SUPPLEMENTARY DATA

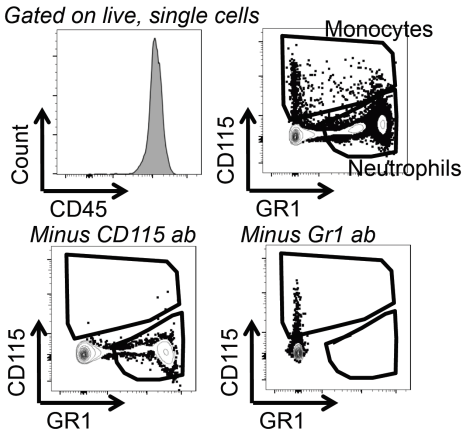
A novel strategy to prevent advanced atherosclerosis and lower blood glucose in a mouse model of metabolic syndrome

Jenny E. Kanter, Farah Kramer, Shelley Barnhart, Jeffrey M. Duggan, Masami Shimizu- Albergine, Vishal Kothari, Alan Chait, Stephan D. Bouman, Jessica A. Hamerman, Bo F. Hansen, Grith S. Olsen and Karin E. Bornfeldt

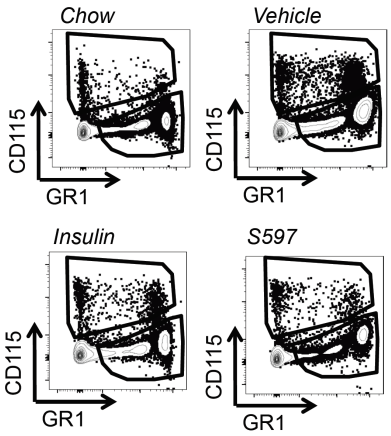
Supplementary Figure S1. Gating strategies for flow cytometry. (*A-B*) Staining strategy for blood leukocytes and representative flow cytometry contour plots. (*C*) Gating strategy and representative flow cytometry plots for identifying LT-HSC and eGMP in bone marrow cells. All gating was determined based on staining controls containing all antibodies except one (flow minus). For analysis of bone marrow progenitor cell populations, bone marrow cells were harvested from two femurs and two tibias per mouse and erythrocytes were lysed using ACK lysis buffer (Lonza, Basel, Switzerland). Lineagenegative progenitor cells were enriched from bone marrow using a mouse Lineage Cell Depletion Kit (Miltenyi Biotec, San Diego, CA). For cell surface staining, lineagenegative progenitors were stained with PE-labeled anti-CD16/32 mAb or a combination of mouse and rat IgG at 4° C for 10 minutes, followed by staining with biotin-conjugated mAbs to CD11B, F4/80, GR1, CD11C, CD3, and NK1.1 at 4°C for 20 minutes. These lineage markers were used for negative gating during flow cytometry. Lineage-negative cells were subsequently surface stained with fluorescently-labeled mAbs to CD34, Sca- 1, CD135, Streptavidin, and CD117 at 4°C for 60-90 min. Data were acquired using a BD LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar). Long- Term Hematopoietic Stem Cells (LT-HSCs) were identified as Lineage- (Lin-) $CD117^{+}$ $Sca\text{-}1^{+}$ $CD34^{-}$ $CD135^{-}$ cells.

SUPPLEMENTARY DATA

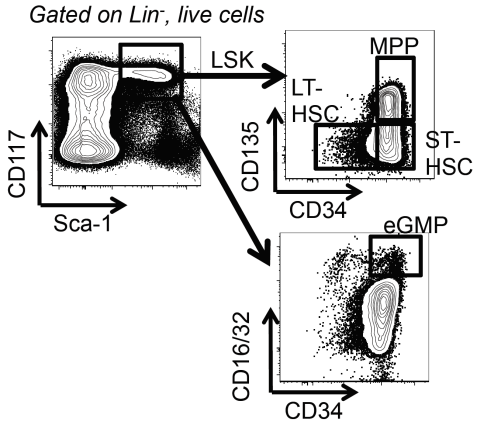
A. Staining strategy for blood leukocytes



