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Supplemental Information

A Receptor of the Immunoglobulin Superfamily Regulates Adaptive Thermogenesis

Carmen Hurtado del Pozo, Henry H. Ruiz, Lakshmi Arivazhagan, Juan Francisco Aranda, Cynthia Shim, Peter Daya, Julia Derk, Michael MacLean, Meilun He, Laura Frye, Randall H. Friedline, Hye Lim Noh, Jason K. Kim, Richard A. Friedman, Ravichandran Ramasamy, and Ann Marie Schmidt

Supplemental Information

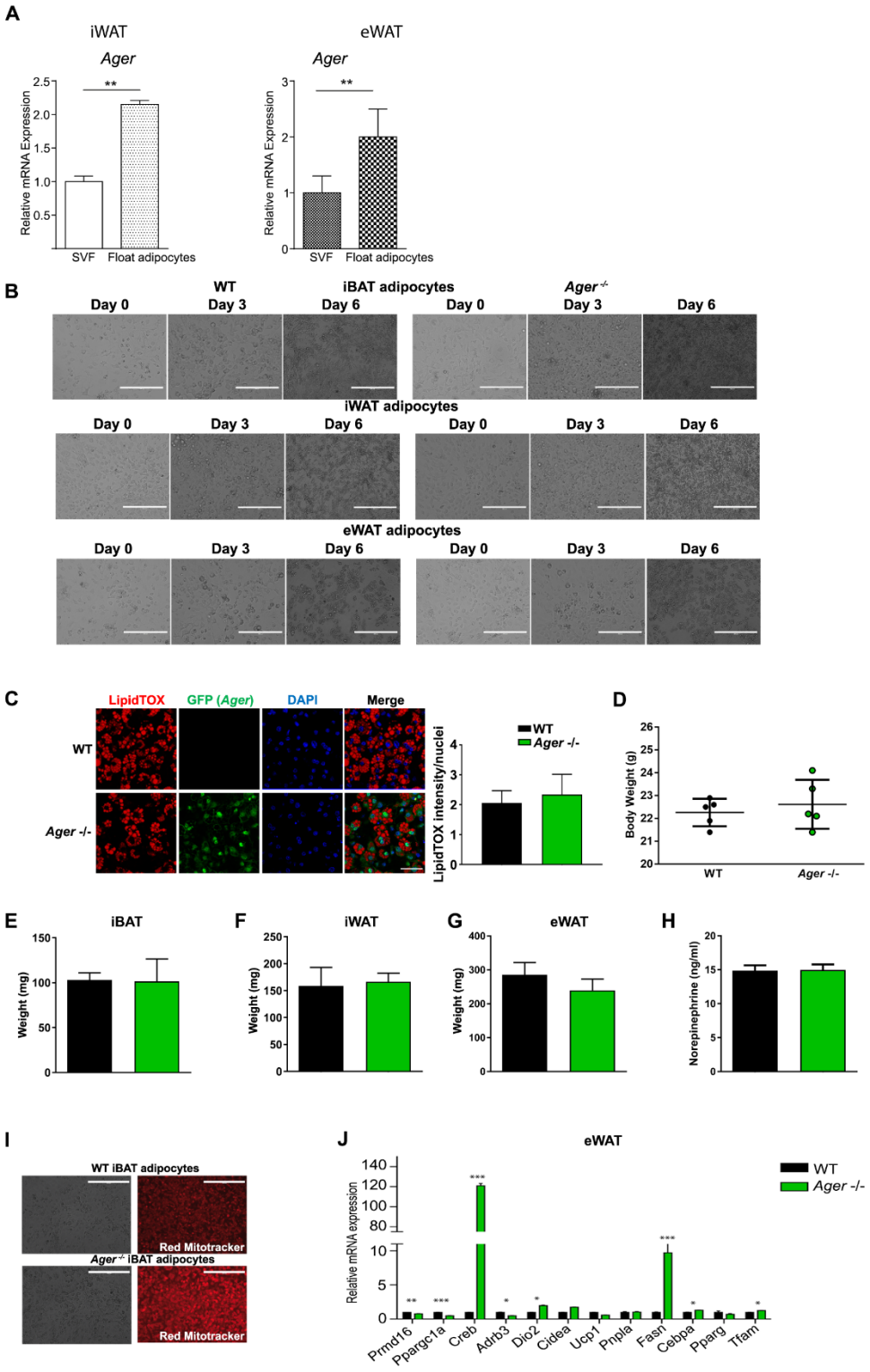


Figure S1. RAGE, primary adipocyte differentiation and adiposity. Related to Figure 1. (A) iWAT (left) and eWAT (right) were retrieved from WT mice and subjected to centrifugation for separation of floating adipocytes and

