SUPPLEMENTARY DATA

Supplementary Table 1. Primary antibodies for Western blot.

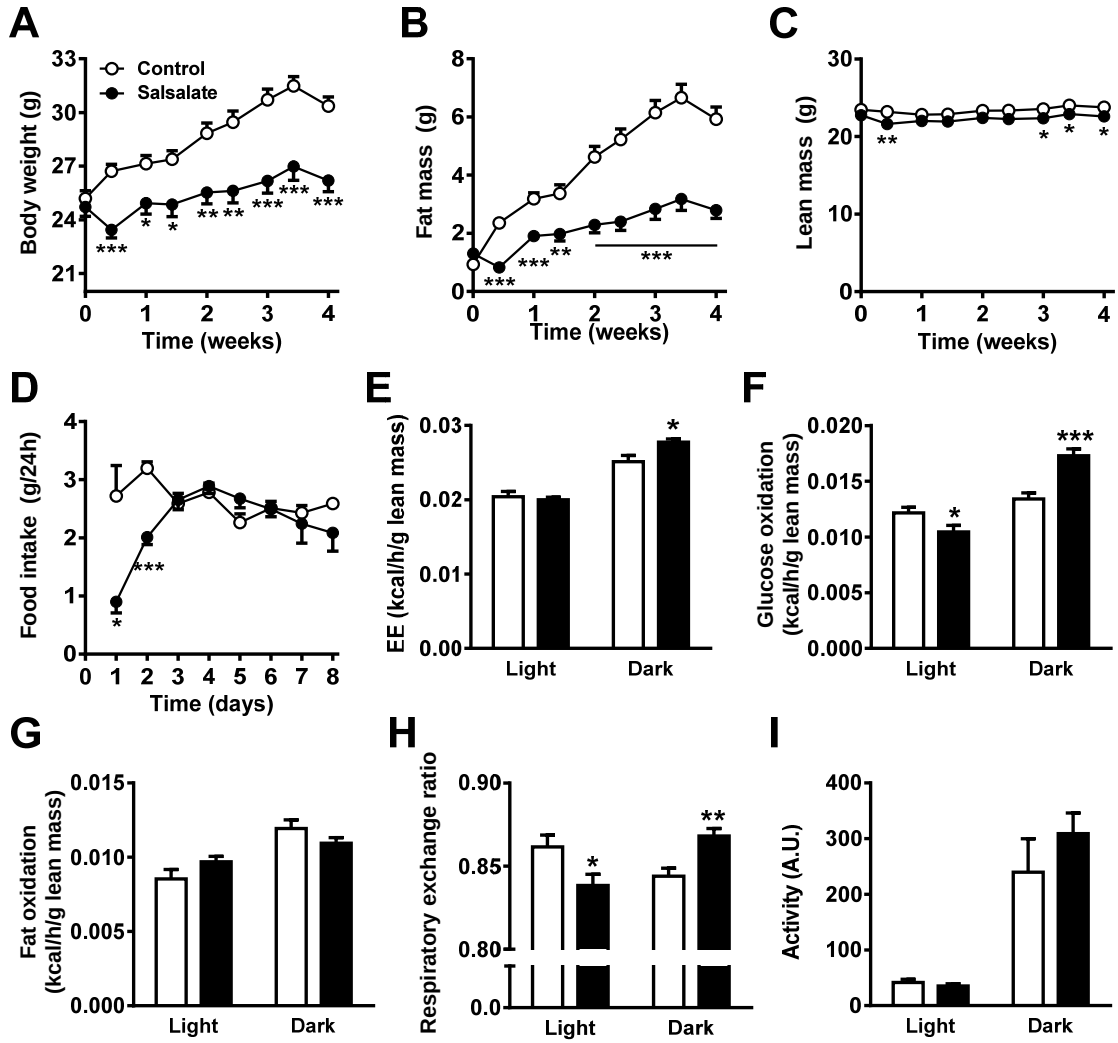
Primary antibody	Residue	Supplier	Reference	Dilution
ACC	-	Cell Signaling	#3662	1:1000
pACC	Ser79	Cell Signaling	#3661	1:1000
AMPKα	-	Cell Signaling	#2532	1:1000
pAMPKα	Thr172	Cell Signaling	#2535	1:1000
pHSL	Ser563	Cell Signaling	#4139	1:1000
pHSL	Ser565	Cell Signaling	#4137	1:1000
PKA substrates	Ser/Thr	Cell Signaling	#9621	1:1000
α/β Tubulin	-	Cell Signaling	#2148	1:1000

Supplementary Table 2. Primer sequences of forward and reverse primers (5' → 3').

