JEM

Supplemental material

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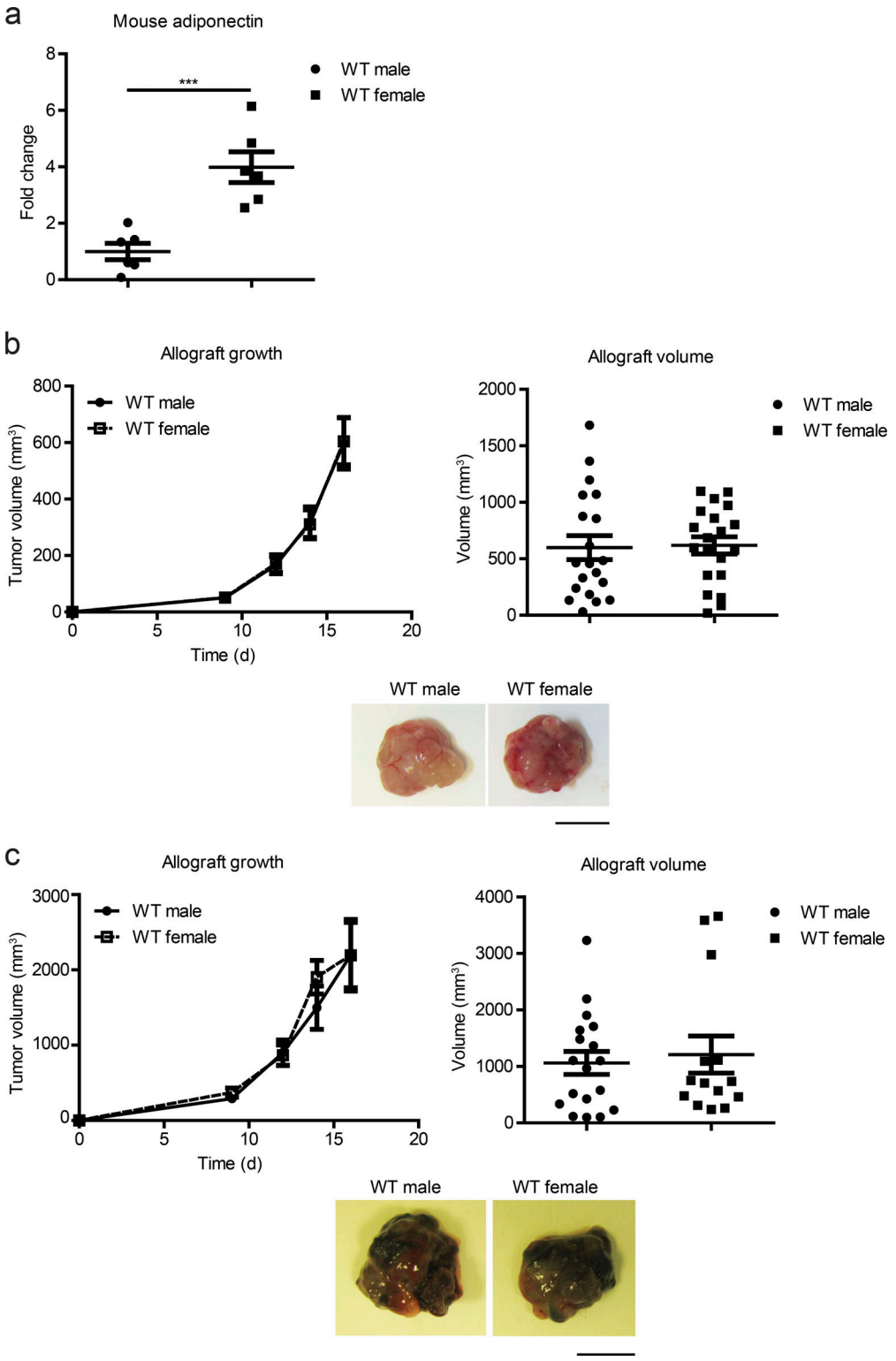