SVF. Real-time qPCR was performed for detection of relative *Ager* mRNA transcripts. The mean relative mRNA expression±SEM is reported in adipocytes derived from N=5 mice/group. Data analysis was performed using independent samples two-tailed Student t-test. **(B)** Representative micrographs of primary adipocytes differentiated from WT or *Ager* null iBAT (top), iWAT (middle) and eWAT (bottom) SVF of N=4 mice/group. Differential interference contrast micrographs of primary cells throughout differentiation (on day 0, 3 and 6). Scale bar: 400 µm. **(C)** Primary adipocytes of eWAT retrieved from WT and *Ager* null mice were differentiated over 7 days and stained with LipidTOX and DAPI. GFP fluorescence denotes *Ager* null cells as *Ager* is replaced by *Gfp* in these mice. Scale bar: 20 µm. Data are plotted as LipidTOX mean intensity/nuclei. The mean±SEM is reported. Data were analyzed using independent samples two-tailed Student's t-test. There were no statistically significant differences between the groups. **(D)** Body weight was recorded in 8-week old male WT or *Ager* null mice fed standard chow at room temperature. The mean±SEM is reported in N=5 mice/group and analyzed using independent samples two-tailed Student's t-test. **(E)** iBAT, **(F)** iWAT and **(G)** eWAT were retrieved from 8-week old standard chow fed WT and *Ager* null mice and weighed. The mean tissue weight±SEM is reported in N=5 mice/group and analyzed using independent samples two-tailed Student's t-test. **(H)** Plasma norepinephrine (NE) levels were measured in 8-week old WT or *Ager* null mice fed standard chow at room temperature. The mean NE levels±SEM are reported in N=5 mice/group and analyzed using independent samples two-tailed Student's t-test. **(I)** MitoTracker staining was performed in WT and *Ager* null primary iBAT adipocytes at day 8 of differentiation; representative micrograph is shown. Scale bar: 400 µm. **(J)** eWAT was retrieved from 8-week old WT or *Ager* null mice and subjected to RT-qPCR to assess the relative mRNA expression of *Prdm16, Ppargc1a*, *Creb, Adrb3, Dio2, Cidea, Ucp1, Pnpla2, Fasn, Cebpa, Pparg* and *Tfam*. The mean relative mRNA expression±SEM is reported for adipocytes derived from N=5 mice/group. Data for each gene were separately analyzed using independent samples two-tailed Student's t-test. In this figure, *$p<0.05$, **$p<0.01$ and ***$p<0.001$. For results presented in C-H & J, WT (black bars/circles) and *Ager* null (green bars/circles).

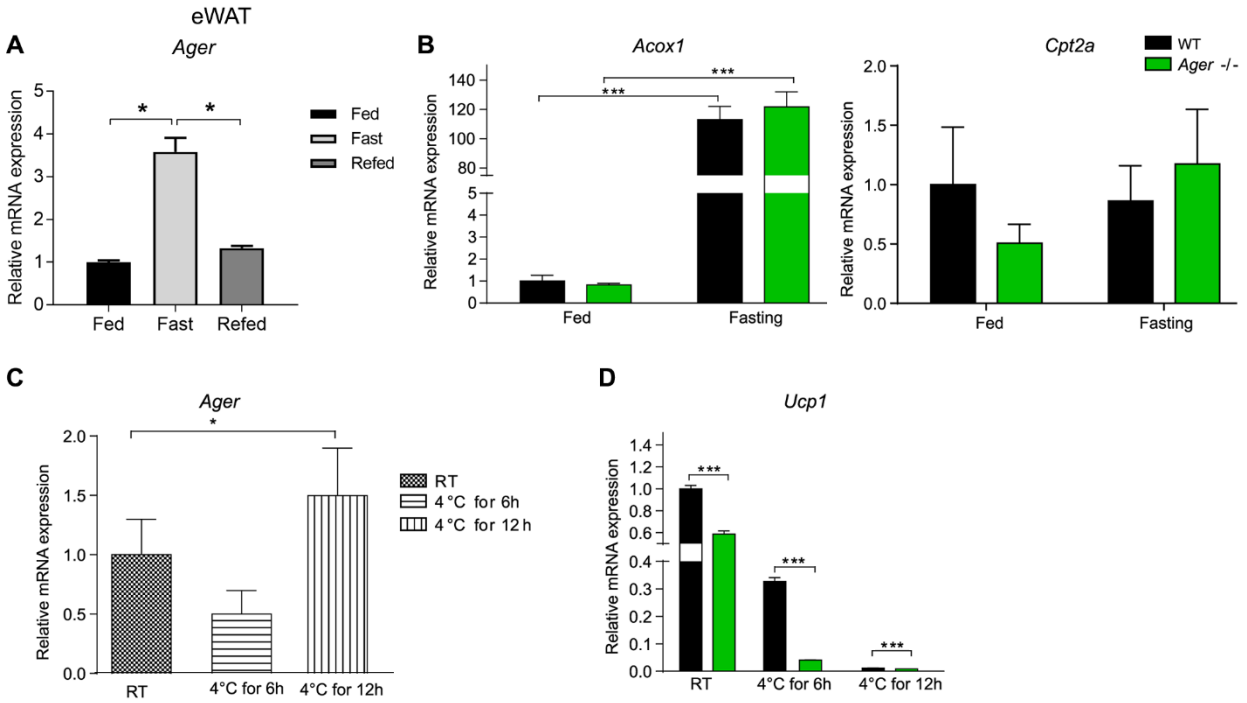


Figure S2. RAGE and effects on fasting and cold challenge. Related to Figure 2. (A) eWAT was retrieved from 11-week old WT mice in a fed, fasted (24 hours) or 24 hours refed state and subjected to RT-qPCR for detection of relative *Ager* mRNA expression. The mean relative mRNA expression±SEM is reported in N=5 mice/group. Data were analyzed using an independent samples one-way ANOVA followed by a *post hoc* Tukey's HSD test. **(B)** The relative expression of fatty acid oxidation-related genes *Acox1* and *Cpt2a* mRNA was measured by RT-qPCR in eWAT of fed and fasted (24 hours) 11-week old WT and *Ager* null mice. The mean relative mRNA expression±SEM is reported in N=4 mice/group. Data were analyzed using independent samples two-way ANOVA followed by *post hoc* Bonferroni test where appropriate. **(C)** eWAT was retrieved from WT mice immediately after exposure to 6 or 12 hours 4°C cold challenge and subjected to RT-qPCR for detection of relative *Ager* mRNA expression. The mean relative mRNA expression±SEM is reported in N=5 mice/group. Data were analyzed using independent samples one-way ANOVA followed by *post hoc* Tukey's HSD test. **(D)** Cold challenge was performed as in (C) for WT and *Ager* null male mice and immediately after sacrifice, eWAT was retrieved and subjected to RT-qPCR for detection of relative *Ucp1* mRNA expression. The mean±SEM is reported in N=5 mice/group. Data were analyzed using independent samples two-way ANOVA followed by *post hoc* Sidak HSD test; ***p<0.001. In this figure, *p<0.05 & ***p<0.001. For results presented in B and D, WT (black bars) and *Ager* null (green bars).

