Stat3-induced S1PR1 expression is critical for persistent Stat3 activation in tumors

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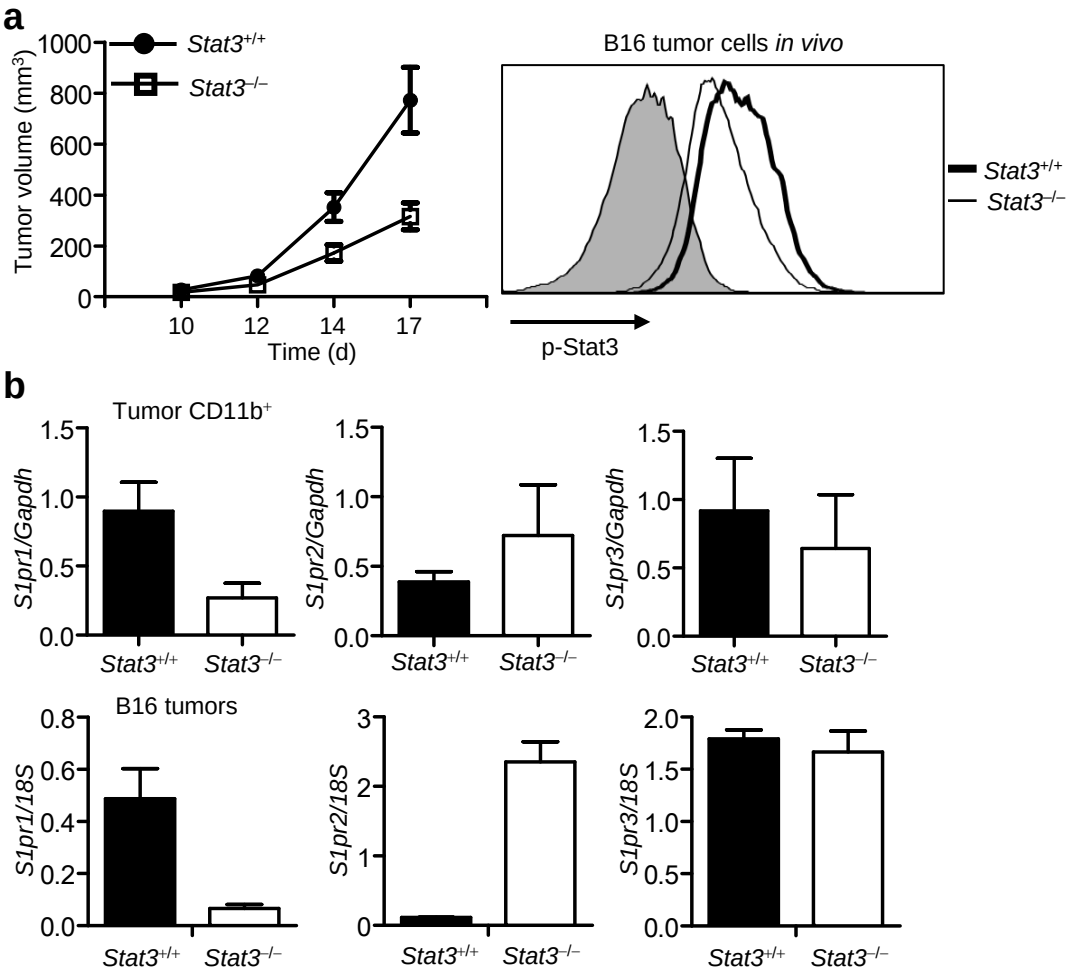
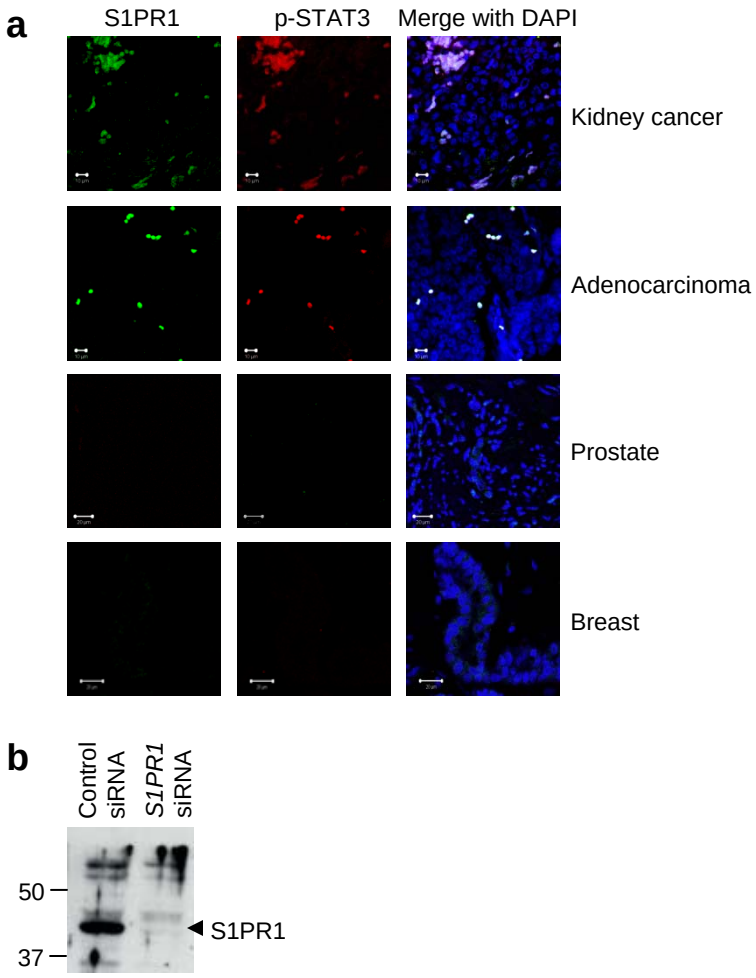
Supplementary Table 1 PCR-based microarray