Figure S1. **Adiponectin quantification and specificity of gender differences in HCC. (a)** Circulating levels of adiponectin were measured in 11–12-wk-old female and male mice. Data are normalized to male mice and shown as means ± SEM; ***, P < 0.001; Student´s *t* test; *n* = 6. **(b)** Representative allografts and tumor volume quantification in WT male and female mice during the experiment and at sacrifice 3 wk after subcutaneous injection with 5 × 10^5 MC-38 cells (colon adenocarcinoma–derived cells) in each flank. Data are shown as means ± SEM; nonsignificant differences were found; two-way ANOVA coupled with Bonferroni's multiple comparisons test (allograft growth); Student's *t* test (allograft volume); *n* = 20 tumors (10 mice per genotype). Bar, 1 cm. **(c)** Representative allografts and tumor volume quantification in WT male and female mice during the experiment and at sacrifice 2 wk after subcutaneous injection with 5 × 10^5 B16-F10 cells (melanoma-derived cells) in each flank. Data are shown as means ± SEM; nonsignificant differences were found; two-way ANOVA coupled with Bonferroni's multiple comparisons test (allograft growth); Student's *t* test (allograft volume); WT male *n* = 18 tumors (10 mice); WT female *n* = 14 tumors (7 mice). Bar, 1 cm.