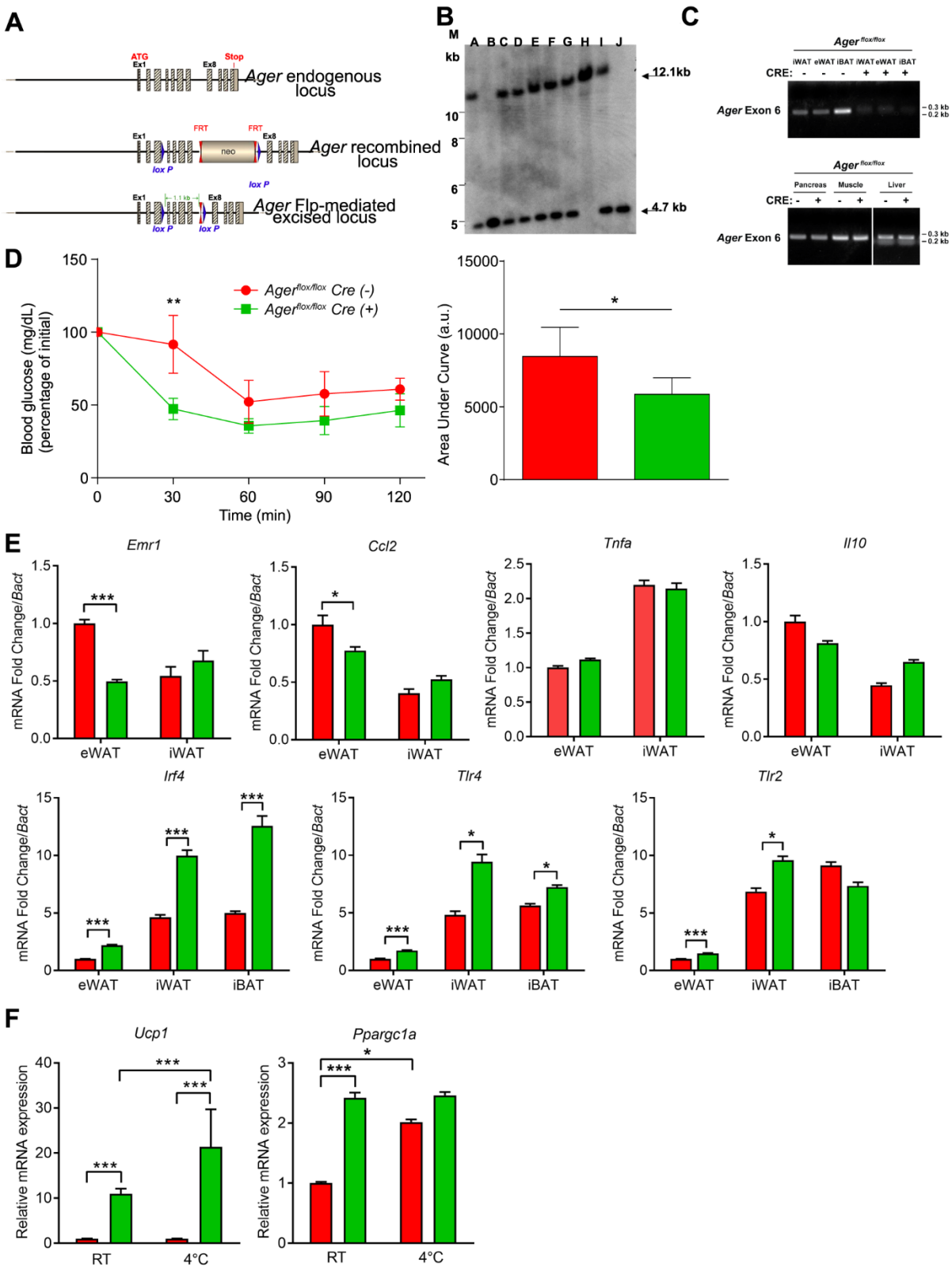


Figure S3. Generation of *Ager* floxed mice and characterization of adipose tissue inflammation and thermogenic programing. Related to Figure 3. (A) Bioinformatics analysis of *Ager* cDNA sequence