Gene	Well	2^-ΔCt		Fold change	
		Stat3 $^{+/+}$	*Stat3* $^{-/-}$	Stat3+/+/Stat3-/-	
Angpt1	A01	3.4E-03	4.3E-04	**7.78**	1.00
Angpt2	A02	3.4E-03	4.3E-04	**7.78**	1.00
Anpep	A03	3.4E-03	4.3E-04	**7.78**	1.00
Bai1	A04	3.4E-03	4.3E-04	**7.78**	1.00
Ccl11	A05	3.4E-03	4.3E-04	**7.78**	1.00
Ccl2	A06	3.4E-03	4.3E-04	**7.78**	1.00
Cdh5	A07	3.4E-03	4.3E-04	**7.78**	1.00
Col18a1	A08	3.4E-03	4.3E-04	**7.78**	1.00
Col4a3	A09	3.4E-03	4.3E-04	**7.78**	1.00
Csf3	A10	3.4E-03	4.3E-04	**7.78**	1.00
Ctgf	A11	3.4E-03	4.3E-04	**7.78**	1.00
Cxcl1	A12	3.4E-03	4.3E-04	**7.78**	1.00
Cxcl2	B01	1.2E-01	1.0E-02	**11.31**	1.45
Cxcl5	B02	3.4E-03	4.3E-04	**7.78**	1.00
Ecgf1	B03	3.4E-03	4.3E-04	**7.78**	1.00
Edg1	B04	9.8E-02	4.3E-04	**227.54**	**29.25**
Efna1	B05	3.4E-03	4.3E-04	**7.78**	1.00
Efnb2	B06	3.4E-03	4.3E-04	**7.78**	1.00
Egf	B07	3.4E-03	4.3E-04	**7.78**	1.00
Eng	B08	3.4E-03	4.3E-04	**7.78**	1.00
Epas1	B09	3.4E-03	4.3E-04	**7.78**	1.00
Ephb4	B10	3.4E-03	4.3E-04	**7.78**	1.00
Ereg	B11	3.4E-03	4.3E-04	**7.78**	1.00
F2	B12	3.4E-03	4.3E-04	**7.78**	1.00
Fgf1	C01	3.4E-03	4.3E-04	**7.78**	1.00
Fgf2	C02	3.4E-03	4.3E-04	**7.78**	1.00
Fgf6	C03	3.4E-03	4.3E-04	**7.78**	1.00
Fgfr3	C04	3.4E-03	4.3E-04	**7.78**	1.00
Figf	C05	3.8E-03	4.3E-04	**8.75**	1.13
Flt1	C06	3.4E-03	4.3E-04	**7.78**	1.00
Fzd5	C07	3.4E-03	4.3E-04	**7.78**	1.00
Gna13	C08	4.5E-03	4.3E-04	**10.34**	1.33
Hand2	C09	3.4E-03	4.3E-04	**7.78**	1.00
Hgf	C10	3.4E-03	8.1E-03	0.41	-0.31
Hif1a	C11	3.4E-03	4.3E-04	**7.78**	1.00
Ifng	C12	8.3E-03	5.3E-03	1.58	**0.20**
Igf1	D01	3.4E-03	4.3E-04	**7.78**	1.00
Il1b	D02	2.6E-02	4.3E-04	**60.97**	**7.84**
Il6	D03	2.6E-02	4.3E-04	**61.39**	**7.89**
Itgav	D04	4.8E-03	4.3E-04	**11.16**	1.43
Itgb3	D05	3.4E-03	4.3E-04	**7.78**	1.00
Jag1	D06	3.4E-03	4.3E-04	**7.78**	1.00
Kdr	D07	4.1E-03	4.3E-04	**9.51**	1.22
Lama5	D08	3.4E-03	4.3E-04	**7.78**	1.00
Lect1	D09	3.4E-03	4.3E-04	**7.78**	1.00
Lep	D10	3.4E-03	4.3E-04	**7.78**	1.00
Mapk14	D11	2.1E-02	4.3E-04	**49.18**	**6.32**