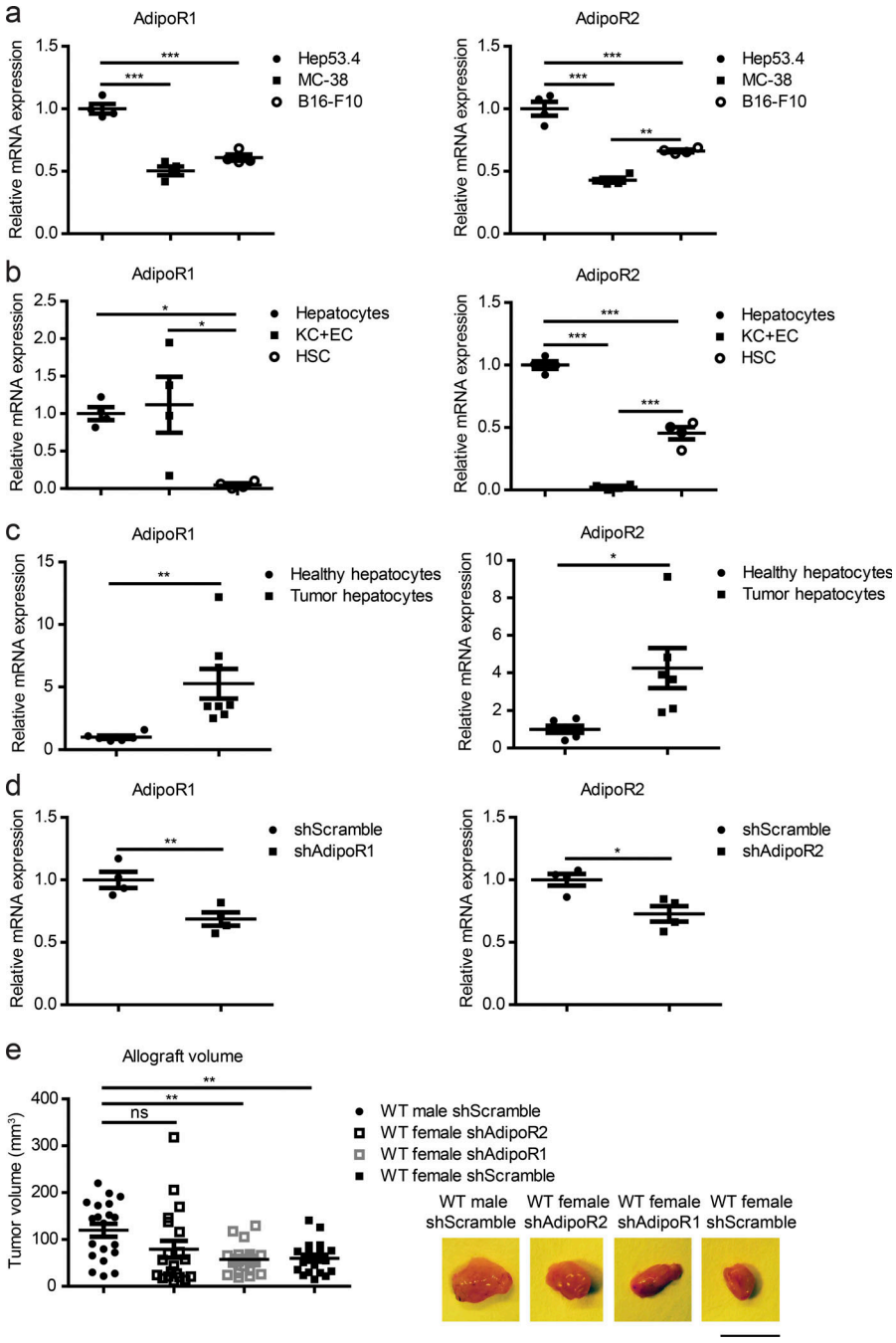

Figure S2. **Analysis of adiponectin receptors expression and their role in tumor growth. (a)** qRT-PCR analysis of adiponectin receptors 1 and 2 (*AdipoR1* and *AdipoR2*) in Hep53.4, MC-38, or B16-F10 tumor cells. mRNA expression was normalized to the amount of *Gapdh* mRNA in each sample. Data are normalized to Hep53.4 cells and shown as means ± SEM; **, P < 0.01; ***, P < 0.001; one-way ANOVA coupled with Bonferroni's multiple comparisons test; *n* = 4. **(b)** qRT-PCR analysis of *AdipoR1* and *AdipoR2* in different liver cell populations. mRNA expression was normalized to the amount of *Gapdh* mRNA in each sample. Data are normalized to hepatocytes and shown as means ± SEM; *, P < 0.05; ***, P < 0.001; one-way ANOVA coupled with Bonferroni's multiple comparisons test; *n* = 4. **(c)** qRT-PCR analysis of *AdipoR1* and *AdipoR2* in healthy hepatocytes and hepatocytes derived from hepatic tumors of C57BL/6J mice treated with DEN at P14 and 300 µg/liter TAA administered in the drinking water for 26 wk. mRNA expression was normalized to the amount of *Gapdh* mRNA in each sample. Data are normalized to healthy hepatocytes and shown as means ± SEM; *, P < 0.05; **, P < 0.01; Student's *t* test with Welch's correction; healthy hepatocytes *n* = 6; tumor hepatocytes *n* = 6–8. **(d)** qRT-PCR analysis of *AdipoR1* in Hep53.4 treated with a shRNA against AdipoR1 (shAdipoR1) or a scrambled control sequence, and analysis of *AdipoR2* in Hep53.4 cells treated with a shRNA against AdipoR2 (shAdipoR2) or a scrambled control sequence. mRNA expression was normalized to the amount of *Gapdh* mRNA in each sample. Data are normalized to shScramble cells and shown as means ± SEM; *, P < 0.05; **, P < 0.01; Student's *t* test; *n* = 4. **(e)** Representative allografts and tumor volume quantification in WT mice with hepatic tumors lacking AdipoR1 or AdipoR2 expression. Mice received subcutaneous injections with 1 × 10^6 Hep53.4 cells per flank, previously transduced with shRNA targeting AdipoR1, AdipoR2 or a control sequence (shScramble). Mice were sacrificed 3 wk after Hep53.4 cell injection. Data are shown as means ± SEM; **, P < 0.01; nonsignificant differences (ns); one-way ANOVA coupled with Bonferroni's multiple comparisons test; WT *n* = 20 tumors (10 mice per group), except WT female shAdipoR1 *n* = 18 tumors (10 mice). Bar, 1 cm.