(NM_007425) indicated that the exon / intron organization of the gene revealed 11 exons separated by 10 introns. Schematic illustrating *Ager* targeting strategy leading to the insertion of a *lox*P site together with an FRT flanked neomycin selection cassette within the intron 7 and a single distal *lox*P within the intron 3 of *Ager*. This was followed by homologous recombination in embryonic stem (ES) cells (directly into C57BL/6), blastocyst injection, generation and identification of chimeric mice and breeding with *Flp* deleter mice to remove the neomycin cassette. Breeding with Cre recombinase mice thus excises the *lox*P flanked *Ager* genomic region (exons 4 to 7), thereby deleting functional RAGE. **(B)** Southern blot validation of *Ager* heterozygous and homozygous null mice. Southern blotting was performed on Kpn1 digested genomic DNA from WT or homozygous or heterozygous *Ager* deleted mice. In this figure, the hybridization figure corresponding to the *Ager* deleted allele is 12.1kb and signal corresponding to the *Ager* WT allele is 4.7 kb. Thus, in this Southern blot, lane H indicates homozygous *Ager* null mouse; Lanes A, C-G and I indicate heterozygous *Ager* deleted mice, and Lanes B and J are WT mice. **(C)** *Ager* floxed mice were bred with Adiponectin (*Adipoq*) *Cre* recombinase mice. Genomic DNA was prepared from eWAT, iWAT, iBAT, pancreas, skeletal muscle and liver of $Ager^{flox/flox}$ *Adipoq Cre* (-) or *Cre* (+) mice and subjected to PCR to assess specific detection of *Ager* exon 6. The primers were designed between exon 5 and exon 6 (as the flox sequences are designed between exons 3 and 7) as follows: Primer Pair: Forward Primer: GGTACCCTGCACCCAACTAC and Reverse Primer: TTGATGGCCCTGGGATTGAC (Product length: 302 base pairs). Note that only in the *Cre* (+) mice was *Ager* selectively deleted in eWAT, iWAT and iBAT, but not in pancreas, skeletal muscle or liver whereas in *Cre* (-) mice. **(D)** Insulin tolerance test was performed in the indicated $Ager^{flox/flox}$ *Cre* (-) or *Cre* (+) fed HFD and reported as % change in level of glucose from baseline and the area under the curve. The mean percent change±SEM is reported in N=4-5 mice/group. Data were analyzed using a repeated measures two-way ANOVA followed by a *post hoc* Bonferroni test or an independent samples Student t-test (for AUC). **(E)** $Ager^{flox/flox}$ *Cre* (-) or *Cre* (+) mice were fed a HFD for 12 weeks and eWAT, iWAT or iBAT were subjected to RT-qPCR for detection of mRNA transcripts expression for *Emr1*, *Ccl2*, *Tnfa*, *Il10*, *Irf4*, *Tlr4* and *Tlr2*. The mean relative mRNA expression±SEM is reported in N=4 mice/group. Data analysis was performed using an independent samples Student t-test. **(F)** Mice were housed at room temperature or 4°C for 24 hours. eWAT was immediately retrieved at the end of the study and subjected to RT-qPCR for detection of relative *Ucp1* and *Ppargc1a* mRNA expression. The mean relative mRNA expression±SEM is reported in N=4 mice/group. Data were analyzed using independent samples two-way ANOVA followed by a post-hoc *Sidak* test. In this figure, *$p<0.05$,

**p<0.01 and ***p<0.001. For results presented in D-F, *Ager*$^{flox/flox}$ *Cre* (-) (red bars) and *Ager*$^{flox/flox}$ *Cre* (+) (green bars).

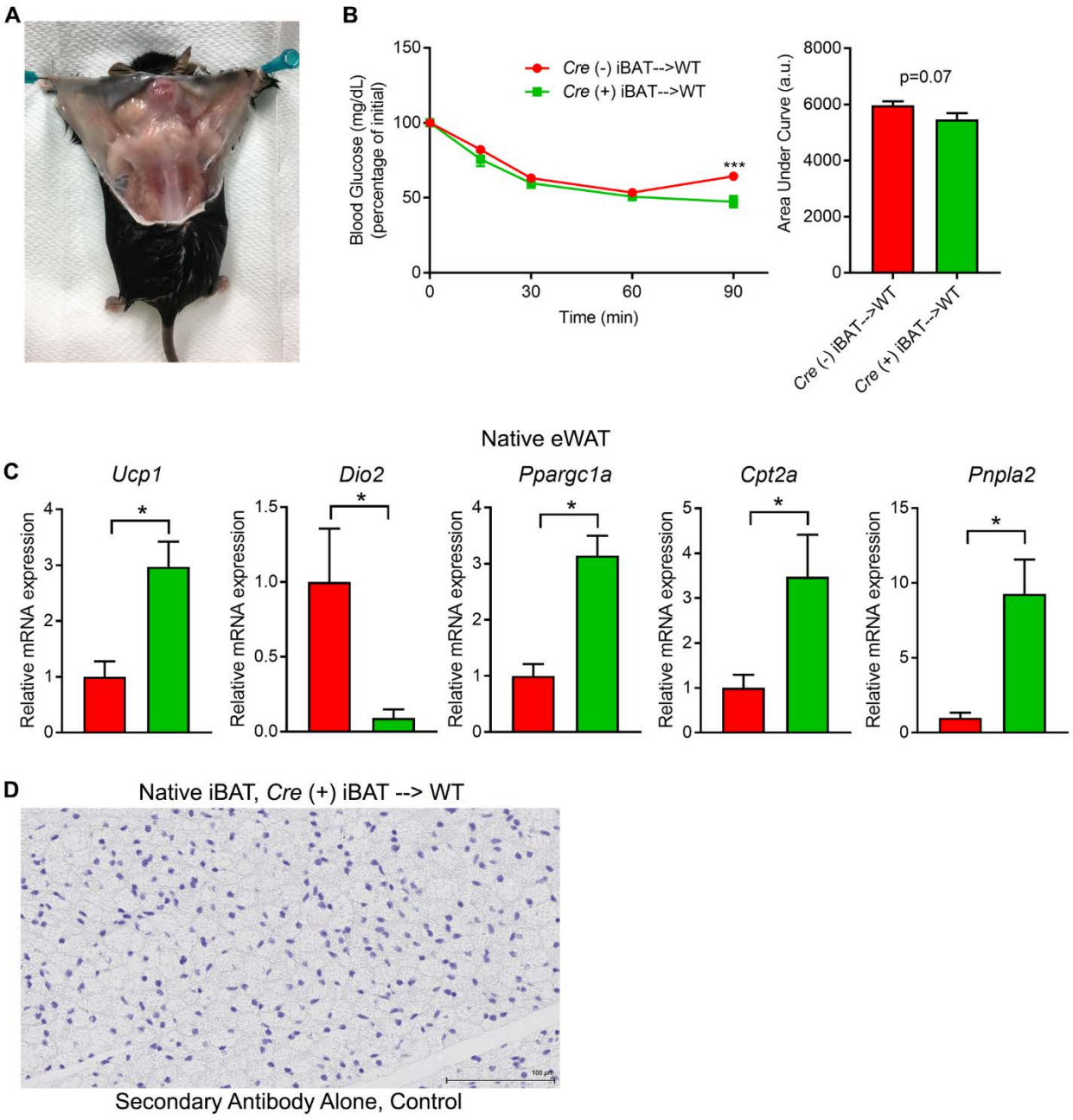


Figure S4. iBAT transplantation: effect of iBAT transplant devoid of adipocyte *Ager* to lean, male WT mice. Related to Figure 4. At age 6 weeks, standard chow fed WT mice were surgically transplanted with iBAT derived from either *Ager*$^{flox/flox}$ *Cre* (-) vs. *Ager*$^{flox/flox}$ *Cre* (+) donor mice. 4 days later, mice were switched to HFD for an additional 20 weeks. **(A)** Representative photograph of iBAT transplanted graft. **(B)** Insulin tolerance test (ITT) was performed after 13 weeks of high fat feeding and the results plotted as percent change in level of blood glucose from baseline and as the area under the curve. For the percent change in glucose excursion, the mean±SEM is reported in N=10-11 mice/group. Data were analyzed using repeated measures two-way ANOVA followed by a *post hoc*