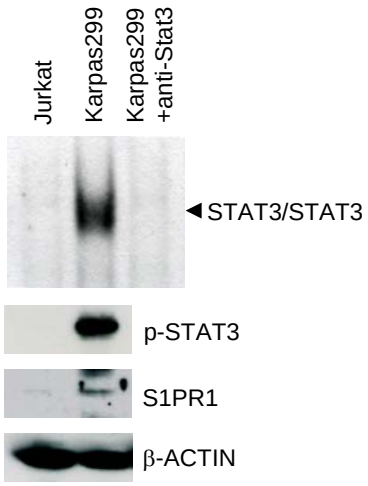
Mdk	D12	3.4E-03	4.3E-04	**7.78**	1.00
Mmp19	E01	3.4E-03	4.3E-04	**7.78**	1.00
Mmp2	E02	3.4E-03	4.3E-04	**7.78**	1.00
Mmp9	E03	3.4E-03	4.3E-04	**7.78**	1.00
Npr1	E04	3.4E-03	4.3E-04	**7.78**	1.00
Nrp1	E05	3.4E-03	4.3E-04	**7.78**	1.00
Nrp2	E06	3.4E-03	4.3E-04	**7.78**	1.00
Pdgfa	E07	3.4E-03	4.3E-04	**7.78**	1.00
Pecam1	E08	2.0E-02	1.2E-02	1.77	**0.23**
Pgf	E09	3.4E-03	4.3E-04	**7.78**	1.00
Plau	E10	3.4E-03	4.3E-04	**7.78**	1.00
Plg	E11	3.4E-03	4.3E-04	**7.78**	1.00
Plxdc1	E12	3.4E-03	4.3E-04	**7.78**	1.00
Ptgs1	F01	3.4E-03	4.3E-04	**7.78**	1.00
Serpinf1	F02	3.4E-03	4.3E-04	**7.78**	1.00
Smad5	F03	3.4E-03	4.3E-04	**7.78**	1.00
Sphk1	F04	3.4E-03	4.3E-04	**7.78**	1.00
Stab1	F05	3.4E-03	4.3E-04	**7.78**	1.00
Tbx1	F06	3.4E-03	4.3E-04	**7.78**	1.00
Tbx4	F07	3.4E-03	4.3E-04	**7.78**	1.00
Tek	F08	3.4E-03	4.3E-04	**7.78**	1.00
Tgfa	F09	3.4E-03	4.3E-04	**7.78**	1.00
Tgfb1	F10	4.6E-02	2.5E-02	1.83	**0.23**
Tgfb2	F11	3.4E-03	4.3E-04	**7.78**	1.00
Tgfb3	F12	3.4E-03	4.3E-04	**7.78**	1.00
Tgfbr1	G01	3.4E-03	4.3E-04	**7.78**	1.00
Thbs1	G02	3.4E-03	4.3E-04	**7.78**	1.00
Thbs2	G03	3.4E-03	4.3E-04	**7.78**	1.00
Timp1	G04	3.4E-03	4.3E-04	**7.78**	1.00
Timp2	G05	3.4E-03	4.3E-04	**7.78**	1.00
Tmprss6	G06	3.4E-03	4.3E-04	**7.78**	1.00
Tnf	G07	6.3E-03	4.3E-04	**14.52**	1.87
Tnfaip2	G08	7.2E-03	4.3E-04	**16.80**	2.16
Tnfsf12	G09	3.4E-03	8.6E-04	**3.92**	0.50
Vegfa	G10	1.3E-02	4.3E-04	**29.24**	**3.76**
Vegfb	G11	4.5E-03	2.0E-03	2.31	0.30
Vegfc	G12	3.4E-03	4.3E-04	**7.78**	1.00



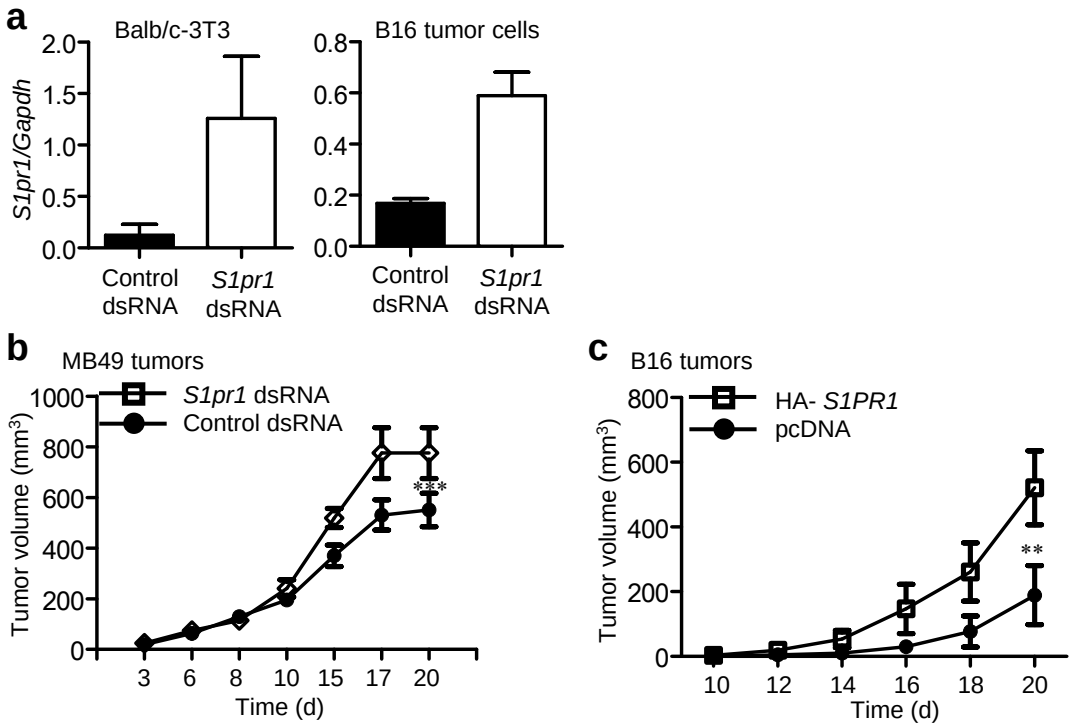
Supplementary Figure 1 Lacking *Stat3* in myeloid cells inhibits tumor growth and Stat3 activity. (**a**) *Left*, Growth curve of B16 tumors grown in mice with *Stat3*$^{+/+}$ and *Stat3*$^{-/-}$ myeloid compartments. Data represent means ± s.e.m., $n = 6$. *Right*, tumor Stat3 activity was determined by intracellular staining with phospho-Stat3-specific antibody (p-Stat3) followed by flow cytometry. Tumor cells were gated as CD11b^{-}, FSC^{high}, SSC^{high} (due to high content of melanin granules). (**b**) Stat3 upregulates *S1pr1* expression, but not other S1P receptors. Real-time RT-PCR was used to detect *S1pr* mRNA levels in either B16 tumor-infiltrating CD11b^{+} myeloid cells or in the whole tumors grown in mice with *Stat3*$^{+/+}$ and *Stat3*$^{-/-}$ hematopoietic cells. Data represent means ± s.d., $n = 3$.