Gene	Forward primer	Reverse Primer
36b4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Acc1	AACGTGCAATCCGATTTGTT	GAGCAGTTCTGGGAGTTTCG
Acc2	AGATGGCCGATCAGTACGTC	GGGGACCTAGGAAAGCAATC
Adrb3	TGAAACAGCAGACAGGGACA	AGTCTGTCAGCTTCCCTCCA
Cd36	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
Cpt1a	AGGAGACAAGAACCCCAACA	AAGGAATGCAGGTCCACATC
Elovl3	TGTTGGCCAGACCTACATGA	ATCCGTGTAGATGGCAAAGC
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Fasn	CACAGGCATCAATGTCAACC	TTTGGGAAGTCCTCAGCAAC
Fizz1	CCTGCCCTGCTGGGATGACT	GGGCAGTGGTCCAGTCAACGA
Mcp1	GCATCTGCCCTAAGGTCTTCA	TTCACTGTCACACTGGTCACTCCTA
Nos2	TCCTGGACATTACGACCCCT	CTCTGAGGGCTGACACAAGG
Ppargc1α	TGCTAGCGGTTCTCACAGAG	AGTGCTAAGACCGCTGCATT
Pparα	CAACCCGCCTTTTGTCATAC	CCTCTGCCTCTTTGTCTTCG
Scd1	GCTCTACACCTGCCTCTTCGGGAT	TCCAGAGGCGATGAGCCCCG
Srebp1c	CTGGCTGAGGCGGGATGA	TACGGGCCACAAGAAGTAGA
Ucp1	TCAGGATTGGCCTCTACGAC	TGCATTCTGACCTTCACGAC
Ym1	ACAATTAGTACTGGCCCACCAGGAA	TCCTTGAGCCACTGAGCCTTCA
β2m	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATA

SUPPLEMENTARY DATA

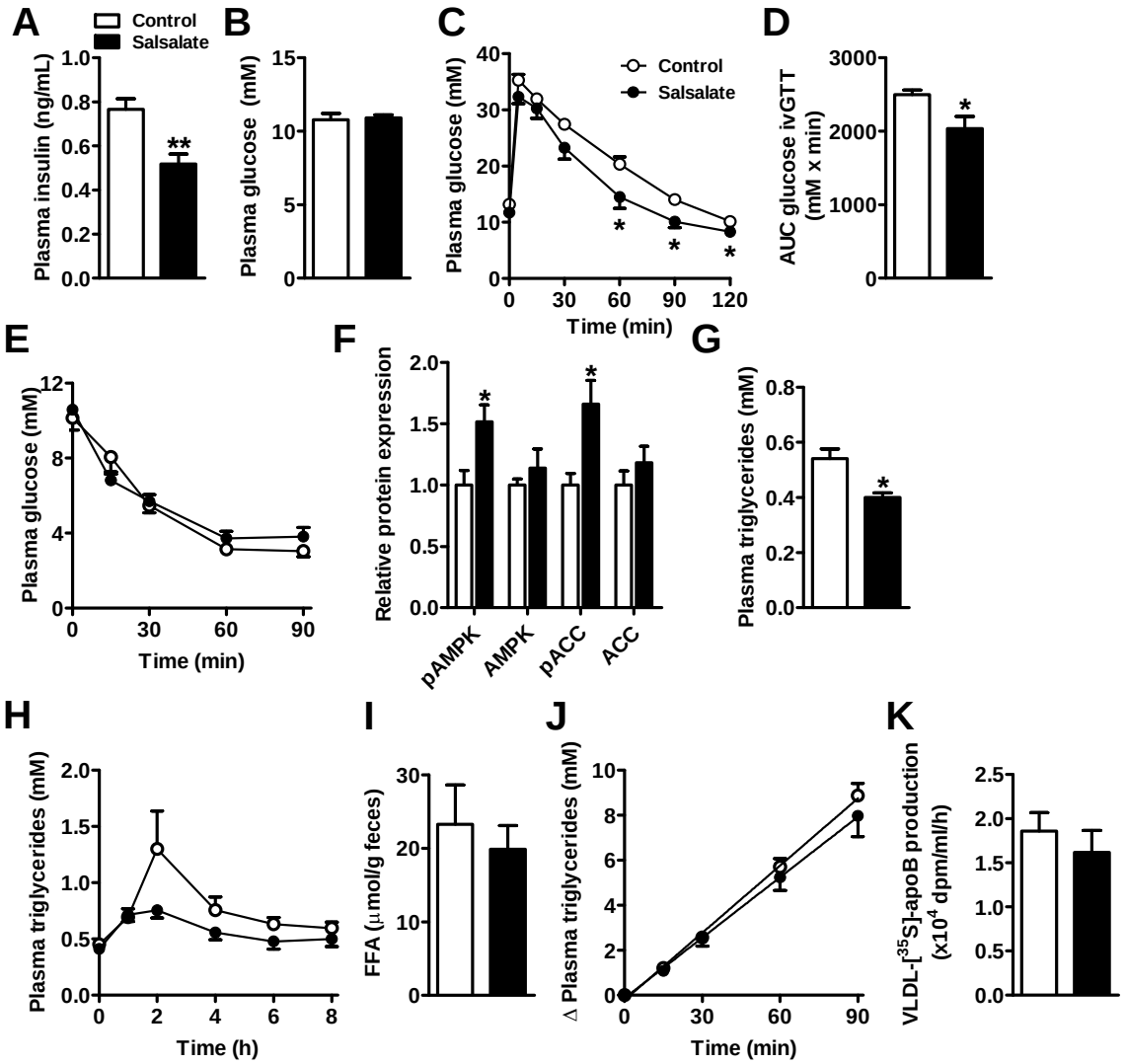
Supplementary Figure 1. Salsalate prevents obesity and fat mass accumulation in wild type mice fed a high fat diet. 10-week old male wild type mice (C57Bl/6J background) were fed a high fat diet (HFD) without (open circles) or with (closed circles) salsalate for 4 weeks (A-D). Body weight (A), fat mass (B) and lean mass (C) were monitored throughout the experiment. Food intake was measured daily in the first week of the experiment (D). From day 5 to day 9 of salsalate treatment, mice were housed in fully automatic metabolic cages (LabMaster System; TSE Systems, Bad Homburg, Germany), which measured oxygen uptake (V_{O2}), carbon dioxide production (V_{CO2}) and caloric intake. Total energy expenditure (E) was calculated from V_{O2} and V_{CO2} using the Weir equation, and glucose oxidation (F) and fat oxidation (G) were calculated from V_{O2} and V_{CO2} as described previously (1). Respiratory exchange ratio (H) was also calculated from V_{O2} and $V_{CO2.}$ Physical activity (I) was measured with infrared sensor frames. Measurements (E-H) were corrected for lean mass as determined by EchoMRI (EchoMRI-100, Houston, Texas, USA). EE = energy expenditure. Values represent means ± SEM (n=7-8). * p<0.05, ** p<0.01, *** p<0.001 *vs* control.