Bonferroni test or an independent samples Student t-test (for AUC); ***p<0.001 & p=0.07, as indicated. **(C)** At sacrifice, eWAT was retrieved from the recipient mice and subjected to RT-qPCR to assess the relative mRNA expression of *Ucp1*, *Dio2*, *Ppargc1a*, *Cpt2a* and *Pnpla2*. The mean relative mRNA expression±SEM is reported in N=4-6 mice/group. Data analysis using an independent samples Student t-test or in the event that mean variances were statistically different, a *post hoc* Mann-Whitney U test was used. For results presented in B&C, $Ager^{flox/flox}$ *Cre* (-) (red bars) and $Ager^{flox/flox}$ *Cre* (+) (green bars). **(D)** Representative image of secondary antibody alone, control, for UCP1 immunohistochemistry in native iBAT post-transplantation with $Ager^{flox/flox}$ *Cre* (+) iBAT. Scale bar: 100 µm. In this figure, *p<0.05 & ***p<0.001.

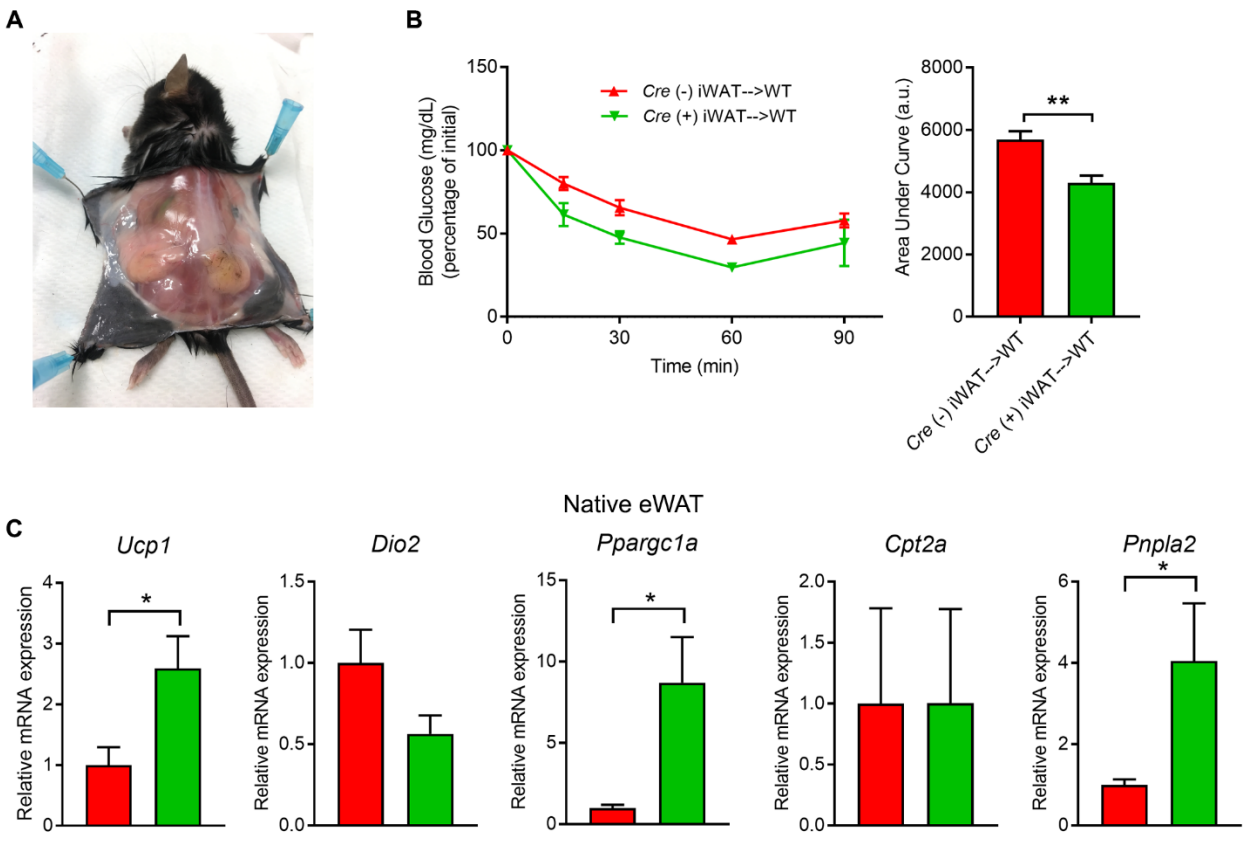


Figure S5. iWAT transplantation: effect of *Ager* devoid iWAT transplant to lean, male WT mice. Related to Figure 5. At age 6 weeks, standard chow fed WT mice were surgically transplanted with iWAT derived from either *Ager*$^{flox/flox}$ *Cre* (-) vs. *Ager*$^{flox/flox}$ *Cre* (+) donor mice. 4 days later, mice