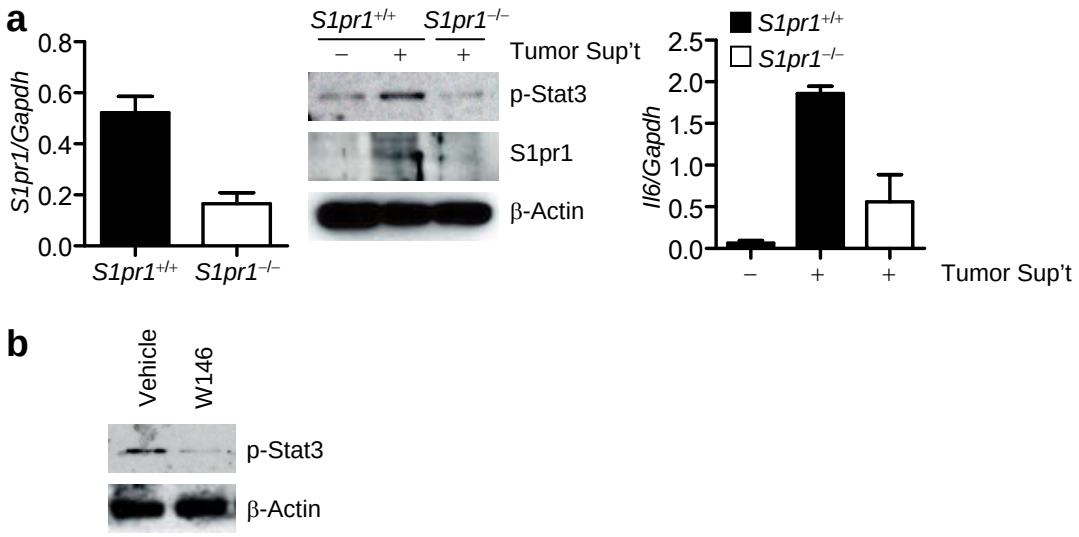
Supplementary Figure 2 S1PR1 expression is reduced in cells with low/no STAT3 activity. (**a**) Immunohistochemical analysis of S1PR1 and p-STAT3 in human tumors (kidney cancer and adenocarinoma; scale bars, 10 μm) as well as in normal tissues (prostate and breast; scale bars, 20 μm). (**b**) Western blotting showing endogenous S1PR1 protein levels in DU145 human prostate cancer cells transfected with either control siRNA or *S1PR1* siRNA. The location of 50 and 37kDa molecular weight markers was indicated. Estimated molecular weight of S1PR1 is around 38 to 40 KDa according to the manufacturer's product information (Santa Cruz Biotechnology). The antibody used for detecting S1RP1 is clone H-60.



Supplementary Figure 3 EMSA measuring STAT3 DNA-binding activity to the *S1PR1* promoter (upper panel) in indicated human tumor cells. Supershifting with a STAT3-specific antibody was included (3rd lane) to verify that the band corresponded to STAT3 protein-DNA complex. Lower bands are Western blotting showing levels of phospho-STAT3 and S1PR1 in the indicated tumor cells.



Supplementary Figure 4 Increasing *S1pr1* expression in tumor cells promotes tumor growth. (**a**) Real-time RT-PCR showing a 21-nucleotide dsRNA complementary to the mouse *S1pr1* promoter increased *S1pr1* expression. After trying several dsRNAs complimentary to the promoter region of the mouse *S1pr1*, we identified dsRNA (5'-UGUCCUCUGUCCUCUAAGAUUTT-3' and 5'-AAUCUUAGAGGACAGAGGACATT-3') to be effective in upregulating *S1pr1* expression, when transfected into both fibroblasts and tumor cells. Data represent mean ± s.d., $n = 3$. (**b**) MB49 tumor cells transfected with control and *S1pr1* dsRNA were implanted into male C57BL/6 mice. Tumor growth was monitored at the indicated time points. Means ± s.e.m., $n = 6$; ***, $P < 0.001$. (**c**) Overexpression of *S1PR1* in B16 tumor cells increases tumor growth *in vivo*, confirming results obtained from MB49 tumors (**Fig. 3**). B16 tumor cells transfected with either pcDNA or HA-*S1PR1* were subcutaneously injected into C57BL/6 mice. Tumor volume was measured at the indicated times. Results were shown as means ± s.e.m., $n = 4$. **, $P < 0.01$.