B. Representative blood leukocyte contour plot

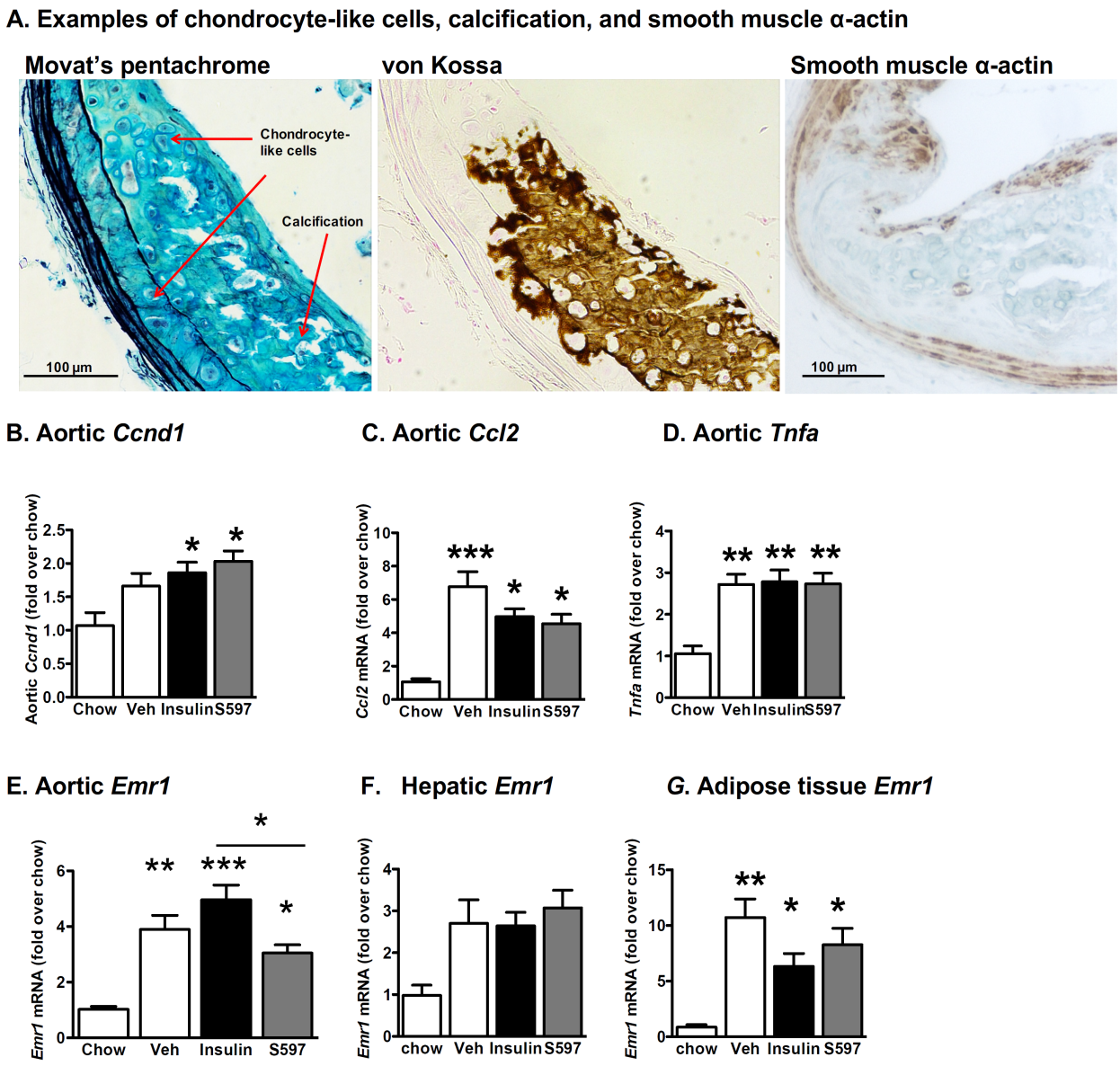


C. Staining and gating on eGMP and LT-HSC



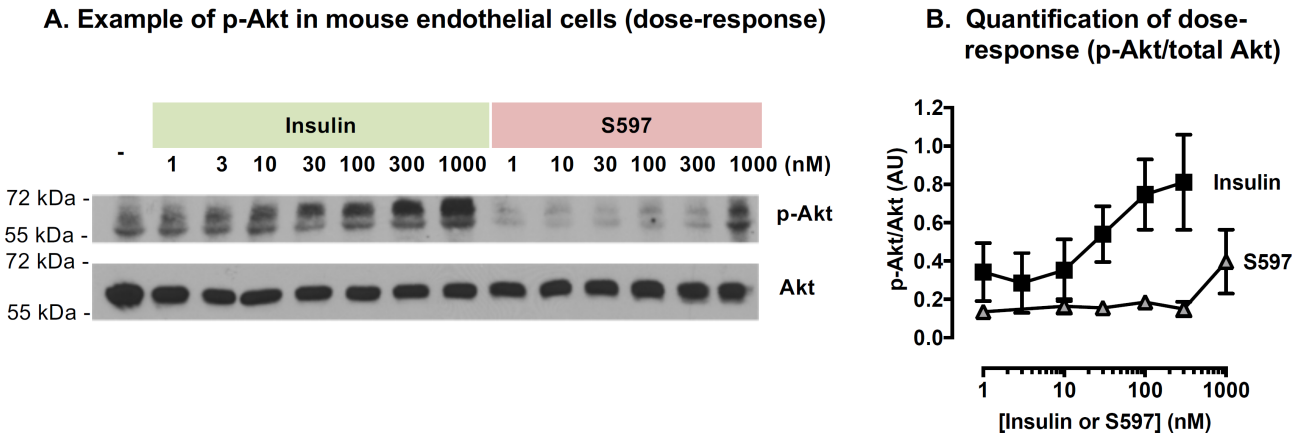
SUPPLEMENTARY DATA

Supplementary Figure S2. Arterial lesion morphology and inflammatory markers. (*A*) Examples of markers of advanced lesions in the brachiocephalic artery. Chondrocyte-like cells and calcification determined by Movat's pentachrome stain (left-most panel) and adjacent section stained with von Kossa for calcification (middle panel). A high magnification photo of a section stained for a-smooth muscle actin, present both in the lesion's fibrous cap and in the media (right-most panel). (*B-E*) Aortic *Ccnd1* (cyclin D1), *Ccl2*, *Tnfa* and *Emr1* (F4/80) mRNA. Data were normalized to *Rn18s* values and presented using the ΔΔCT method with the chow group as control. Similar results were obtained when the data were normalized to *Rpn32* or *Gapdh*. (*F*) Hepatic *Emr1* mRNA levels. (*G*) Epididymal adipose tissue *Emr1* mRNA levels. Data are expressed as mean ± SEM, n=5 chow, DDC groups vehicle n=14, insulin n=13, S597 n=13. *p<0.05, **p<0.01, *** p<0.001 compared to chow or as indicated, one-way ANOVA.



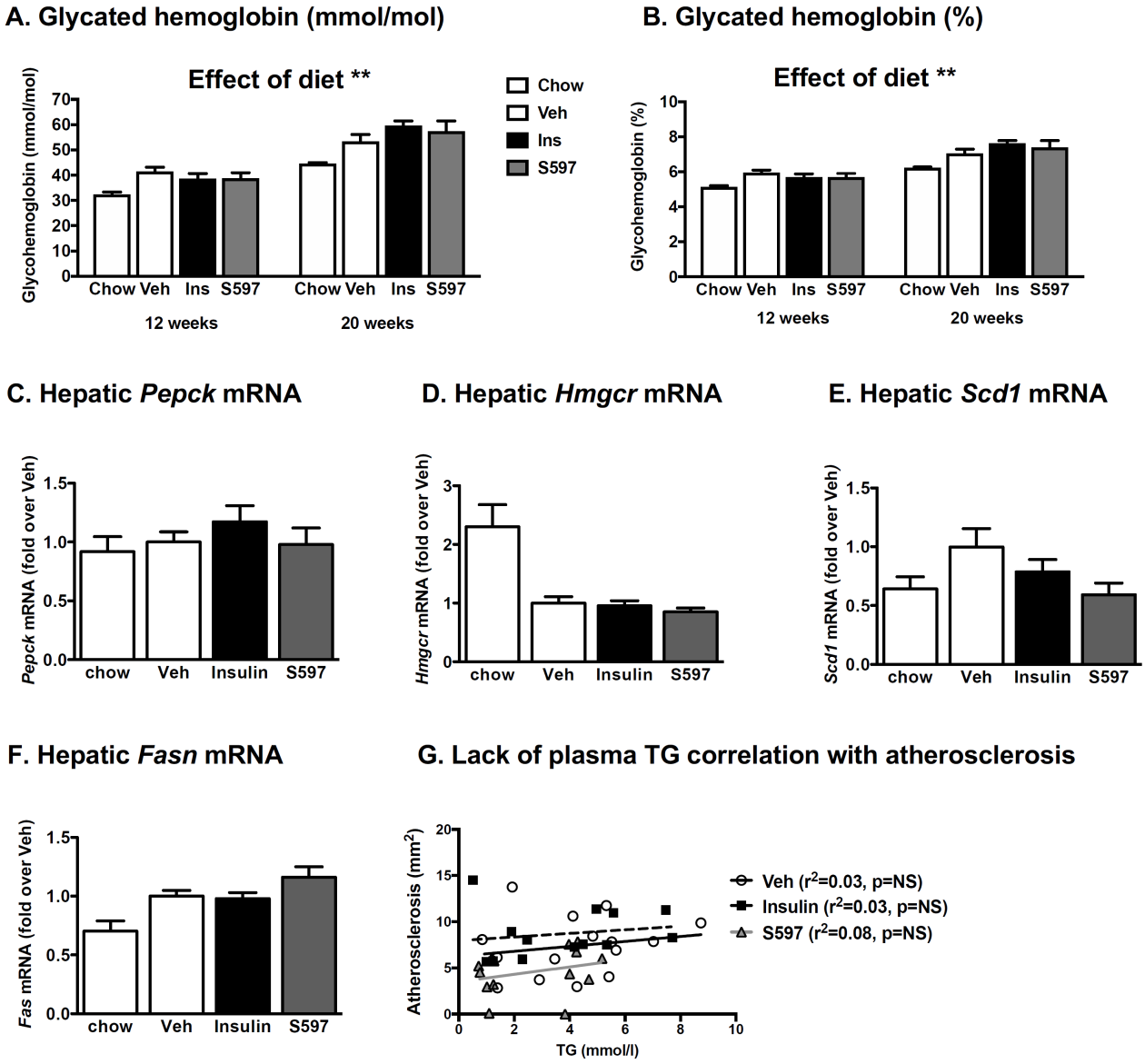
SUPPLEMENTARY DATA

Supplementary Figure S3. S597 does not activate Akt until very high concentrations are reached in mouse endothelial cells. Mouse endothelial cells were isolated from hearts, and Diabetes stimulated with the indicated concentrations of insulin or S597 for 15 minutes. Levels of p-Akt (Ser473) and total Akt were detected by Western blot analysis (*A*). (*B*) Results were quantified and expressed as mean ± SEM (n=3).