SUPPLEMENTARY DATA

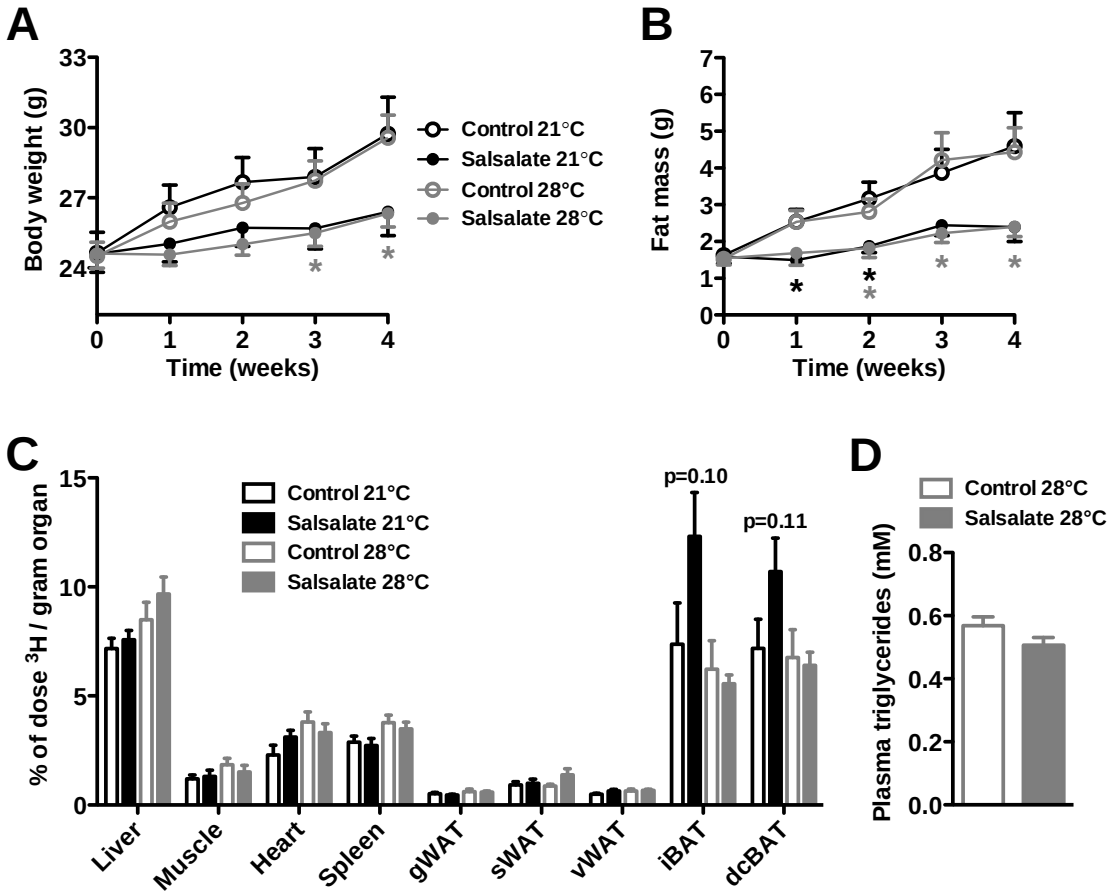
Supplementary Figure 2. Salsalate improves glucose and triglyceride metabolism in wild type mice fed a high fat diet. 10-week old male wild type mice were fed a high fat diet (HFD) without (open circles/bars) or with (closed circles/bars) salsalate for 4 weeks. Blood samples from 6 h-fasted mice were collected by tail bleeding at different time points and plasma insulin (A), glucose (B) and triglyceride (G) levels were determined. After 4 weeks, 6 h fasted mice were i.v. injected with glucose and additional blood samples were taken at 5, 15, 30, 60, 90 and 120 min after injection (C-D). After 4 weeks, an insulin tolerance test was performed by injecting 6 h fasted mice i.p.with insulin (NovoRapid, Novo Nordisk, Denmark; 1 U/kg whole body mass) and measuring glucose at t = 15, 30, 60 and 90 min (E). Protein content in muscle was determined by Western blot (F). Postprandial triglyceride (TG) response was measured after 3 weeks of salsalate treatment. Animals were fasted for 6 h and a basal blood sample was drawn before an intragastric load of 200 µL olive oil (Carbonell, Traditional, Cordoba, Spain) was given. Blood samples were drawn 1, 2, 4, 6 and 8 h after the bolus via tail vein bleeding and plasma TG levels were measured (H). Feces was collected during the 3rd week of salsalate treatment. Feces was then weighed, freeze-dried and ground, and fecal fatty acids were determined by methyl esterification. To this end, 750 µL CH_3OH/NaOH (10 M)/H_2O (3:1:1 v/v) was added to the feces and samples were incubated and mixed in a thermomixer (600 rpm, 90°C) for 1 h. Then, 1050 µL of HCl (6 M)/hexane (3:7.7 v/v) was added before samples were vortexed and spun down 1 min at 14,000 rpm. The upper hexane layer was dried using N_2 and redissolved in 2% Triton X-100. Fatty acids were measured using the NEFA C kit (Wako Diagnostics, Instruchemie, Delfzijl) (I). After 4 weeks of salsalate treatment, hepatic VLDL production was determined. Mice were fasted for 4 h and anesthetized by intraperitoneal injection of 6.25 mg/kg acepromazine (Alfasan, Weesp), 6.25 mg/kg midazolam (Roche, Mijdrecht), and 0.31 mg/kg fentanyl (Janssen Pharmaceuticals, Tilburg). Mice were injected intravenously with Tran[^{35}S] label (20 µCi/mouse; MP Biomedicals, Eindhoven) to label newly produced apolipoprotein B (apoB). After 30 min, at t = 0 min, Triton WR-1339 (Sigma-Aldrich) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Blood samples were drawn before (t = 0) and at 15, 30, 60, and 90 min after injection of Triton and used for determination of plasma TG concentration (J). After 120 min, mice were exsanguinated via the retro-orbital plexus. VLDL was isolated from serum after density gradient ultracentrifugation at $d < 1.006$ g/ml by aspiration (2) and examined for incorporated ^{35}S-activity as a measure of ApoB production rate (K). AUC = area under the curve; FFA = free fatty acids. Values represent means ± SEM (n=5-8). * $p<0.05$, ** $p<0.01$ vs control.