Supplementary Figure 5 *S1pr1* expression in immune cells is required for elevated Stat3 activity (p-Stat3), as well as expression of *Il6* in tumors. (**a**) *Left*, Real-time RT-PCR. *Middle*, Western blotting showing tumor supernatant-induced p-Stat3 with and without *S1pr1* ablation. Data represent means ± s.d., $n = 2$. *Right*, Real-time RT-PCR showing *Il6* expression in *S1pr1*$^{+/+}$ and *S1pr1*$^{-/-}$ myeloid cells exposed to tumor supernatant. Data represent means ± s.d., $n = 2$. (**b**) Western blotting showing the effects of the S1PR1 antagonist, W146, on Stat3 activity in myeloid cells directly isolated from B16 tumors. Whole cell lysates were prepared after 2 h of W146 treatment.

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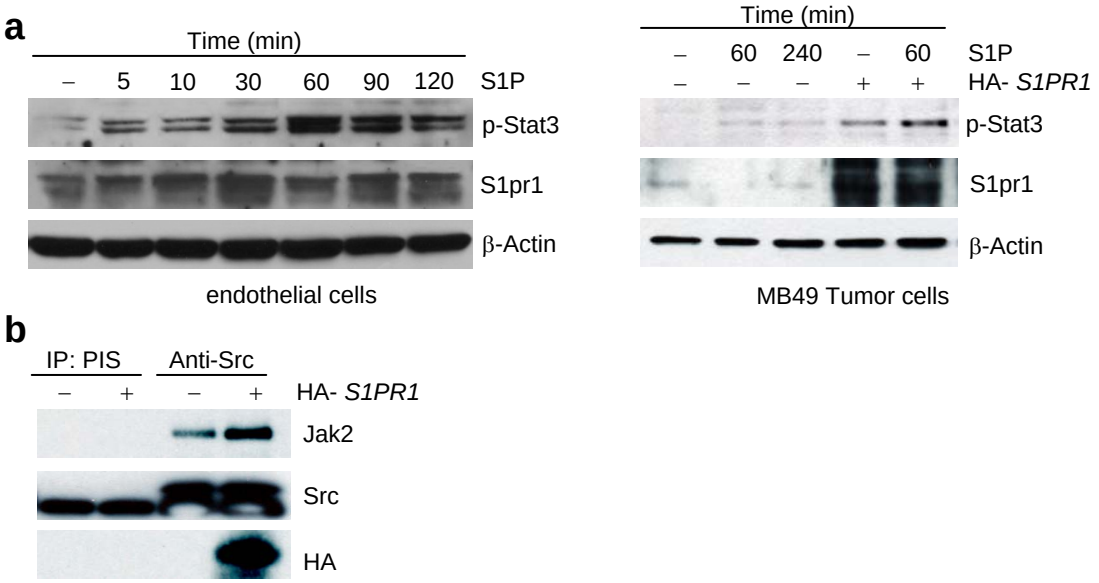
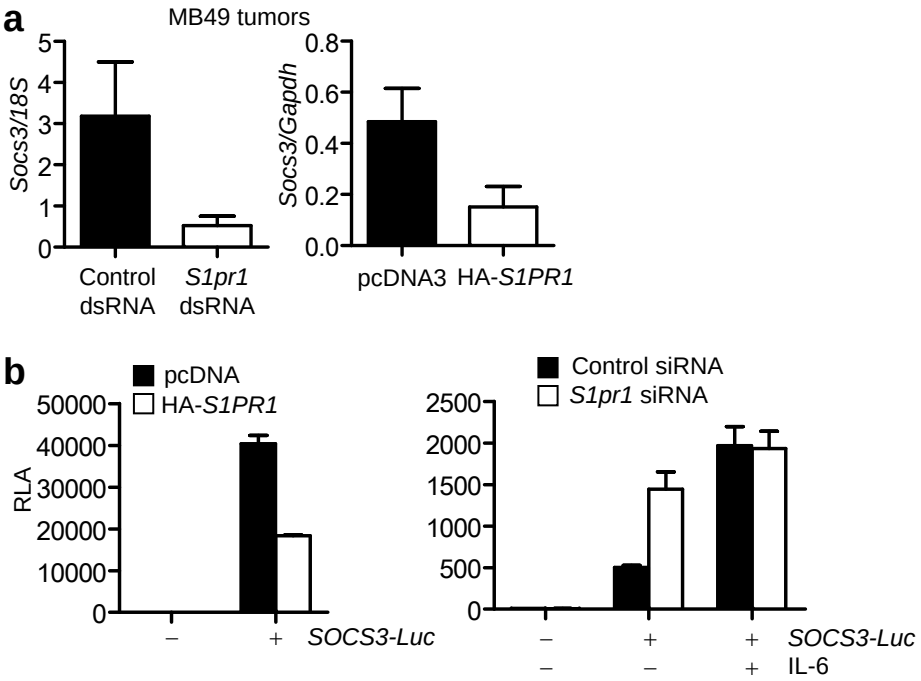
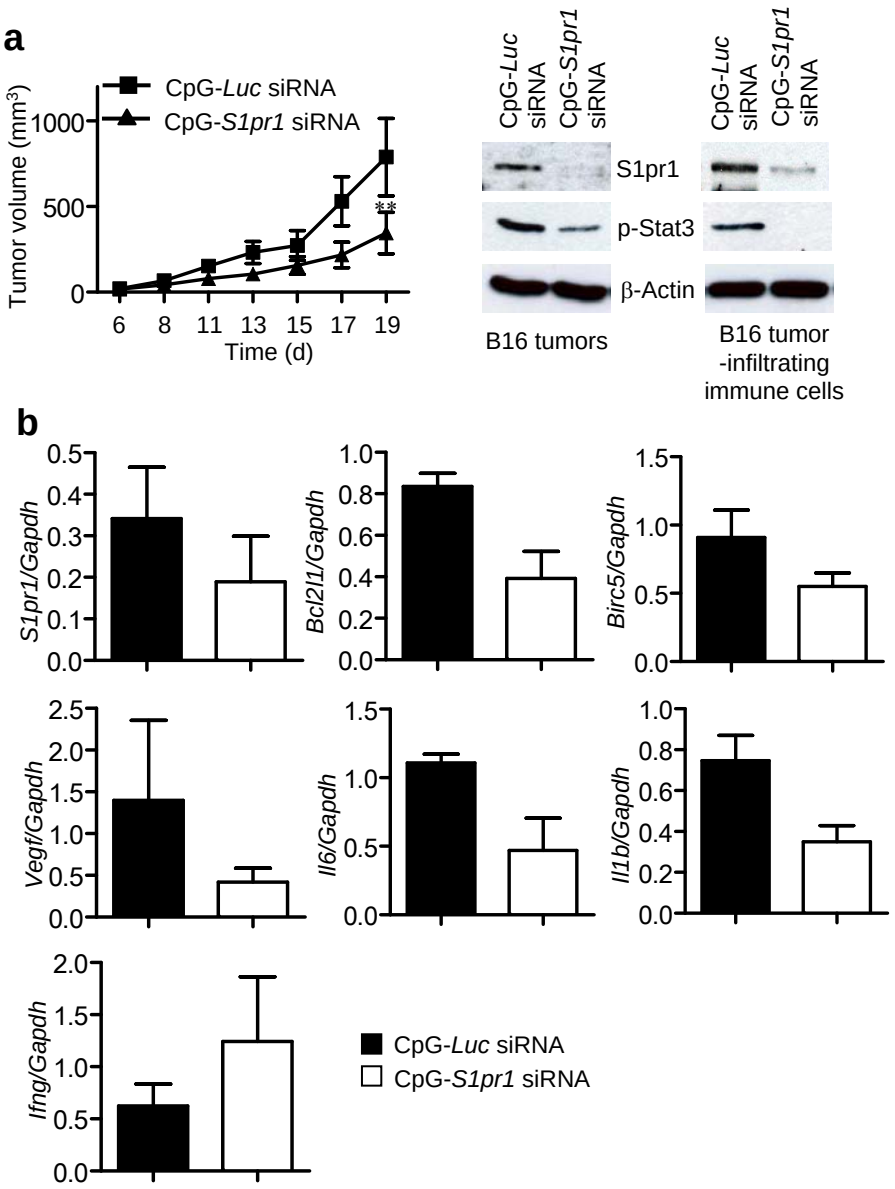
Supplementary Figure 5