were switched to HFD for an additional 20 weeks. **(A)** Representative photograph of iWAT transplanted graft. **(B)** Insulin tolerance test (ITT) was performed after 13 weeks of high fat feeding and the results plotted as percent change in level of blood glucose from baseline and as the area under the curve. For the percent change in glucose excursion, the mean±SEM is reported in N=5-6 mice/group. Data were analyzed using repeated measures two-way ANOVA followed by a *post hoc* Bonferroni test or an independent samples Student t-test (for AUC). **(C)** At sacrifice, eWAT was retrieved from the recipient mice and subjected to RT-qPCR to assess the relative mRNA expression of *Ucp1*, *Dio2*, *Ppargc1a*, *Cpt2a* and *Pnpla2*. The mean relative mRNA expression±SEM is reported in N=3-4 mice/group. Data analysis was performed using an independent samples Student t-test or in the event that mean variances were statistically different, a *post hoc* Mann-Whitney U test was used. For results presented in B&C, *Ager*$^{flox/flox}$ *Cre* (-) (red bars) and *Ager*$^{flox/flox}$ *Cre* (+) (green bars). In this figure, *p<0.05 & **p<0.01.

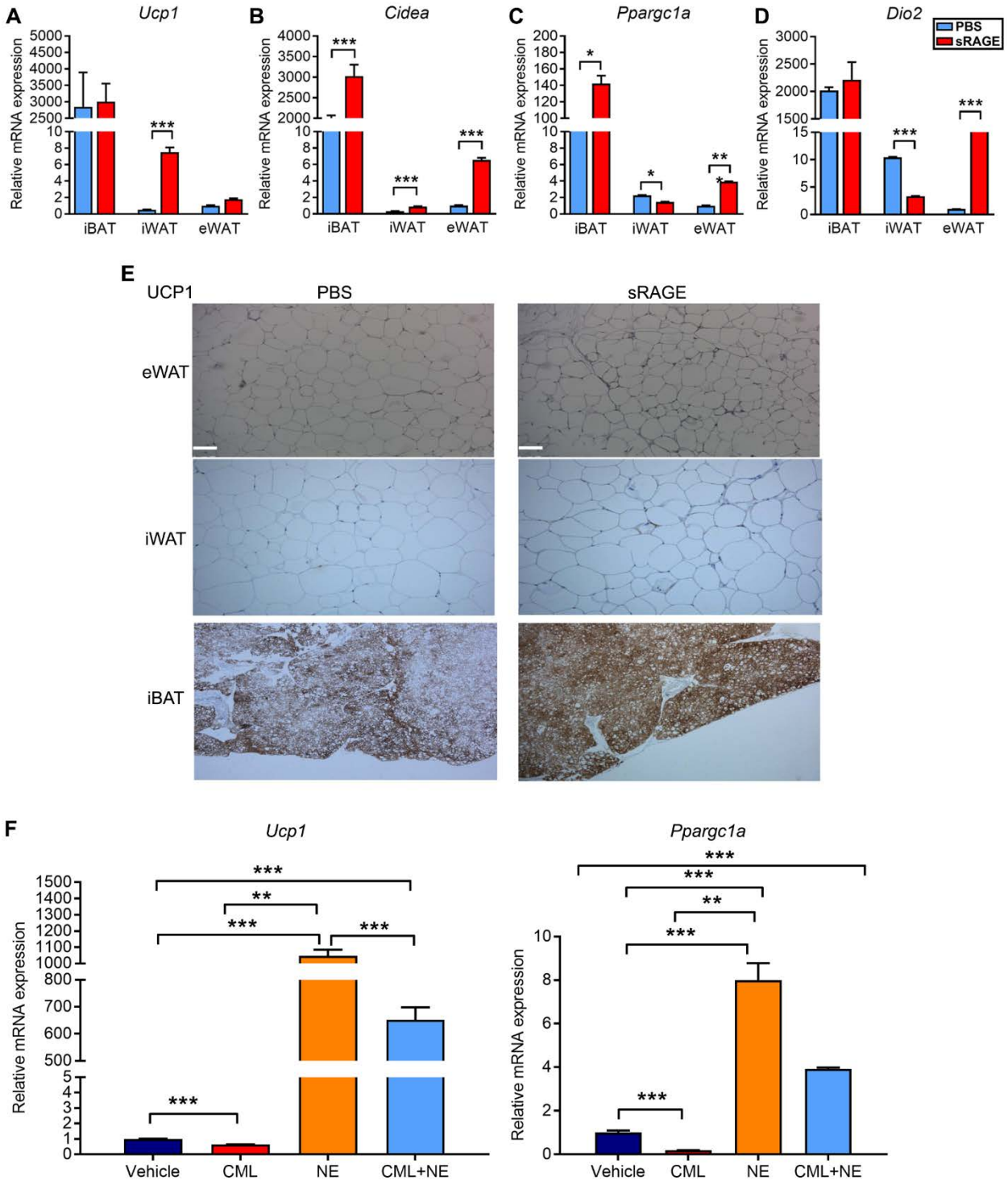
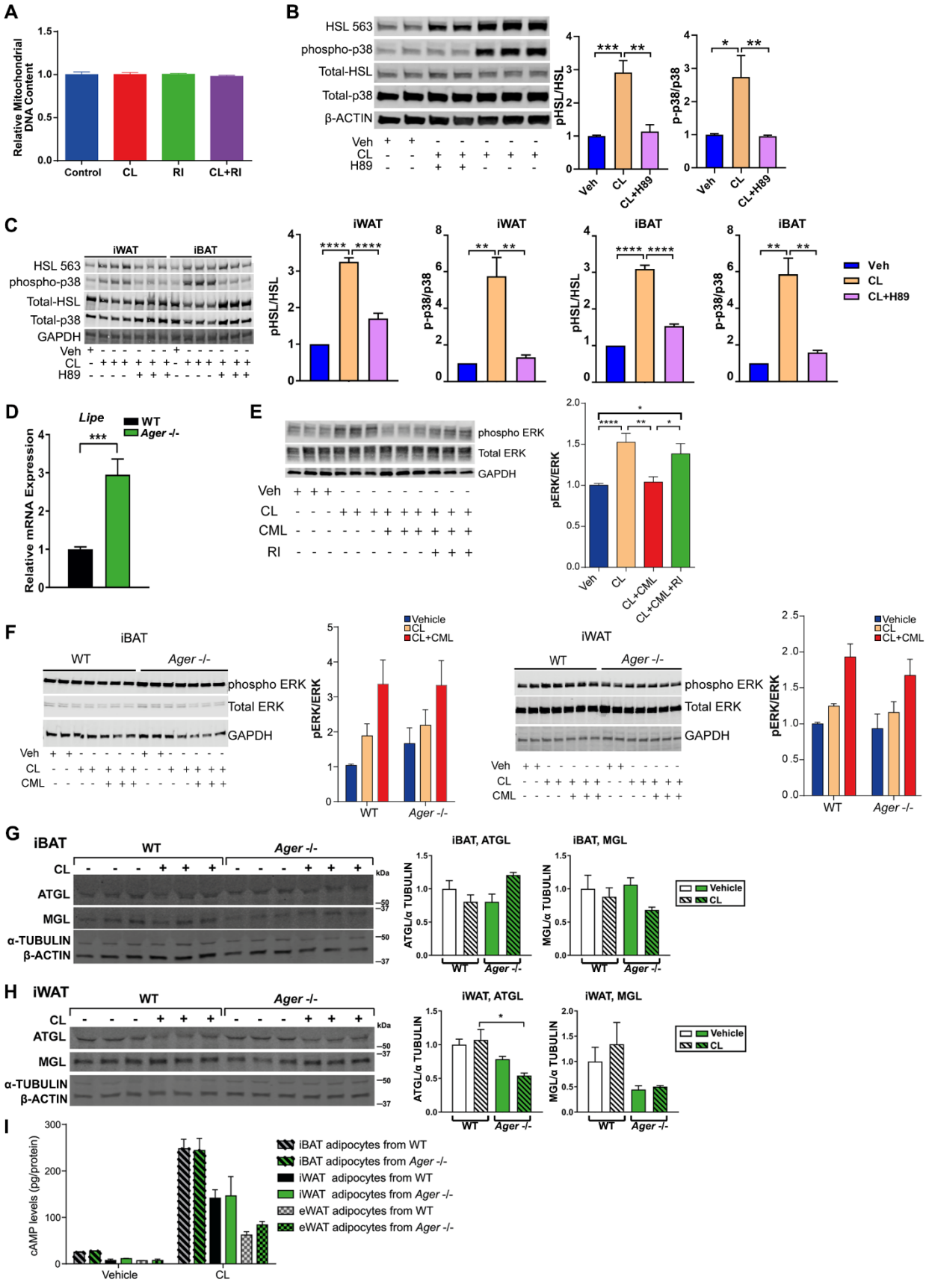