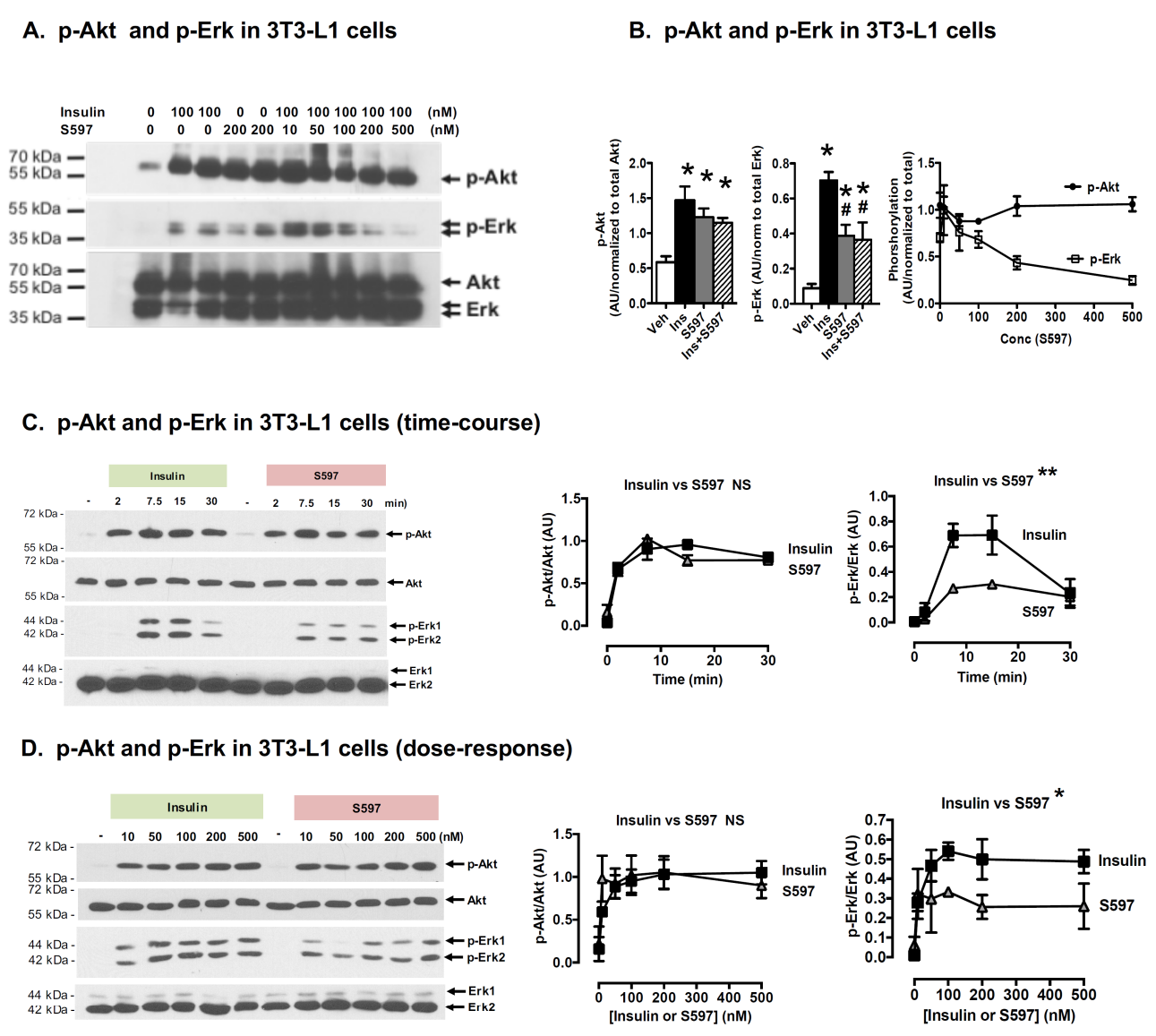
SUPPLEMENTARY DATA

Supplementary Figure S4. S597 does not significantly affect glycated hemoglobin or hepatic expression of genes involved in lipid metabolism. (*A-B*) Glycated hemoglobin was measured in plasma at the end of the study. (*C-F*) Livers were isolated at the end of the 22-week study and subjected to reverse transcription and real-time PCR. After each assay, a dissociation curve was run to confirm specificity of PCR amplicons. All primer reactions were analyzed on agarose gels for correct size and the presence of a single reaction product. Resulting Ct values were normalized to *Rn18s*, *Rpn32* and *Gapdh*, and the ΔΔCt method was then used to express values as fold-over control samples. All samples were run in at least duplicates, and statistical analysis was performed on $2^{-(\Delta Ct)}$ values. (*C*) Hepatic *Pepck* mRNA, (*D*) *Hmgcr* mRNA, (*E*) *Scd1* mRNA, and (*F*) *Fasn* mRNA levels. (*G*) Lack of correlation between plasma triglycerides and atherosclerosis. A subset of mice had received insulin 10 minutes prior to euthanasia. Data are expressed as mean ± SEM, chow n=5, DDC groups vehicle n=14, insulin n=13, S597 n=13, unless otherwise specified. There were no significant differences between vehicle-treated mice, insulin-treated mice and S597-treated mice. ** p<0.01 by two-way ANOVA.



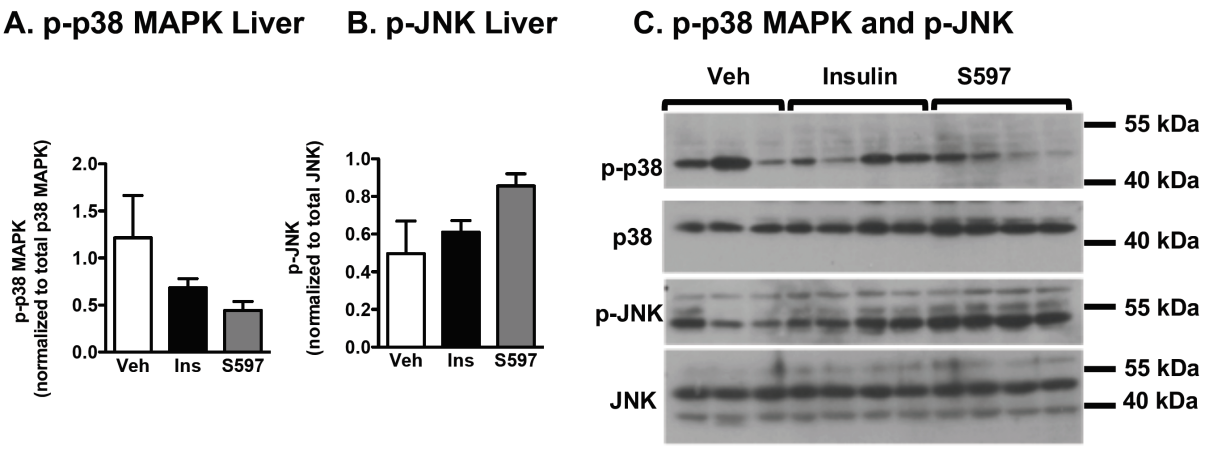
SUPPLEMENTARY DATA

Supplementary Figure S5. S597 preferentially activates Akt over Erk at a wide range of concentrations and different time-points, and inhibits insulin-induced Erk activation in 3T3-L1 cells. (*A-B*) Differentiated 3T3-L1 cells were stimulated with vehicle (Veh), 100 nM insulin, 200 nM S597, or a combination of 100 nM insulin and 200 nM S597 (*A*) or increasing concentrations of S597 in nM (*B*) for 7.5 min. Western blots were used to detect phosphorylated Akt (Ser473) and Erk (Thr202/Tyr204) and total Akt and Erk. Statistical analysis was performed by one-way ANOVA; *p<0.05 versus Veh and #p<0.05 versus insulin. Results are expressed as mean ± SEM (n=3). (*C-D*) Time-course studies (*C*) and dose-response curves (*D*) demonstrate that S597 and insulin are equally effective in activating Akt but that S597 is significantly less able to activate Erk over a wide range of concentrations and time-points. Results are expressed as mean ± SEM (n=3). Statistical analysis was performed by two-way ANOVA; *p<0.05, **p<0.01 S597 versus insulin.