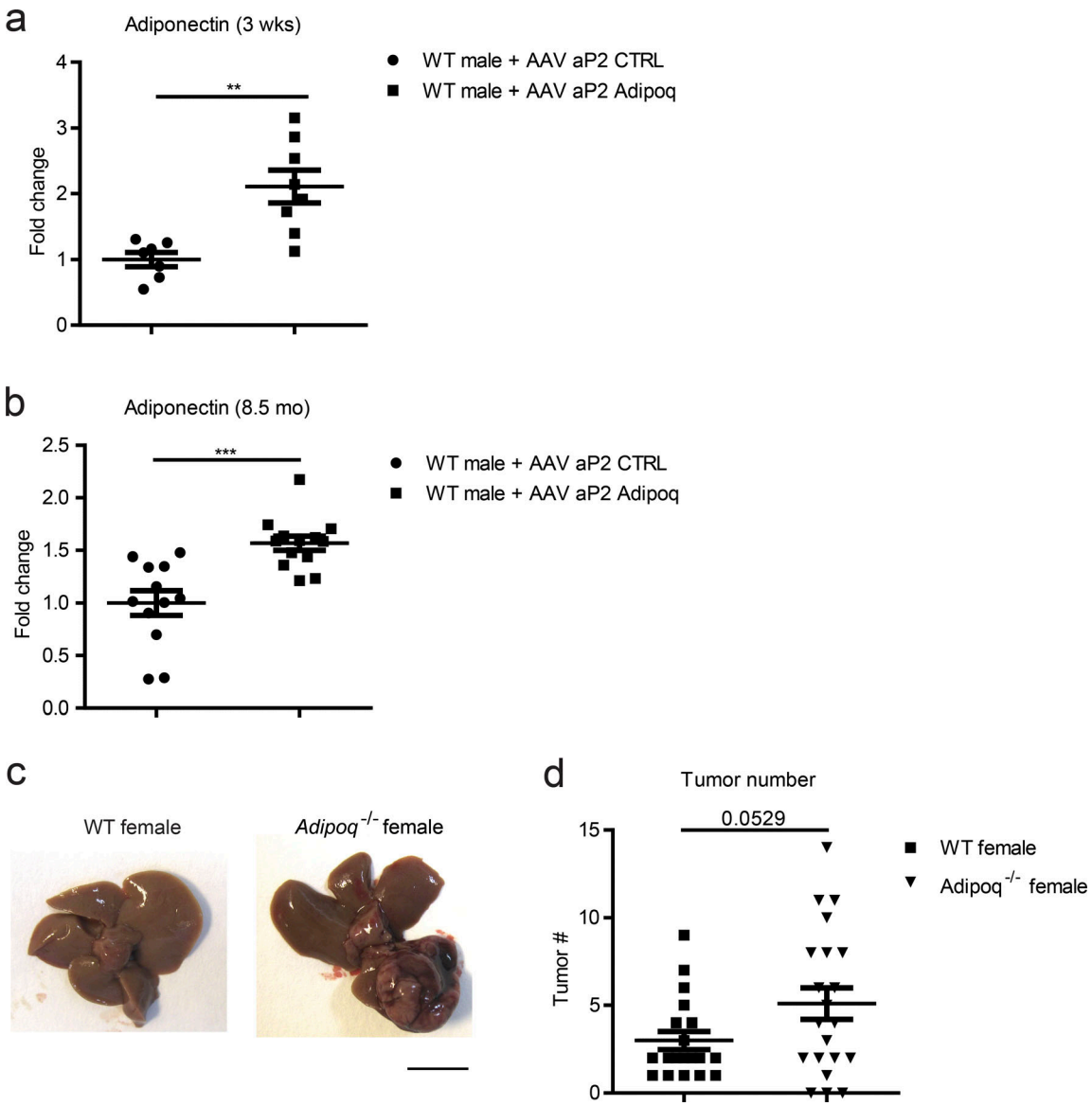

Figure S3. **Adiponectin quantification and overexpression in mice and effect of adiponectin deficiency in female mice. (a and b)** 6–7-wk-old WT male mice were injected with adeno-associated virus carrying a control sequence (WT male + AAV aP2 CTRL) or the adiponectin gene under control of the aP2 promoter (WT male + AAV aP2 Adipoq) at P1 and received an i.p. DEN injection (50 mg/kg body weight) 14 d later. AAV, adeno-associated virus; CTRL, control. **(a)** Quantification of circulating levels of adiponectin 3 wk after virus injection. Data are shown as means ± SEM; **, P < 0.01; Student´s *t* test with Welch's correction; WT male + AAV aP2 CTRL *n* = 7; WT male + AAV aP2 Adipoq *n* = 8. **(b)** Quantification of circulating levels of adiponectin 8.5 mo after virus injection. Data are shown as means ± SEM; ***, P < 0.001; Student's *t* test; WT male + AAV aP2 CTRL *n* = 12; WT male + AAV aP2 Adipoq *n* = 13. **(c)** HCC development 8 mo after i.p. injection with DEN (50 mg/kg) on P14 in WT and *Adipoq*$^{-/-}$ female mice. Bar, 1 cm. **(d)** Tumor number was determined at sacrifice. Data are shown as means ± SEM; Student's *t* test with Welch's correction; WT female *n* = 19; *Adipoq*$^{-/-}$ female *n* = 21.