Figure S6. Effects of soluble RAGE *in vivo* and RAGE ligands on expression and induction of *Ucp1* and *Ppargc1a* in eWAT-derived adipocytes. Related to Figure 6. (A-D) WT mice were fed a HFD beginning at age 6 weeks; simultaneously, vehicle (phosphate buffered saline, PBS) or recombinant sRAGE (100 µg/day) was administered once daily for an additional 18 weeks. At sacrifice, eWAT, iWAT and iBAT were retrieved and

subjected to RT-qPCR for detection of **(A)** *Ucp1*, **(B)** *Cidea*, **(C)** *Ppargc1a* and **(D)** *Dio2* mRNA expression. The mean relative mRNA expression±SEM is reported in N=5 mice/group. Data were analyzed using independent samples Student t-test. **(E)** Representative images of eWAT, iWAT and iBAT stained for UCP1 after 18 weeks HFD and treatment with PBS vs. sRAGE. Scale bar: 100 µm. **(F)** Primary adipocytes were differentiated from eWAT SVF of WT mice and incubated with vehicle or RAGE ligand CML-AGE (300 µg/ml) for 16 hours alone or with NE (2 nM) for the final 6 hours of incubation. RT-qPCR for detection of relative *Ucp1* or *Ppargc1a* mRNA expression was performed. The mean relative mRNA expression±SEM is reported from cells originating from N=5 mice/group. Data were analyzed using independent samples one-way ANOVA followed by a *post hoc* Tukey's HSD test. In this figure, *$p<0.05$, **$p<0.01$ and ***$p<0.001$.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Relative Mitochondrial DNA Content
Control
CL
RI
CL+RI
B
HSL 563
phospho-p38
Total-HSL
Total-p38
β-ACTIN
Veh
CL
H89
pHSL/HSL
p-p38/p38
Veh
CL
CL+H89
C
iWAT
iBAT
HSL 563
phospho-p38
Total-HSL
Total-p38
GAPDH
Veh
CL
H89
pHSL/HSL
p-p38/p38
Veh
CL
CL+H89
D
Lipe
WT
Ager -/-
Relative mRNA Expression
E
phospho ERK
Total ERK
GAPDH
Veh
CL
CML
RI
pERK/ERK
Veh
CL
CL+CML
CL+CML+RI
F
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iWAT
WT
Ager -/-
phospho ERK
Total ERK
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Vehicle
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CL+CML
G
iBAT
WT
Ager -/-
CL
ATGL
MGL
α-TUBULIN
β-ACTIN
kDa
iBAT, ATGL
iBAT, MGL
ATGL/α TUBULIN
MGL/α TUBULIN
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CL
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I
cAMP levels (pg/protein)
iBAT adipocytes from WT
iBAT adipocytes from Ager -/-
iWAT adipocytes from WT
iWAT adipocytes from Ager -/-
eWAT adipocytes from WT
eWAT adipocytes from Ager -/-
Vehicle
CL