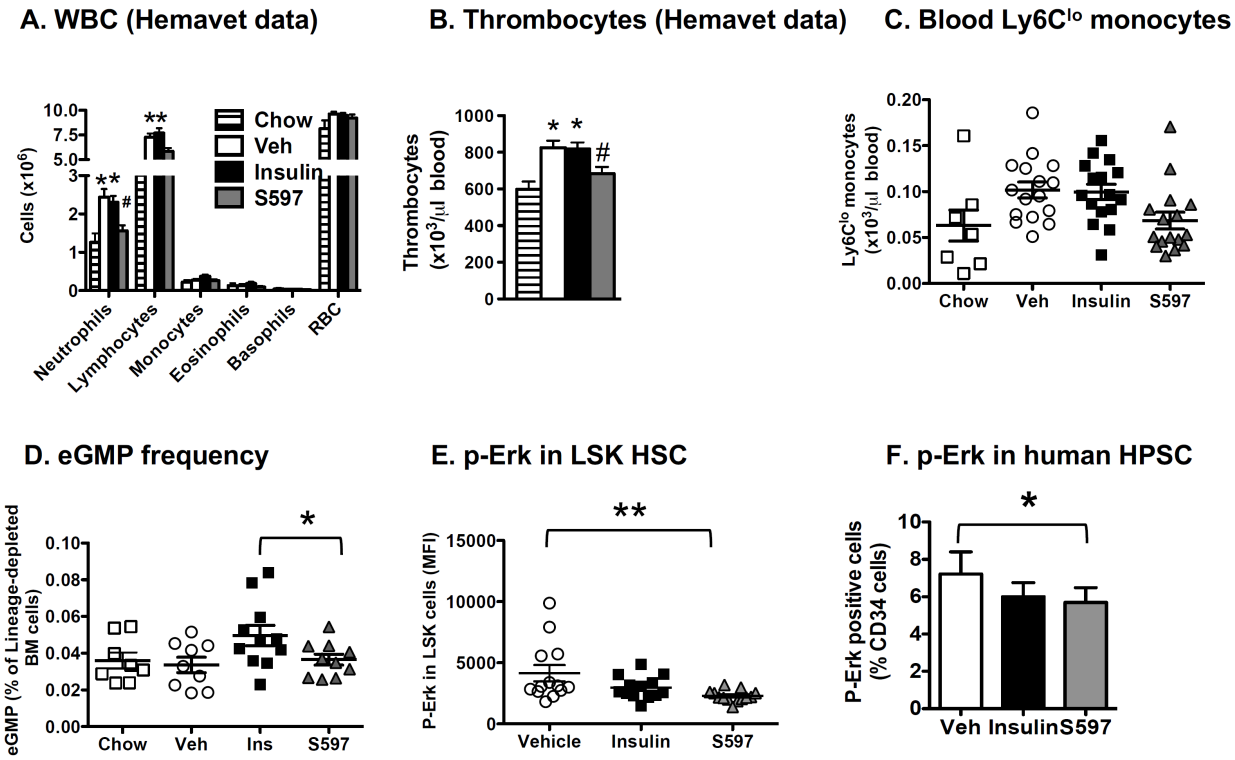
SUPPLEMENTARY DATA

Supplementary Figure S6. **S597 does not activate JNK or p38 MAPK.** Mice were injected acutely with vehicle, insulin or S597 5 minutes before euthanasia, and hepatic p-p38 MAPK (Thr180/182) (*A*) and p-JNK (Thr183/Tyr185) (*B*) were analyzed by Western blot (n=3- 4). There were no significant effects of insulin or S597. (*C*) Representative Western blots.



SUPPLEMENTARY DATA

Supplementary Figure S7. S597 prevents leukocytosis associated with the metabolic syndrome phenotype. (*A-B*) Mice were fed the DDC for 4 weeks and were then injected with insulin or S597 twice-daily for 4 weeks. Blood was collected from the retro-orbital plexus and analyzed using an automated Hemavet white blood cell counter (n=8 chow, n=15 for all DDC groups). Data are expressed as mean ± SEM. * $p<0.05$ compared to chow, # $p<0.05$ compared to vehicle treated mice, one-way ANOVA. (*C*) Blood Ly6C^{lo} monocytes determined by flow cytometry and normalized to Hemavet data. (*D*) Emergency granulocyte-macrophage progenitor (eGMP) cell frequency in bone marrow determined by flow cytometry. Hematopoietic progenitors were isolated from Lineagelive BM cells. Long-Term Hematopoietic Stem Cells (LT-HSCs) were identified as Lineage- CD117+ Sca-1+ CD34- CD135- cells, and eGMPs were identified as Lineage$^-$ CD117$^+$ Sca-1$^+$ CD16/32^{hi} CD34$^+$ cells. (*E*) Phospho-Erk in LSK (Lineage- CD117$^+$ Sca- 1$^+$) hematopoietic stem cells that had been sorted and then stimulated with insulin or S597. Data were analyzed by one-way ANOVA; *$p<0.05$; **$p<0.01$ as indicated. (*F*) Human CD34-positive progenitor cells (similar to mouse LT-HSC), from 4 donors each analyzed in 2-5 replicates were stimulated in the presence of vehicle, insulin or S597 for 7.5 minutes, and analyzed for intracellular p-Erk by flow cytometry (average from each donor is presented and data analyzed with paired two-tailed t-test; *$p<0.05$).



SUPPLEMENTARY DATA

Supplementary Table 1. S597 is an insulin receptor ligand. Radioligand binding assays, using the ligands shown in Table S1 were used. Non-specific binding was determined by co-incubation with receptor unlabeled ligand. The ability of 1 μM S597 to compete with ligand binding was determined. The results are presented as the mean percent inhibition of specific binding in duplicate assays. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. A significant response (≥ 50% inhibition) was evident only for S597 binding to the insulin receptor.

Receptor/binding site	Source	Ligand (specific and non-specific binding)	% Inhibition S597
Adenosine A_1	Human recombinant CHO cells	1.0 nmol/L [^{3}H] DPCPX 100 μmol/L R(-)-PIA	3
Adenosine A_{2A}	Human recombinant HEK-293 cells	0.050 μmol/L [^{3}H] CGS-21680 50.0 μmol/L NECA	17
Adenosine A_3	Human recombinant CHO-K1 cells	0.50 nmol/L [^{125}I] AB-MECA 1.0 μmol/L IB-MECA	-1
Adrenergic α_{1A}	Wistar rat submaxillary gland	0.25 nmol/L [^{3}H] Prazosin 10.0 μmol/L Phentolamine	-4
Adrenergic α_{1B}	Wistar rat liver	0.25 nmol/L [^{3}H] Prazosin 10.0 μmol/L Phentolamine	-8
Adrenergic α_{1D}	Human recombinant HEK-293 cells	0.60 nmol/L [^{3}H] Prazosin 10.0 μmol/L Phentolamine	-3
Adrenergic α_{2A}	Human recombinant insect Sf9 cells	1.0 nmol/L [^{3}H] MK-912 10.0 μmol/L WB-4101	6
Adrenergic α_{2C}	Human recombinant insect Sf9	1.0 nmol/L [^{3}H] MK-912 10.0 μmol/L WB-4101	-1
Adrenergic β_1	Human recombinant CHO-K1 cells	0.030 nmol/L [^{125}I] Cyanopindolol 100 μmol/L S(-)-Propranolol	9
Adrenergic β_2	Human recombinant CHO cells	0.20 nmol/L [^{3}H] CGP-12177 10.0 μmol/L ICI-118551	12
Adrenergic β_3	Human recombinant HEK-293 cells	0.50 nmol/L [^{125}I] Cyanopindolol 1.0 mmol/L Alprenolol	6
Adrenomedullin AM_1	Human recombinant insect Sf9 cells	0.040 nmol/L [^{125}I] Adrenomedullin (13-52) 3.0 μmol/L Adrenomedullin (22-52)	11
Adrenomedullin AM_2	Human recombinant CHO-K1 cells	0.040 nmol/L [^{125}I] Adrenomedullin (13-52)	21