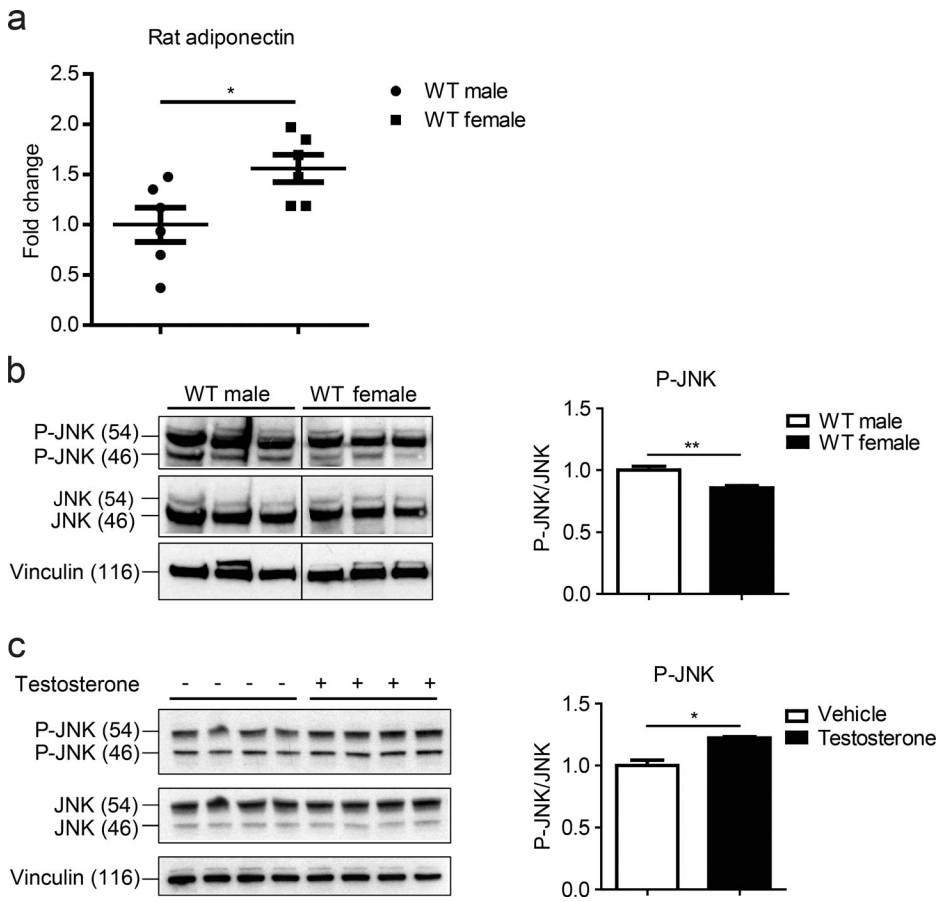

Figure S4. **Gender disparity in rat adiponectin, mice adipose tissue, and human adipocytes JNK phosphorylation. (a)** Circulating levels of adiponectin were measured in WT male and female rats. Data are normalized to male rats. Data are shown as means ± SEM; *, P < 0.05; Student's *t* test; *n* = 6. **(b)** Immunoblot analysis and quantification of phospho-JNK and JNK in adipose tissue from WT male and female mice. Vinculin protein expression was monitored as a loading control. Data are shown as means ± SEM; **, P < 0.01; Student's *t* test; WT male *n* = 3; WT female *n* = 4. **(c)** Immunoblot analysis and quantification of phospho-JNK and JNK in human differentiated adipocytes after treatment with testosterone (1,200 nM for 30 min). Vinculin protein expression was monitored as a loading control. Data are shown as means ± SEM; *, P < 0.05; Student's *t* test with Welch's correction; *n* = 4.