SUPPLEMENTARY DATA



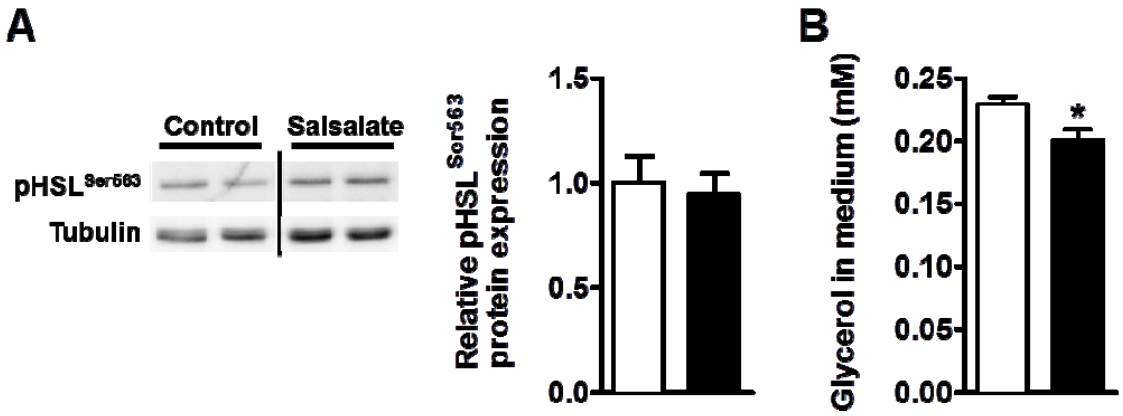
SUPPLEMENTARY DATA

Supplementary Figure 3. Effect of salsalate at thermoneutrality 10-week old male wild type mice were housed at 21°C (black circles/bars) or 28°C (grey circles/bars) and fed a high fat diet (HFD) without (open circles/bars) or with (closed circles/bars) salsalate for 4 weeks. Body weight (A) and fat mass (B) were monitored throughout the experiment. A clearance experiment was performed in which 6 h-fasted mice were i.v. injected with [^{3}H]TO-labeled lipoprotein-like emulsion particles. After 15 min, mice were sacrificed and uptake of [^{3}H]TO-derived activity was determined in the organs (C). After 4 weeks of treatment, blood samples from 6 h-fasted mice were collected by tail bleeding and plasma triglyceride levels were determined (D). Values represent means ± SEM (n=7-8). * $p<0.05$ *vs* control. *(g,s,v)WAT*, gonadal, subcutaneous, visceral white adipose tissue; *(i,dc)BAT*, interscapular, dorsocervical brown adipose tissue.