SUPPLEMENTARY DATA

		3.0 µmol/L Adrenomedullin (22-52)	
Aldosterone	Wistar rat kidney	4.50 nmol/L [^{3}H] D-Aldosterone 3.0 µmol/L D-Aldosterone	-5
Androgen (Testosterone) AR	Rat recombinant E. coli	1.50 nmol/L [^{3}H] Mibolerone 10.0 µmol/L Mibolerone	1
Angiotensin AT_1	Human recombinant CHO-K1 cells	0.020 nmol/L [^{125}I] (Sar^{1},Ile^{8})-Angiotensin II 10.0 µmol/L Angiotensin II	5
Angiotensin AT_2	Human recombinant CHO-K1 cells	0.050 nmol/L [^{125}I] CGP-42112A 10.0 µmol/L (Sar1, Ile8)-Angiotensin II	0
APJ	Human recombinant CHO-K1 cells	0.15 nmol/L [^{125}I] (Glp^{65}, Nle^{75},Tyr^{77})-Apelin-13 1.0 µmol/L (Pyr1)-Apelin-13	-4
Atrial Natriuretic Factor (ANF)	Duncan Hartley derived guinea pig adrenal gland	0.016 n mol/L [^{125}I] ANF (rat) 0.10 µmol/L ANF (rat)	-6
Bombesin BB1	Human recombinant CHO cells	0.050 nmol/L [^{125}I] (Tyr4)-Bombesin 1.0 µmol/L Neuromedin B	-6
Bombesin BB2	Human recombinant HEK-293 cells	0.050 nmol/L [^{125}I] (Tyr4)-Bombesin 1.0 µmol/L GRP (human)	-9
Bombesin BB3	Human recombinant Balb 3T3 cells	0.10 nmol/L [^{125}I](D-Tyr^{6},β-Ala^{11},Phe^{13},Nle^{14})-BN (6-14) 1.0 µmol/L (D-Phe^{6},β-Ala^{11},Phe^{13},Nle^{14})-BN (6-14)	-4
Bradykinin B_1	Human IMR-90 cells	0.50 nmol/L [^{3}H] (Des-Arg10)-Kallidin 10.0 µmol/L (Des-Arg^{9}, Leu^{8})-Bradykinin	0
Bradykinin B_2	Human recombinant Chem-1 cells	0.50 nmol/L [^{3}H] Bradykinin 5.0 µmol/L Bradykinin	0
Calcitonin	Human T-47D cells	0.030 nmol/L [^{125}I] Calcitonin (salmon) 0.5 µmol/L Calcitonin (salmon)	0
Calcitonin Gene-Related Peptide $CGRP_1$	Human recombinant Chem-1 cells	0.030 nmol/L [^{125}I] CGRP (human) 1.0 µmol/L α-CGRP (human)	23
Calcium Channel L-Type, Benzothiazepine	Wistar rat brain	2.0 nmol/L [^{3}H] Diltiazem 10.0 µmol/L Diltiazem	12
Calcium Channel L-Type, Dihydropyridine	Wistar rat cerebral cortex	0.10 nmol/L [^{3}H] Nitrendipine 1.0 µmol/L Nifedipine	23
Calcium Channel L-Type, Phenylalkylamine	Wistar rat brain	0.40 nmol/L [3H](-)-Desmethoxyverapamil (D-888) 10.0 µmol/L Methoxyverapamil (D-600)	8
Calcium Channel N-Type	Wistar rat frontal brain	10 pmol/L [^{125}I] ω-Conotoxin GVIA 0.10 µmol/L ω-Conotoxin GVIA	-2
Cannabinoid CB_1	Human recombinant Chem-1 cells	2.0 nmol/L [^{3}H] SR141716A 10.0 µmol/L R(+)-WIN-55,212-2	-10
Cannabinoid CB_2	Human recombinant CHO-K1 cells	2.40 nmol/L [^{3}H] WIN-55,212-2 10.0 µmol/L R(+)-WIN-55,212-2	4
Chemokine CCR1	Human recombinant Chem-2 cells	0.10 nmol/L [^{125}I] MIP-1α 0.10 µmol/L MCP-3	-2
Chemokine CCR2B	Human recombinant CHO-K1 cells	0.10 nmol/L [^{125}I] MCP-1 0.030 µmol/L MCP-1	0
Chemokine CCR4	Human recombinant Chem-1 cells	0.050 nmol/L [^{125}I] TARC 0.10 µmol/L TARC	7
Chemokine CCR5	Human recombinant HEK-293T cells	0.060 nmol/L [^{125}I] MIP-1α 0.10 µmol/L MIP-1β	1
Chemokine CX3CR1	Human recombinant Chem-1 cells	0.020 nmol/L [^{125}I] Fractalkine 10 nmol/L Fractalkine	1
Chemokine CXCR2 (IL-$8R_B$)	Human recombinant CHO-K1 cells	0.015 nmol/L [^{125}I] IL-8 10 nmol/L IL-8	10
Cholecystokinin CCK_1 (CCK_A)	Human recombinant 1321-N1 cells	0.80 nmol/L [^{3}H] L-364,718 1.0 µmol/L Devazepide (L-364,718)	-1
Cholecystokinin CCK_2 (CCK_B)	Human recombinant Chem-1 cells	0.050 nmol/L [^{125}I] CCK-8 1.0 µmol/L Sincalide	10
Colchicine	Wistar rat brain	2.50 nmol/L [^{3}H] Colchicine 100 µmol/L Colchicine	0
Corticotropin Releasing Factor CRF1	Human recombinant CHO-K1 cells	0.050 nmol/L [^{125}I] (Tyr0)-CRF (ovine) 0.10 µmol/L Urocortin (human)	-5
Dopamine D_1	Human recombinant CHO cells	1.40 nmol/L [^{3}H] SCH-23390 10.0 µmol/L (+)-Butaclamol	7
Dopamine D_{2S}	Human recombinant CHO cells	0.16 nmol/L [^{3}H] Spiperone 10.0 µmol/L Haloperidol	29
Dopamine D_3	Human recombinant CHO cells	0.70 nmol/L [^{3}H] Spiperone 25.0 µmol/L S(-)-Sulpiride	0
Dopamine $D_{4.2}$	Human recombinant CHO-K1 cells	0.50 nmol/L [^{3}H] Spiperone 10.0 µmol/L Haloperidol	0
Dopamine D_5	Human recombinant CHO cells	2.0 nmol/L [^{3}H] SCH-23390 10.0 µmol/L Flupentixol	2
Endothelin ET_A	Human recombinant CHO-K1 cells	0.030 nmol/L [^{125}I] Endothelin-1 0.10 µmol/L Endothelin-1	-5
Endothelin ET_B	Human recombinant CHO-K1 cells	0.10 nmol/L [^{125}I] Endothelin-1 0.10 µmol/L Endothelin-1	-4
Epidermal Growth Factor (EGF)	Human A431 cells	0.080 nmol/L [^{125}I] EGF (human)	1

SUPPLEMENTARY DATA

		0.10 µmol/L EGF (human)	
Estrogen ERα	Human recombinant insect Sf9 cells	0.50 nmol/L [^{3}H] Estradiol 1.0 µmol/L Diethylstilbestrol	21
Estrogen ERβ	Human recombinant insect Sf9 cells	0.50 nmol/L [^{3}H] Estradiol 1.0 µmol/L Diethylstilbestrol	7
G Protein-Coupled Receptor GPR103	Human recombinant HEK-293 cells	0.075 nmol/L [^{125}I] QRFP-43 0.5 µmol/L Orphan GPCR SP9155 agonist P518	10
$GABA_A$, Chloride Channel, TBOB	Wistar rat cerebral cortex	3.0 nmol/L [^{3}H] TBOB 200 µmol/L Picrotoxin	11
$GABA_A$, Flunitrazepam, Central	Wistar rat brain (minus cerebellum)	1.0 nmol/L [^{3}H] Flunitrazepam 10.0 µmol/L Diazepam	14
$GABA_A$, Muscimol, Central	Wistar rat brain (minus cerebellum)	1.0 nmol/L [^{3}H] Muscimol 0.10 µmol/L Muscimol	30
$GABA_{B1A}$	Human recombinant CHO cells	4.0 nmol/L [^{3}H] CGP-54626 3.0 mmol/L GABA	3
$GABA_{B1B}$	Human recombinant CHO cells	4.0 nmol/L [^{3}H] CGP-54626 3.0 mmol/L GABA	2
Gabapentin	Wistar rat brain cortex	0.020 µmol/L [^{3}H] Gabapentin 100 µmol/L Gabapentin	3
Galanin GAL1	Human recombinant HEK-293 cells	0.012 nmol/L [^{125}I] Galanin (porcine) 1.0 µmol/L Galanin (human)	3
Galanin GAL2	Human recombinant CHO-K1 cells	0.070 nmol/L [^{125}I] Galanin (human) 2.0 µmol/L Galanin (human)	-6
Glucocorticoid	Human recombinant insect cells	5.0 nmol/L [^{3}H] Dexamethasone 10.0 µmol/L Dexamethasone	-4
Glutamate, AMPA	Wistar rat cerebral cortex	5.0 nmol/L [^{3}H] AMPA 1.0 mmol/L L-Glutamic acid	3
Glutamate, Kainate	Wistar rat brain (minus cerebellum)	5.0 nmol/L [^{3}H] Kainic acid 1.0 mmol/L L-Glutamic acid	10
Glutamate, NMDA, Agonism	Wistar rat cerebral cortex	2.0 nmol/L [^{3}H] CGP-39653 1.0 mmol/L L-Glutamic acid	-3
Glutamate, NMDA, Glycine	Wistar rat cerebral cortex	0.33 nmol/L [^{3}H] MDL 105,519 10.0 µmol/L MDL 105,519	14
Glutamate, NMDA, Phencyclidine	Wistar rat cerebral cortex	4.0 nmol/L [^{3}H] TCP 1.0 µmol/L Dizocilpine ((+)-MK-801)	7
Glutamate, NMDA, Polyamine	Wistar rat cerebral cortex	2.0 nmol/L [^{3}H] Ifenprodil 10.0 µmol/L Ifenprodil	-4
Glycine, Strychnine-Sensitive	Wistar rat spinal cord	10 nmol/L [^{3}H] Strychnine 1.0 mmol/L Glycine	-14
Growth Hormone Secretagogue (GHS, Ghrelin)	Human recombinant CHO-K1 cells	0.030 nmol/L [^{125}I] Ghrelin (human) 0.10 µmol/L Ghrelin (human)	3
Histamine H_1	Human recombinant CHO-K1 cells	1.20 nmol/L [^{3}H] Pyrilamine 1.0 µmol/L Pyrilamine	2
Histamine H_2	Human recombinant CHO-K1 cells	0.10 nmol/L [^{125}I] Aminopotentidine 3.0 µmol/L Tiotidine	17
Histamine H_3	Human recombinant CHO-K1 cells	0.40 nmol/L [^{3}H] N-α-Methylhistamine (NAMH) 1.0 µmol/L R(-)-α-Methylhistamine (RAMH)	10
Histamine H_4	Human recombinant CHO-K1 cells	8.20 nmol/L [^{3}H] Histamine 1.0 µmol/L Histamine	-1
Imidazoline I_2, Central	Wistar rat cerebral cortex	2.0 nmol/L [^{3}H] Idazoxan 1.0 µmol/L Idazoxan	-6
Inositol Trisphosphate IP_3	Wistar rat cerebellum	2.50 nmol/L [^{3}H] 1,4,5-IP_3 1.0 µmol/L 1,4,5-IP_3	-1
Insulin	Wistar rat liver	0.030 nmol/L [^{125}I] Insulin 1.0 µmol/L Insulin	74
Interleukin IL-1	Mouse 3T3-SWISS cells	0.10 nmol/L [^{125}I] Interleukin-1β 10.0 µmol/L Interleukin-1β	0
Interleukin IL-6	Human U266 cells	0.080 nmol/L [^{125}I] IL-6 10 nmol/L IL-6	-9
Leptin	Mouse recombinant BafL46 cells	0.10 nmol/L [^{125}I] Leptin 0.10 µmol/L Leptin	10
Leukotriene, BLT (LTB_4)	Human U937 cells	0.20 nmol/L [^{3}H] LTB_4 2.0 µmol/L LTB_4	-11
Leukotriene, Cysteinyl $CysLT_1$	Human recombinant CHO-K1 cells	0.30 nmol/L [^{3}H] LTD_4 0.30 µmol/L LTD_4	-1
Leukotriene, Cysteinyl $CysLT_2$	Human recombinant CHO-K1 cells	1.30 nmol/L [^{3}H] LTC_4 0.5 µmol/L LTC_4	0
Melanocortin MC_1	Human recombinant CHO-K1 cells	0.040 nmol/L [^{125}I] NDP-α-MSH 1.0 µmol/L NDP-α-MSH	3
Melanocortin MC_3	Human recombinant HEK-293	0.035 nmol/L [^{125}I] NDP-α-MSH 3.0 µmol/L NDP-α-MSH	9
Melanocortin MC_4	Human recombinant HEK-293	0.020 nmol/L [^{125}I] NDP-α-MSH 3.0 µmol/L NDP-α-MSH	3
Melanocortin MC_5	Human recombinant HEK-293	0.035 nmol/L [^{125}I] NDP-α-MSH	0