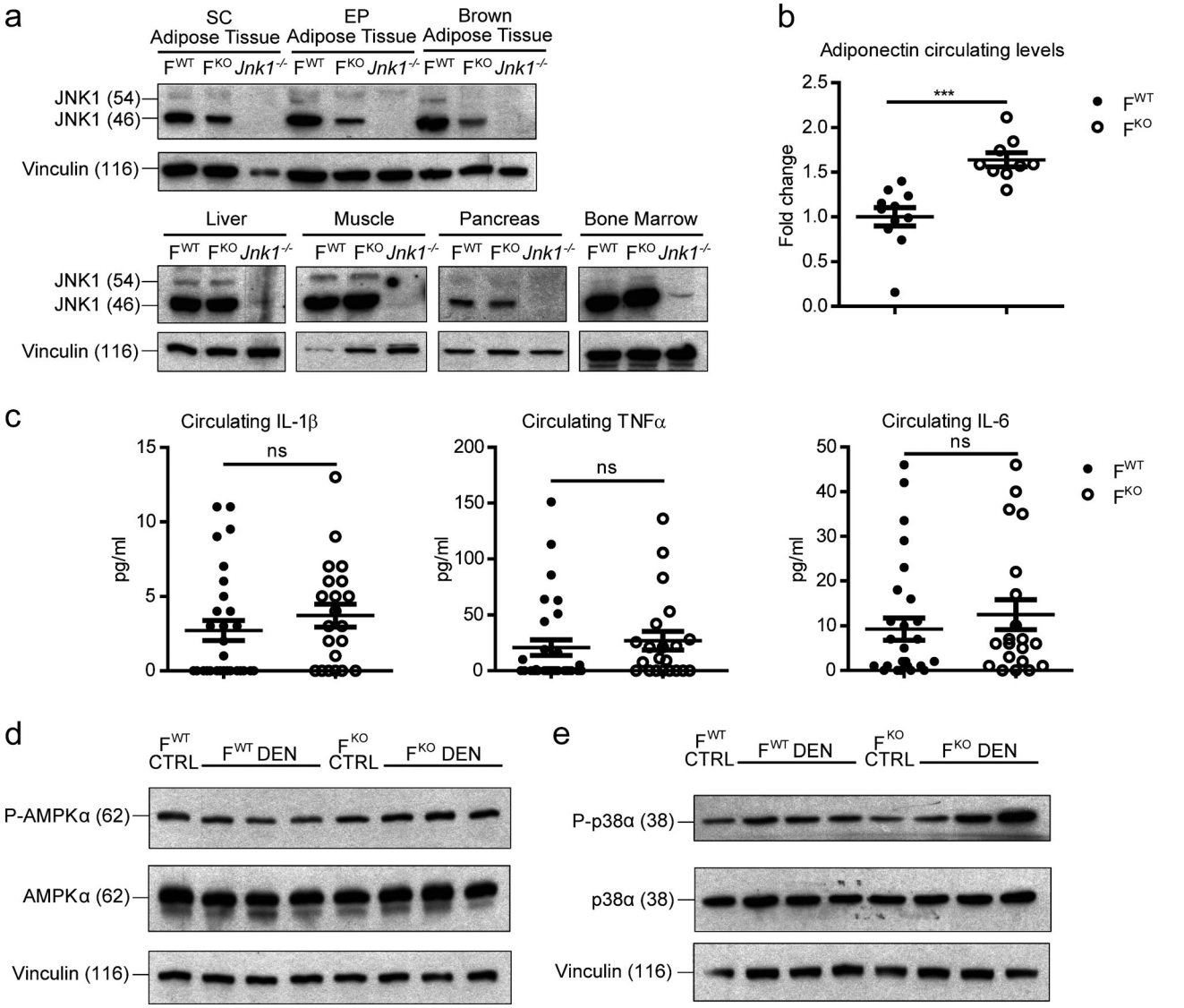

Figure S5. **Deletion of JNK1, cytokines levels in F^{WT} and F^{KO} mice, and adiponectin protection through AMPKα and p38α activation. (a)** Control (F^{WT}) and adipose tissue JNK1-deficient (F^{KO}) mice were sacrificed at 10 wk, and different tissues were extracted and analyzed by immunoblotting. Tissues from *Jnk1*$^{-/-}$ mice were used as a control. EP, epididymal; SC, subcutaneous. Vinculin protein expression was monitored as a loading control. **(b)** Circulating levels of adiponectin were measured in control (F^{WT}) and adipose tissue JNK1-deficient (F^{KO}) mice. Data are normalized to F^{WT} adiponectin levels and are shown as means ± SEM; ***, $P < 0.001$; Student's *t* test; F^{WT} $n = 11$; F^{KO} $n = 9$. **(c)** Control (F^{WT}) and adipose tissue JNK1-deficient (F^{KO}) mice were injected i.p. with DEN (50 mg/kg) on P14. Serum was analyzed after 8 mo on a Luminex platform to measure the levels of TNFα, IL-1β, and IL-6 adipokines. Data are shown as means ± SEM; nonsignificant differences were found (ns); Student's *t* test; $n = 20–30$. **(d and e)** F^{WT} and F^{KO} mice were injected i.p. with DEN (50 mg/kg) or saline (CTRL) on P14. **(d)** Immunoblot analysis of phospho-AMPKα, AMPKα and vinculin in livers obtained 15 d after DEN injection. **(e)** Immunoblot analysis of phospho-p38α, p38α, and vinculin in livers obtained 1 mo after DEN injection.

Table S1. **Characteristics of women and men**

Variable	Women (*n* = 9)	Men (*n* = 10)	P value
Age (yr)	49.7 (14.3)	58.1 (14.3)	0.278
Hypertension (n)	1 (11.1)	2 (20)	0.542
Diabetes mellitus (n)	0	0	–
BMI (kg/m^2)	25.8 (3.5)	26.8 (4.5)	0.905
Fasting blood sugar (mg/dl)	86.1 (12.8)	99 (10.5)	0.046
AST (IU/liter)	24.3 (12.9)	21.1 (5.1)	0.798
