mm^2 area for each section.

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