SUPPLEMENTARY DATA

		3.0 µmol/L NDP-α-MSH	
Melatonin MT_1	Human recombinant CHO-K1 cells	0.050 nmol/L [^{125}I] 2-Iodomelatonin 1.0 µmol/L 6-Chloromelatonin	-8
Melatonin MT_2	Human recombinant CHO-K1 cells	0.050 nmol/L [^{125}I] 2-Iodomelatonin 1.0 µmol/L 6-Chloromelatonin	3
Motilin	Human recombinant HEK-293 cells	0.10 nmol/L [^{125}I] Motilin (human, porcine) 1.0 µmol/L Motilin (human, porcine)	5
Muscarinic M_1	Human recombinant CHO-K1 cells	0.80 nmol/L [^{3}H] N-Methylscopolamine 1.0 µmol/L Atropine	2
Muscarinic M_2	Human recombinant CHO-K1 cells	0.80 nmol/L [^{3}H] N-Methylscopolamine 1.0 µmol/L Atropine	4
Muscarinic M_3	Human recombinant CHO-K1 cells	0.80 nmol/L [^{3}H] N-Methylscopolamine 1.0 µmol/L Atropine	5
Muscarinic M_4	Human recombinant CHO-K1 cells	0.80 nmol/L [^{3}H] N-Methylscopolamine 1.0 µmol/L Atropine	7
Muscarinic M_5	Human recombinant CHO cells	0.80 nmol/L [^{3}H] N-Methylscopolamine 1.0 µmol/L Atropine	-3
N-Formyl Peptide Receptor FPR1	Human recombinant Chem-2 cells	0.030 nmol/L [^{125}I] WKYMVm 1.0 µmol/L fMLF	-4
N-Formyl Peptide Receptor-Like FPRL	Human recombinant CHO cells	0.025 nmol/L [^{125}I] WKYMVm 1.0 µmol/L WKYMVm	7
Neuromedin U NMU_1	Human recombinant HEK-293 cells	0.037 nmol/L [^{125}I] Neuromedin U-25 (porcine) 10.0 µmol/L Neuromedin U-8 (porcine)	2
Neuromedin U NMU_2	Human recombinant HEK-293 cells	0.037 nmol/L [^{125}I] Neuromedin U-25 (porcine) 10.0 µmol/L Neuromedin U-8 (porcine)	-2
Neuropeptide Y Y_1	Human SK-N-MC cells	0.015 nmol/L [^{125}I] Peptide YY 1.0 µmol/L Neuropeptide Y (human, rat)	8
Neuropeptide Y Y_2	Human KAN-TS cells	10 pmol/L [^{125}I] Peptide YY 1.0 µmol/L Neuropeptide Y (13-36) (porcine)	1
Neurotensin NT_1	Human recombinant HEK-293 EBNA	0.020 nmol/L [^{125}I] Neurotensin 1.0 µmol/L Neurotensin	1
Nicotinic Acetylcholine	Human IMR-32 cells	0.10 nmol/L [^{125}I] Epibatidine 300 µmol/L (-)-Nicotine	-1
Nicotinic Acetylcholine α1, Bungarotoxin	Human RD cells	0.60 nmol/L [^{125}I] α-Bungarotoxin 1.0 µmol/L α-Bungarotoxin	0
Nicotinic Acetylcholine α7, Bungarotoxin	Wistar rat brain (minus cerebellum)	0.60 nmol/L [^{125}I] α-Bungarotoxin 1.0 µmol/L α-Bungarotoxin	1
NPBW2/GPR8	Human recombinant Chem-1 cells	0.060 nmol/L [^{125}I] NPW-23 1.0 µmol/L (Des-Br)NPB-23	10
Opiate δ_1 (OP1, DOP)	Human recombinant HEK-293 cells	1.30 nmol/L [^{3}H] Naltrindole 1.0 µmol/L Naltrindole	7
Opiate κ (OP2, KOP)	Human recombinant HEK-293 cells	0.60 nmol/L [^{3}H] Diprenorphine 10.0 µmol/L Naloxone	10
Opiate µ (OP3, MOP)	Human recombinant CHO-K1 cells	0.60 nmol/L [^{3}H] Diprenorphine 10.0 µmol/L Naloxone	-7
Orphanin ORL_1	Human recombinant HEK-293 cells	0.60 nmol/L [^{3}H] Nociceptin 1.0 µmol/L Orphanin-FQ	1
Phorbol Ester	ICR mouse brain	3.0 nmol/L [^{3}H] PDBu 1.0 µmol/L PDBu	5
Platelet Activating Factor (PAF)	Human platelets	0.12 nmol/L [^{3}H] PAF 1.0 µmol/L PAF	3
Platelet-Derived Growth Factor (PDGF)	Mouse 3T3 cells	0.020 nmol/L [^{125}I] PDGF 0.10 nmol/L PDGF	-4
Potassium Channel [K_A]	Wistar rat cerebral cortex	10 pmol/L [^{125}I] α-Dendrotoxin 10 nmol/L α-Dendrotoxin	0
Potassium Channel [K_{ATP}]	Hamster pancreatic HIT-T15 beta cells	5.0 nmol/L [^{3}H] Glyburide 1.0 µmol/L Glyburide	22
Potassium Channel [SK_{CA}]	Wistar rat brain	5.0 pmol/L [^{125}I] Apamin 0.10 µmol/L Apamin	0
Potassium Channel hERG	Human recombinant HEK-293 cells	1.50 nmol/L [^{3}H] Astemizole 10.0 µmol/L Astemizole	4
Progesterone PR-B	Human recombinant insect Sf9 cells	1.40 nmol/L [^{3}H] Progesterone 1.0 µmol/L Progesterone	18
Prostanoid CRTH2	Human recombinant CHO-K1 cells	1.0 nmol/L [^{3}H] Prostaglandin D_2 (PGD_2) 1.0 µmol/L Prostaglandin D_2 (PGD_2)	-9
Prostanoid DP	Human recombinant Chem-1 cells	2.0 nmol/L [^{3}H] Prostaglandin D_2 (PGD_2) 1.0 µmol/L Prostaglandin D_2 (PGD_2)	19
Prostanoid EP_2	Human recombinant HEK-293 cells	4.0 nmol/L [^{3}H] Prostaglandin E_2 (PGE_2) 10.0 µmol/L Prostaglandin E_2 (PGE_2)	-1
Prostanoid EP_4	Human recombinant Chem-1 cells	1.0 nmol/L [^{3}H] Prostaglandin E_2 (PGE_2) 10.0 µmol/L Prostaglandin E_2 (PGE_2)	0
Purinergic P_{2X}	New Zealand derived albino rabbit urinary bladder	8.0 nmol/L [^{3}H] α, β-Methylene-ATP 100 µmol/L β, γ-Methylene ATP	7
Purinergic P_{2Y}	Wistar rat brain	0.10 nmol/L [^{35}S] ATP-αS	26