Supplementary Figure 6 S1P/S1PR1-induced Stat3 activation is persistent and mediated by Jak2 and Src tyrosine kinases. (**a**) Western blotting indicating S1P-S1PR1 signaling activates Stat3 in endothelial cells (left) and in *S1PR1*-expressing MB49 tumor cells. 100 nM S1P was used to treat the cells in serum-free medium for the indicated time periods (min). (**b**) Comparing Jak2 and Src kinase association levels in tumor cells with or without elevated S1PR1 expression by Western blotting following immunoprecipitation with either Src-specific antibody or pre-immune serum (PIS).



Supplementary Figure 7 *S1PR1* overexpression does not activate but inhibits *SOCS3* expression and promoter activity. (**a**) Real-time RT-PCR showing *Socs3* mRNA levels in MB49 tumors *in vivo,* with or without an increase in *S1PR1* expression. Data represent means ± SD, n = 3. (**b**) Luciferase assays to measure *SOCS3* promoter activity in MB49 tumor cells with various levels of S1PR1, and also in the presence or absence of IL-6. Cells were treated with either vehicle or IL-6 (20 ng/ml) for 6 h (right panel). RLA: the ratio of *Firefly* to *Renilla* luciferase activity. Results were shown as means ± s.e.m. of RLA performed in triplicates. Data represent one of 3 experiments.



Supplementary Figure 8 Additional tumor model to demonstrate that targeting *S1pr1* reduces tumor growth, decreases Stat3 activity and changes Stat3 target gene expression. (**a**) Treatment of B16 tumors with CpG-*S1pr1* siRNA results in decreased Stat3 activation, leading to impaired tumor growth. The first treatment (peritumoral injection) was given when tumors were 2-4 mm in diameter, followed by more treatments every other day. RNA and protein were prepared from tumors on day 20. Results were shown as means $\pm$ s.e.m., $n = 6$; **, $P < 0.01$ (left). S1pr1 protein expression and p-Stat3 levels in tumors were shown in the right panel. (**b**) Regulation of Stat3 downstream genes in B16 tumors received CpG-*S1pr1* siRNA treatment. Shown are real-time RT-PCR data using total RNA collected from B16 tumors treated with either CpG-*Luc* siRNA or CpG-*S1pr1* siRNA. Results were shown as means $\pm$ s.d., $n = 3$.