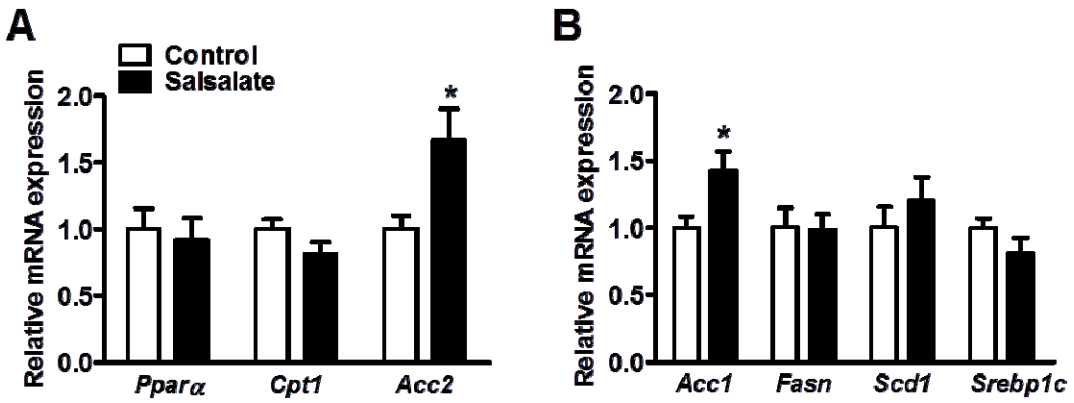


SUPPLEMENTARY DATA

Supplementary Figure 4. Effect of salsalate on 3T3-L1 cells. 3T3-L1 cells were cultured and differentiated into white adipocytes. White adipocytes were treated with 300 µM salsalate for 8 h. Protein content was determined by Western blot (A) and glycerol concentration in the culture medium was measured (B). Values represent means ± SD (n=3). * $p<0.05$ *vs* control.