SUPPLEMENTARY DATA

		10.0 µmol/L ADP-βS	
Retinoid X Receptor RXRα	Human recombinant E. coli	5.0 nmol/L [^{3}H] 9-cis-Retinoic acid 1.0 µmol/L 9-cis-Retinoic acid	-20
Rolipram	Wistar rat brain	1.80 nmol/L [^{3}H] Rolipram 10.0 µmol/L Rolipram	7
Ryanodine RyR3	Wistar rat cerebral cortex	3.0 nmol/L [^{3}H] Ryanodine 10.0 µmol/L Ryanodine	-6
Serotonin (5-Hydroxytryptamine) 5-HT$_{1A}$	Human recombinant CHO-K1 cells	1.50 nmol/L [^{3}H] 8-OH-DPAT 10.0 µmol/L Metergoline	-11
Serotonin (5-Hydroxytryptamine) 5-HT$_{1B}$	Wistar rat cerebral cortex	10 pmol/L [^{125}I] Cyanopindolol 10.0 µmol/L Serotonin (5-HT)	7
Serotonin (5-Hydroxytryptamine) 5-HT$_{2B}$	Human recombinant CHO-K1 cells	1.20 nmol/L [^{3}H] Lysergic acid diethylamide (LSD) 10.0 µmol/L Serotonin (5-HT)	3
Serotonin (5-Hydroxytryptamine) 5-HT$_{2C}$	Human recombinant CHO-K1 cells	1.0 n ol/L [^{3}H] Mesulergine 1.0 µmol/L Mianserin	-4
Serotonin (5-Hydroxytryptamine) 5-HT$_{3}$	Human recombinant HEK-293 cells	0.69 nmol/L [^{3}H] GR-65630 10.0 µmol/L MDL 72222	12
Serotonin (5-Hydroxytryptamine) 5-HT$_{4}$	Duncan Hartley derived guinea pig striatum	0.70 nmol/L [^{3}H] GR-113808 30.0 µmol/L Serotonin (5-HT)	2
Serotonin (5-Hydroxytryptamine) 5-HT$_{5A}$	Human recombinant CHO-K1 cells	1.70 nmol/L [^{3}H] Lysergic acid diethylamide (LSD) 100 µmol/L Serotonin (5-HT)	5
Serotonin (5-Hydroxytryptamine) 5-HT$_{6}$	Human recombinant HeLa cells	1.50 nmol/L [^{3}H] Lysergic acid diethylamide (LSD) 5.0 µmol/L Serotonin (5-HT)	1
Sigma σ_1	Human Jurkat cells	8.0 nmol/L [^{3}H] Haloperidol 10.0 µmol/L Haloperidol	6
Sigma σ_2	Wistar rat brain	3.0 nmol/L [^{3}H] Ifenprodil 10.0 µmol/L Ifenprodil	0
Somatostatin sst1	Human recombinant CHO-K1 cells	0.10 nmol/L [^{125}I] Tyr^{11}-Somatostatin 14 1.0 µmol/L Somatostatin-14	-16
Somatostatin sst2	Human recombinant CHO-K1 cells	0.030 nmol/L [^{125}I] Tyr^{11}-Somatostatin 14 1.0 µmol/L Somatostatin-14	-6
Somatostatin sst3	Human recombinant CHO-K1 cells	0.10 nmol/L [^{125}I] Tyr^{11}-Somatostatin 14 1.0 µmol/L Somatostatin-14	3
Somatostatin sst4	Human recombinant Chem-1 cells	0.10 nmol/L [^{125}I] Tyr^{11}-Somatostatin 14 1.0 µmol/L Somatostatin-14	5
Somatostatin sst5	Human recombinant Chem-1 cells	0.10 nmol/L [^{125}I] Tyr^{11}-Somatostatin 14 1.0 µmol/L Somatostatin-14	2
Tachykinin NK1	Human recombinant CHO cells	0.80 nmol/L [^{3}H] Substance P 10.0 µmol/L L-703,606	7
Tachykinin NK2	Human recombinant CHO cells	0.50 nmol/L [^{3}H] SR-48968 2.0 µmol/L MEN-10,376	1
Tachykinin NK3	Human recombinant CHO cells	0.060 nmol/L [^{125}I] MePhe^{7}-Neurokinin B 50.0 µmol/L Senktide	-7
Thyroid Hormone	Wistar rat liver	0.030 nmol/L [^{125}I] Triiodothyronine 1.0 µmol/L Triiodothyronine	0
Thyrotropin Releasing Hormone (TRH)	Wistar rat brain	3.0 nmol/L [^{3}H] Me-TRH 10.0 µmol/L TRH	2
Transforming Growth Factor-β (TGF-β)	Mouse 3T3 cells CCL-92	0.013 nmol/L [^{125}I] TGF-β_1 0.10 nmol/L TGF-β_1	-19
Transporter, Adenosine	Duncan Hartley derived guinea pig cerebral cortex	0.50 nmol/L [^{3}H] Nitrobenzylthioinosine 5.0 µmol/L Nitrobenzylthioinosine	4
Transporter, Choline	Wistar rat brain striatum	0.75 nmol/L [^{3}H] Hemicholinium-3 1.0 µmol/L Hemicholinium-3	-14
Transporter, Dopamine (DAT)	Human recombinant CHO-K1 cells	0.15 nmol/L [^{125}I] RTI-55 10.0 µmol/L Nomifensine	1
Transporter, GABA	Wistar rat cerebral cortex	6.0 nmol/L [^{3}H] GABA 10.0 µmol/L NO-711	6
Transporter, Monoamine	New Zealand derived albino rabbit platelets	2.0 nmol/L [^{3}H] Ketanserin 10.0 µmol/L Tetrabenazine	-11
Transporter, Norepinephrine (NET)	Human recombinant MDCK cells	0.20 nmol/L [^{125}I] RTI-55 10.0 µmol/L Desipramine	-10
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	Human recombinant HEK-293 cells	0.40 nmol/L [^{3}H] Paroxetine 10.0 µmol/L Imipramine	3
Tumor Necrosis Factor (TNF), Non-Selective	Human U937 cells	0.028 nmol/L [^{125}I] TNF-α 0.040 µmol/L TNF-α	27
Urotensin II	Human recombinant CHO-K1 cells	0.10 nmol/L [^{125}I] Urotensin II 1.0 µmol/L Urotensin II	10
Vanilloid	Wistar rat spinal cord	0.20 nmol/L [^{3}H] Resiniferatoxin 0.10 µmol/L Resiniferatoxin	-1
Vasoactive Intestinal Peptide VIP$_1$	Human HT29 colon adenocarcinoma cells	10 pmol/L [^{125}I] VIP 1.0 µmol/L VIP	-5
Vasopressin V$_{1A}$	Human recombinant HEK-293 cells	0.030 nmol/L [^{125}I]PhenylacetylTyr(Me)PheGlnAsnArgProArgTyr 1.0 µmol/L (Arg^{8})-Vasopressin	9
Vasopressin V$_{1B}$	Human recombinant CHO cells	1.0 nmol/L [^{3}H] (Arg^{8})-Vasopressin	-1