Supplementary Figure 8

Supplementary Methods

Cell culture and reagents. B16 mouse melanoma cell line, Balb/c-3T3 and NIH-3T3 fibroblasts were obtained from ATCC. Polyclonal antibodies recognizing Stat3, S1PR1 (clones H-60 and A-6), Jak2, Gαq and siRNA against mouse G protein α subunits were purchased from Santa Cruz Biotechnology. Mouse *S1pr1* siRNA from Ambion; phospho-Stat3 Y705, phospho-Jak2, phospho-Src antibodies from Cell Signaling Technology; antibodies against Src, Gαi, Gαo and acetyl-H4 were purchased from Millipore. HA-tagged *S1PR1* were purchased from UMR cDNA Resource Center (University of Missouri-Rolla). Anti-HA (12CA5) was from Roche. IL-6 was obtained from PeproTech and used at 20 ng ml^{-1}. S1P was purchased from Sigma and used at 100 nM unless specified otherwise. W146 was obtained from Avanti Polar Lipids and SEW2871 from Cayman. Both were used at 2 μM. Jak2 inhibitor, AZD 1480 was from AstraZeneca and used at the indicated concentrations.

***In vivo* tumor experiments.** MB49 tumor cells (1×10^6) were implanted subcutaneously into the flank of female mice with $Stat3^{+/+}$ or $Stat3^{-/-}$ hematopoietic cells. To generate mice with *S1pr1* ablation in hematopoietic cells, $S1pr1^{flox/flox}$ mice were crossed with Mx1-Cre mice. Mice were challenged with B16 tumor cells (2×10^5).

For subcutaneous MB49 tumor challenge, either dsRNA encoding *S1pr1* promoter or plasmid expressing HA-tagged *S1PR1* was introduced into MB49 tumor cells by Lipofectamine 2000 (Invitrogen). The sequence for *S1pr1* dsRNA was 5'-UGUCCUCUGUCCUCUAAGAUUTT-3' and 5'-AAUCUUAGAGGACAGAGGACATT-3'. Twenty-four hour after transfection, MB49 (1×10^6) tumor cells were injected subcutaneously into the flank of 6 to 8-week-old wild-type female C57BL/6 mice. Since MB49 tumor cells carry a male antigen (H-Y antigen), they will generate a male-specific

response in female mice by tumor challenge. To generate MB49 tumors in male mice, 1×10^6 MB49 tumor cells or 5×10^5 tumor cells stably expressing either *S1pr1* shRNA or control shRNA were implanted subcutaneously into wild-type male C57BL/6 mice. To produce B16 tumors, 2×10^5 cells were implanted into male C57BL/6 mice.

Mice were sacrificed one day after the last tumor measurement in all cases. Each organ was examined for spontaneous metastasis. Tumor and spleen were processed as described below. Protein and RNA were prepared from isolated immune cells, whole spleens and tumors for various analyses as indicated.

To generate experimental lung metastasis model, B16 tumor cells (1×10^5) were injected intravenously into *S1pr1*$^{+/+}$ or *S1pr1*$^{-/-}$ mice. After 21 d, lungs were removed and washed in Hank's buffered salt solution (HBSS). Number of visible metastatic tumor nodules was enumerated by counting individual nodules. B16 tumor nodules were easily identifiable due to their pigmentation.

To test the therapeutic effect of CpG conjugated siRNA on tumor growth, MB49 or B16 tumor cells were implanted into male C57BL/6 mice and monitored for tumor growth. When tumors became palpable, mice were injected into the peritumoral region with either 0.78 nmole of CpG-*Luc* siRNA or CpG-*S1pr1* siRNA every other day. Mice were sacrificed at endpoint and tissues were harvested for further analyses. siRNA sequences used for this experiments were: Mouse *S1pr1* siRNA (SS), 5'-GAUCGUAUCUUGUUGCAAAUGCCCC-3' and CpG1668-mouse *S1pr1* siRNA (AS),5'-TCCATGACGTTCCTGATGCT-linker-GGGGCAUUUGCAACAAGAUACGAUCCG-3'.

Isolation of immune cells. To isolate tumor-infiltrating immune cells, tumors were gently minced then incubated (30 min, 37 ºC) with collagenase D and DNAse (Roche,

400 U ml^{-1}) solution. Cells were resuspended by repeated pipetting and filtered through a mesh filter. Mononuclear cells were separated by gradient centrifugation using Histopaque (Sigma, 1.083 g ml^{-1}) and kept as tumor-infiltrating immune cells. Tumor $CD11b^+$ or $Gr1^+$ cells were isolated from immune cell mixtures using specific antibodies in combination with EasySep magnetic nanoparticles from StemCell Technologies. Splenic $CD11b^+$ cells were enriched from spleen in the same manner.

Tumor supernatant was prepared from tumor-infiltrating immune cells of MB49 tumors overexpressing either pcDNA or HA-*S1PR1*. Cells (1×10^6) were plated in 24-well plates. Twenty-four hours later, culture medium was collected, filtered to remove cell debris and kept as tumor supernatant. Conditioned medium was prepared by reconstituting fresh cell culture medium with 10% tumor supernatant.