Figure S7. RAGE, intracellular levels of cAMP, expression of downstream lipolytic lipases and ERK MAPK regulation in adipocytes. Related to Figure 7. (A) Relative mitochondrial DNA content in C3H10T1/2 cells differentiated to adipocytes and treated with either CL316,243 (10 μM) for 15 mins and/or the RAGE Inhibitor (RI) (1 μM) for 30 mins was assessed by measurement of expression of mitochondrial gene, NADH subunit 1 (*Nd1*). Data are presented as relative mitochondrial DNA content±SEM and analyzed using independent samples one-way ANOVA. No statistically significant differences were observed. **(B)** C3H10T1/2 cells were differentiated into adipocytes and treated with vehicle or CL316,243 (10 μM) for 15 minutes alone or after pre-treatment with the PKA inhibitor, H89 (20 μM) for 30 mins. Cells were lysed and Western blotting performed for phosphorylated HSL Serine 563, phosphorylated p38, total HSL, total p38 and GAPDH. Band intensities were normalized to the respective total HSL or total p38 MAPK, as indicated, and the relative fold change is presented. The mean fold change±SEM of at least 2 independent studies with 2-3 technical replicates per experiment is shown. Data were analyzed using an independent measures one-way ANOVA followed by a *post hoc* Bonferroni test. **(C)** Primary adipocytes differentiated from iWAT (left) or iBAT (right) SVF of WT and *Ager* null mice were treated with vehicle or CL316,243 (10 μM) for 15 minutes alone or after pre-treatment with the PKA inhibitor, H89 (20 μM) for 30 minutes. Cells were lysed and Western blotting performed for phosphorylated HSL Serine 563, phosphorylated p38 MAPK, total HSL, total p38 MAPK and GAPDH. Band intensities were normalized to the respective total HSL or total p38 MAPK, as indicated, and the relative fold change is presented. The mean fold change±SEM is reported in cells derived from N=4 pooled mice/group in three independent experiments with at least two to three replicates per experiment. Data were analyzed using independent samples one-way ANOVA followed by a *post hoc* Tukey's HSD test. **(D)** Floating adipocytes were retrieved from the iWAT of WT or *Ager* null mice fed a HFD for 3 months and were subjected to RT-qPCR for detection of relative *Lipe* mRNA expression. The mean relative mRNA expression±SEM is reported. Data analysis was performed using independent samples Student's t-test. **(E)** C3H10T1/2 cells were differentiated into adipocytes and treated with vehicle, CML-AGE (300 μg/ml) for 75 minutes alone or after pre-treatment with the RAGE inhibitor (1 μM) for 30 minutes and with CL316,243 (10 μM) for 15 minutes. Cells were lysed and Western blotting performed for phosphorylated ERK MAPK, total ERK MAPK and GAPDH. Band intensities were normalized to total ERK and the relative fold change is presented. The mean fold change±SEM of at least three independent studies with at least three technical replicates per experiment is shown. Data were analyzed

using an independent measures one-way ANOVA followed with a *post hoc* Bonferroni test. **(F)** Primary adipocytes differentiated from iBAT left) or iWAT (right) SVF of WT and *Ager* null mice were treated with CL316,243 (10 μM) for 15 minutes after pre-treatment with RAGE ligand CML-AGE (300 μg/ml) for 1 hour. Cells were lysed and Western blotting performed for detection of phospho-ERK MAPK, total ERK MAPK and GAPDH. Band intensities were normalized to the respective total ERK and the relative fold change is presented as mean±SEM. The mean fold change±SEM is reported in cells derived from N=4 pooled mice/group in three independent studies with two to three technical replicates per experiment. Data were analyzed using independent samples one-way ANOVA followed by a *post hoc* Tukey's HSD test. No statistically significant differences were observed. **(G-H)** Primary adipocytes differentiated from **(G)** iBAT and **(H)** iWAT SVF retrieved from WT and *Ager* null mice were treated with CL316,243 (10 μM) for 15 minutes and Western blotting was performed for detection of ATGL, MGL, α-TUBULIN and β-ACTIN. Detected bands were normalized to α-TUBULIN and the mean fold change from control±SEM is reported. Data were analyzed using independent samples two-way ANOVA followed by a *post hoc* Tukey's HSD test. **(I)** Primary adipocytes were differentiated from iBAT, iWAT and eWAT SVF of WT and *Ager* null mice and stimulated with CL316,243 (10 μM) for 15 min. cAMP levels were measured from the cellular lysates. The mean level±SEM is reported in N=3 independent studies. Data analysis was performed using independent samples two-way ANOVA. Per adipose tissue depot, there were no statistically-significant genotype-dependent differences. In this figure, *$p<0.05$, **$p<0.01$, ***$p<0.001$ & ****$p<0.0001$.

Supplemental Table S1. Related to STAR Methods.
Taqman Primers

	Assay ID	Catalog
Acox1	Mm00446408_m1	4448892
Actb	Mm00607939_s1	4352341E
Adrb3	Mm02601819_g1	4453320
Ager	Mm01134790_g1	4448892
Ccl2	Mm00441242_m1	4453320
Cebp	Mm00514283_s1	4453320
Cidea	Mm00432554_m1	4453320
Creb	Mm00457268_m1	4448892
Cpt2	Mm00487205_m1	4448892
Dio2	Mm00515664_m1	4453320
Emr1	Mm00802529_m1	4453320
Fasn	Mm00662319_m1	4453320
Il10	Mm01288386_m1	4453320
Irf4	Mm00516431_m1	4453320
Pnpla2	Mm00503040_m1	4453320
Prdm16	Mm00712556_m1	4453320
Pparg	Mm00440940_m1	4453320
Ppargc1a	Mm01208835_m1	4453320
Tfam	Mm00447485_m1	4453320
Tlr2	Mm00442346_m1	4453320
Tlr4	Mm00445273_m1	4453320
Tnfa	Mm00443258_m1	4453320
Ucp1	Mm01244861_m1	4453320