SUPPLEMENTARY DATA

		1.0 µmol/L (Arg^8)-Vasopressin	
Vasopressin V_2	Human recombinant CHO cells	4.0 nmol/L [^{3}H] (Arg^8)-Vasopressin 1.0 µmol/L (Arg^8)-Vasopressin	4
Vitamin D_3	Human recombinant insect cells	0.30 nmol/L [^{3}H] 1α, 25-Dihydroxyvitamin D_3 0.5 µmol/L 1α, 25-Dihydroxyvitamin D_3	0

Items meeting criteria for significance (≥50% stimulation or inhibition) are highlighted in grey. gp=Guinea pig; ham=Hamster; hum=Human

Supplementary Reagent Tables
Antibodies for flow cytometry and cell sorting:

Antibody	Clone	Conjugate	Source	Dilution
CD16/32 (FC block)	93	N/A	eBioscience	1:400
Viability dye eFluor450	N/A	N/A	eBioscience	1:1000
CD45	30-F11	FITC	eBioscience	1:1000
CD115	AFS98	PE	eBioscience	1:100
GR1	RB6-8C5	PE-Cy7	eBioscience	1:2000
B220	RA3-6B2	APC	eBioscience	1:500
CD3e	145-2C11	APC	eBioscience	1:500
CD11B	M1/70	PerCP-Cy5.5	eBioscience	1:1000
CD11B	M1/70	Biotin	eBioscience	1:600
F4/80	BM8	Biotin	eBioscience	1:100
GR1	RB6-8C5	Biotin	eBioscience	1:600
CD11C	N418	Biotin	eBioscience	1:100
CD3	eBio500A2	Biotin	eBioscience	1:100
NK1.1	PK136	Biotin	eBioscience	1:100
CD34	RAM34	FITC	eBioscience	1:10
Sca-1	D7	PerCP-Cy5.5	eBioscience	1:100
CD135	A2F10	APC	BioLegend	1:100
CD117	2B8	PE-Cy7	eBioscience	1:100
CD16/32	93	PE	eBioscience	1:100
Streptavidin	N/A	APC-eFluor780	eBioscience	1:100
Sca-1	D7	BV510	BioLegend	1:100
CD48	HM48-1	Af647	BioLegend	1:100
CD150	TC15-12F12.2	Pacific Blue	BioLegend	1:100
p-ERK	197G2	PE	Cell signaling	1:100
CD34	Qbend-10	FITC	Abcam	1:50
CD38	HIT2	Alexa-647	BioLegend	1:20
CD68	FA-11	N/A	BioRad	1:50

SUPPLEMENTARY DATA

Antibodies for Western blot analysis:

Antibody	Description	Product number
Phospho-Akt	polyclonal rabbit anti-mouse Ser473-Akt	9271
Total Akt	polyclonal rabbit anti-mouse Akt	9272
Phospho-Erk1/2	polyclonal rabbit anti-mouse Thr202/Tyr204-Erk	9101
Phospho-GSK3β	monoclonal rabbit anti-human Ser9-GSK3β IgG	5558
Total GSK3β	monoclonal rabbit anti-human GSK3β IgG	9315
Phospho-p38 MAPK	monoclonal rabbit anti-Thr180/182 p38 MAPK; 12F8	4631
Total p38 MAPK	polyclonal rabbit anti-human p38 MAPK	9212
Phospho-JNK	monoclonal mouse anti-Thr183/Tyr185 JNK IgG1, G9	9255
Total JNK	polyclonal rabbit anti-human JNK2	9252

All the above antibodies were obtained from Cell Signaling Technologies and were used according to the manufacturer's instructions for Western blots.

Antibodies for immunohistochemistry:

Antibody	Staining	Description	Source
Mac-2	macrophages	clone M3/38; monoclonal rat anti-mouse IgG2a	Cedarlane Laboratories
Cleaved caspase 3	apoptotic cells	Asp175; polyclonal rabbit anti-human IgG, #9661	Cell Signaling Technology
Smooth muscle α-actin	smooth muscle cells	clone 1A4, monoclonal mouse anti-human IgG2a	Dako
Ki67	proliferating cells	polyclonal rabbit anti-mouse Ki67, #NB110-89719	Novus Biologicals
VCAM-1	VCAM-1	polyclonal rabbit anti-human VCAM-1, H-276, #sc-8304	Santa Cruz Biotechnologies

Other sections were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Roche Applied Science, Indianapolis, IN) after proteinase K treatment as directed by the manufacturer. DNAse treatment (500 units/ml for 30 min at 37°) was used as a positive control for the TUNEL assay. The anti-cleaved caspase 3 (Asp175) (5A1) antibody detects the large fragment (17/19 kDa) of activated caspase 3 resulting from cleavage adjacent to Asp175, and does not crossreact with full length caspase 3 or other cleaved caspases. To detect proliferating cells using the Ki67 antigen, cross sections from paraffin-embedded BCAs were deparaffinized and rehydrated, and then incubated in a hot antigen retrieval buffer (10 mM sodium citrate, 0.05% and Tween 20, pH 6.0) for 40 min. The Ki67 antibody (1 mg/ml) was used at a final dilution of 1:200.

SUPPLEMENTARY DATA

Supplementary Primer Table. Primers for real-time PCR

Gene	Primer sequences	Source
Ccl2	F 5'-TTAAAAACCTGGATCGGAACCAA-3' R 5'-GCATTAGCTTCAGATTTACGGGT-3'	Invitrogen
Ccnd1	F 5'-GCGTACCCTGACACCAATCTC-3' R 5'-CTCCTCTTCGCACTTCTGCTC-3'	Invitrogen
Emr1	F 5'-TGACTCACCTTGTGGTCCTAA-3' R 5'-CTTCCCAGAATCCAGTCTTTCC-3'	Invitrogen
Fasn	F 5'-GGAGGTGGTGATAGCCGGTAT-3' R 5-TGGGTAATCCATAGAGCCCAG-3'	Invitrogen
Gapdh	F 5'-AGGTCGGTGTGAACGGATTTG-3' R 5'-TGTAGACCATGTAGTTGAGGTCA-3'	Invitrogen
Hmgcr	F 5'-TGTTCACCGGCAACAACAAGA-3' R 5'-CCGCGTTATCGTCAGGATGA-3'	Invitrogen
Igf1r	#Mn00802831_m1	Applied Biosystems
Il6	F 5'-TAGTCCTTCCTACCCCAATTTCC-3' R 5'-TTGGTCCTTAGCCACTCCTTC-3'	Invitrogen
Insr	#Mn01211875_m1	Applied Biosystems
Pepck	F 5'-CTGCATAACGGTCTGGACTTC-3' R 5'-CAGCAACTGCCCGTACTCC-3'	Invitrogen
Ppib	#Mn00478295_m1	Applied Biosystems
Rn18s	F 5'-CATTAAATCAGTTATGGTTCCTTTGG-3' R 5'-CCCGTCGGCATGTATTAGCT-3'	Invitrogen
Rpn32	F 5'-TTAAGCGAAACTGGCGGAAAC-3' R 5'-TTGTTGCTCCCATAACCGATG-3'	Invitrogen
Scd1	F 5'-TTCTTGCGATACACTCTGGTGC-3' R 5-CGGGATTGAATGTTCTTGTCGT-3'	Invitrogen
Tnfa	F 5'-CCCTCACACTCAGATCATCTTCT-3' R 5'-GCTACGACGTGGGCTACAG-3'	Invitrogen
Vcam1	F 5'-TGCACAGTCCCTAATGTGTATCC-3' R 5'-GACTTTATGCCCATTTCCTCCA-3'	Invitrogen
Nos2	F 5'-GTTCTCAGCCCAACAATACAAGA-3' R 5'-GTGGACGGGTCGATGTCAC-3'	Invitrogen
Il1b	F 5'-GGGCTGCTTCCAAACCTTTG-3' R 5'-TGATACTGCCTGCCTGAAGCTC-3'	Invitrogen
Il6	F 5'-TAGTCCTTCCTACCCCAATTTCC-3' R 5'-TTGGTCCTTAGCCACTCCTTC-3'	Invitrogen
Ym1	F 5'-AGAAGGGAGTTTCAAACCTGG-3' R 5'-GTCTTGCTCATGTGTGTAAGTG-3'	Invitrogen
Arg1	F 5'-CTCCAAGCCAAAGTCCTTAGAG-3' R 5'-AGGAGCTGTCATTAGGGACATCA-3'	Invitrogen