ALT (IU/liter)	32.5 (32.2)	30.3 (20.7)	0.878
Alkaline phosphatase (IU/liter)	77.4 (21.4)	95.3 (35.3)	0.536
Bilirubin (mg/dl)	0.5 (0.3)	0.9 (0.5)	0.059
Albumin (mg/dl)	4.5 (0.3)	4.5 (0.6)	0.607
Total cholesterol (mg/dl)	205.9 (50)	189.9 (47.9)	0.383
Triglycerides (mg/dl)	118.7 (67.5)	116 (49.7)	0.902
LDL-cholesterol (mg/dl)	121.7 (44.5)	123.7 (43.7)	0.945
HDL-cholesterol (mg/dl)	62.1 (14.6)	42.9 (12.7)	0.035
Adiponectin (µg/ml)	19.69 (2.663)	14.18 (3.620)	0.0016

Variables are presented as mean (SD) or absolute frequency (%) and are compared by means of Mann–Whitney *U* test or χ^2 test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table S2. **qRT-PCR primers**

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
AdipoR1	AATGGGGCTCCTTCTGGTAAC	GGATGACTCTCCAACGTCCCT
AdipoR2	GGCCCATCATGCTATGGAAC	GTGAGGGATCACTCGCCATC
Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGA

Table S3. **Solutions for hepatic perfusion**

Solution (ml)	A	B	C	D
SC-1	100	–	–	–
SC-2	–	100	100	100
DNAse I (stock solution)	–	–	–	1[a]
Collagenase D (mg)	–	–	110	80
Pronase E (mg)	–	40	–	50

[a]DNase I stock solution: 2 mg/ml in GBSS-B.

Table S4. **Stock solutions for hepatic cell isolation**

Stock solution (mg)	SC1	SC2	GBSS-A	GBSS-B
EGTA	95	-	-	-
Glucose	450	-	495.5	495.5
HEPES	1,190	1,190	-	-
KCl	200	200	185	185
$Na_2HPO_4 \cdot 2H_2O$	75.5	75.5	37.5	37.5
NaCl	4,000	4,000	-	4,000
$NaH_2PO_4 \cdot H_2O$	39	39	-	-
$NaHCO_3$	175	175	113.5	113.5
Phenol Red	3	3	3	3
$CaCl_2 \cdot 2H_2O$	-	280	112.5	112.5
KH_2PO_4	-	-	15	15
$MgCl_2 \cdot 6H_2O$	-	-	105	105
$MgSO_4 \cdot 7H_2O$	-	-	35	35
H_2O to (ml)	500	500	500	500

For the density gradient medium, the following solutions were prepared before starting the perfusion of the liver: Nycodenz 1: 5.18 g/total volume 15 ml GBSS-A; Nycodenz 2: 3.63 g/total volume 25 ml GBSS-A.