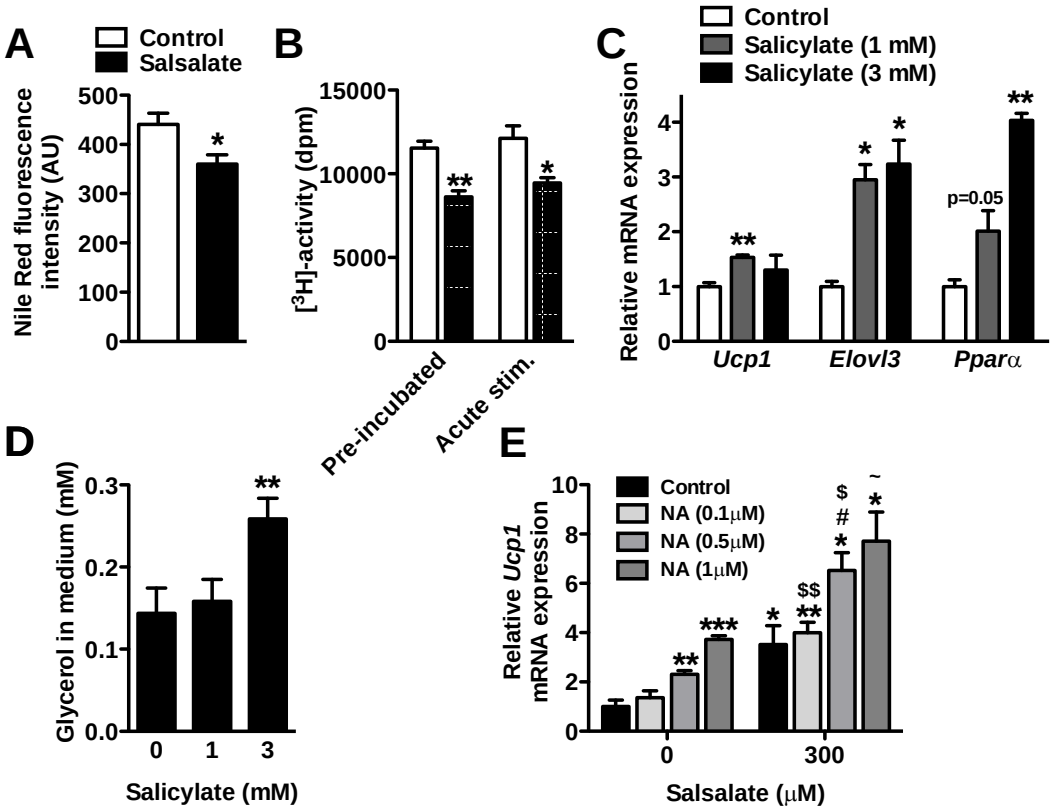


Supplementary Figure 5. Effect of salsalate on genes involved in beta-oxidation and lipogenesis in gWAT of wild type mice fed a high fat diet. 10-week old male wild type mice were fed a high fat diet (HFD) without (open bars) or with (closed bars) salsalate for 4 weeks and gonadal white adipose tissue (gWAT) was collected. mRNA expression was determined by qRT-PCR (A-B). Values represent means ± SEM (n=7-8). * $p<0.05$ *vs* control.



SUPPLEMENTARY DATA

Supplementary Figure 6. *In vitro* effects of salsalate and salicylate on T37i brown adipocytes. T37i cells were cultured and differentiated into mature brown adipocytes. Cells (grown in 0.32 cm^2 wells) were treated with salsalate (300 µM) for 8 h, stained with 0.1 mg/mL Nile Red solution (Molecular probes, N-1142) for 2 h at 37°C and measured with a fluorimeter (Ex. 485 nm, Em. 590 nm). Values represent means ± SEM of 11 independent wells (A). To assess *de novo* lipogenesis, cells (grown in 3.8 cm^2 wells) were incubated at 37°C with HEPES buffer (29.8 g HEPES, 8.78 g NaCl, 4.66 g KCl, 1.12 g D-glucose, 18.8 g BSA, 0.132 g $CaCl_2$ in 1.125 L H_2O, pH 7.3), 100 nM insulin and [^{3}H]glucose (~500,000 cpm). Cells were either pre-incubated with vehicle or salsalate for 6 h or the compound was added together with the [^{3}H]glucose. After 2 h, cells were washed and 600 µL cold $CHCl_3$/CH_3OH (1:1 v/v) was added. Cells were left on ice for 30 min, upon which the supernatant was isolated. Next, 125 µL H_2O was added and the samples were mixed and spun for 10 min at 3,500 rpm. The $CHCl_3$ layer was isolated and evaporated under N_2 and incorporation of [^{3}H]glucose-derived radioactivity was quantified. Values represent means ± SEM of 4-5 independent wells (B). Cells were treated with salicylate (1 or 3 mM) for 8 h and mRNA expression was determined by qRT-PCR (C). In addition, glycerol concentration in the medium was assessed (D). Cells were treated with salsalate (300 µM) in the presence of increasing concentrations of noradrenalin (0.1 – 1 µM) for 8 h and *Ucp1* mRNA expression was determined (E). NA = noradrenalin. Values represent means ± SD of 3-4 independent sets of RNA. * p<0.05, ** p<0.01 *vs* control , $ p<0.05, $$ p<0.01 vs NA, ~ p<0.10 *vs* NA and salsalate.



SUPPLEMENTARY DATA

References

1. van Klinken,JB, van den Berg,SA, Havekes,LM, Willems van,DK: Estimation of activity related energy expenditure and resting metabolic rate in freely moving mice from indirect calorimetry data. *PLoS One* 7:e36162, 2012
2. Redgrave,TG, Roberts,DC, West,CE: Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal Biochem* 65:42-49, 1975