Real-time RT- PCR. Total RNA was prepared using RNeasy kit (Quiagen) following the manufacturer's instructions. RNA (0.5 to 1 μg) was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad), and real-time PCR reactions were performed using iQ SYBR Green supermix (Bio-Rad) on DNA Engine thermal cycler equipped with Chromo4 detector (Bio-Rad). Gene specific primer sets were purchased from SA Bioscience. Either 18S rRNA or *Gapdh* housekeeping genes were used as internal controls to normalize mRNA expression. Each independent experiment was pooled to calculate mean and s.d..

Western blotting and co-immunoprecipitation analysis. Cells were lysed in a modified RIPA buffer containing 50 mM Tris, PH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 and protease inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE and subjected to immunoblotting.

For the interaction study of S1PR1 and protein kinases, 200 μg of protein lysates were pre-cleared with Protein G agarose (Invitrogen) for 30 min at 4 °C. After brief centrifugation, the supernatant was incubated with specific antibodies for overnight. Protein agarose was added for an additional hour to capture immune complexes. Immunoprecipitates were collected by centrifugation, washed three times with modified RIPA buffer and subjected to SDS-PAGE and immunoblotting analysis.

Immunohistochemical/mmunofluorescent staining and confocal microscopy. IHC staining of OCT-embedded frozen tissue slides were performed based on the protocol provided by Cell Signaling Technology. Briefly, the tissue slides were dried at room temperature for 30 min then fixed in 2% paraformaldehyde for 15 min, followed by 5 min in methanol at -20 ºC. After wash, tissue sections were treated with 1% H_2O_2 in methanol for 10 min at room temperature. Tissue was incubated with the primary antibody for overnight at 4 ºC and subjected to ABC/DAB detection method (Vector Laboratories). The expression level of p-Stat3 in tumor tissues was visualized by a Nikon ECLIPSE TE2000-U microscope and imaged using SPOT software.

Human tissue array slides (including both normal and malignant tissues) were used to detect phosphorylated Stat3 and S1PR1 proteins. Briefly, tissue slides were deparaffinized, rehydrated through an alcohol series and boiled in Antigen Unmasking Solution (Vector). After blocking, sections were stained (overnight, 4 °C) with a 1:50 dilution of a primary antibody, followed by incubation with a secondary antibody (Alexa 488 and Alexa 546, Invitrogen), mounted in Vectashield mounting medium containing 4'6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and imaged by confocal microscopy (Zeiss LSM Image Browser software).

Promoter activity assay. To analyze the promoter activity of *S1pr1* genes, 960 bp upstream from transcriptional start site of mouse *S1pr1* genes were inserted into pGL3 plasmid vector to generate pGL3-*S1pr1* wild-type. pGL3-*S1pr1* mutant plasmid (Mut) in which the putative Stat3 binding site was mutated was generated by site-directed mutagenesis. Primers used for mutagenesis was 5'-TCAAAGGCCTCTGAGCTGACCATCATCTGGGGCACGCCTGG-3' and 5'-CCAGGCGTGCCCCAGATGATGGTCAGCTCAGAGGCCTTTGA-3'. The mutation site is underlined.

Cells (2×10^5) were plated the day prior to transfection in 6-well plates. Either wild-type or mutant pGL3-*S1pr1* plasmids were then introduced into cells, together with a *Renilla* luciferase construct, using Lipofectamine 2000. After 48 hours, cells were lysed in 1 x passive lysis buffer and analyzed for dual luciferase activity following the manufacturer's instructions (Promega). For IL-6 treatment, cells were stimulated with 20 ng ml^{-1} of IL-6 for 6 h. Relative Luciferase Activity (RLA) was calculated as a ratio of *Firefly* to *Renilla* luciferase activity. Data represent the means ± s.e.m. of relative luciferase activities performed in triplicate. Data in Figure represents one of three independent experiments.

Transfection. Cells were seeded (5×10^5) in 100 mm plates 24 h before transfection with either *S1pr1* dsRNA or plasmids encoding *S1PR1* using Lipofectamine 2000 (Invitrogen). For some experiments, cells were transfected with *S1pr1* siRNA (Ambion) in the same manner. To establish MB49 tumor cell line stably expressing *S1pr1* shRNA, LinX packaging cells (1×10^6) were transfected with pGFP-V-RS-*S1pr1* shRNA constructs (OriGene). The viral supernatant was collected and added into culture medium of MB49 cells in the presence of polybrene (5 μg ml^{-1}). Twenty-four hour after transduction, fresh media containing 2 μg ml^{-1} puromycin was added for selection.

Puromycin-resistant cell colonies were individually isolated and analyzed for S1PR1 expression. Tumor cells with control shRNA were prepared in the same manner except that cells were transfected with control shRNA provided by the manufacturer.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts (5 μg) were incubated at room temperature for 20 min with ^{32}P-labeled probe (50,000 c.p.m.). The oligonucleotide probe sequence to detect Stat3 binding to *S1pr1* promoter is: 5'-AGGCCTCTGAGCTTCCCAGAATCTGGGGCACAC-3'. To detect IL-6 induced Stat3 activation, cells were treated with IL-6 (20 ng ml^{-1}) for 20 min before harvest, then analyzed for Stat3-binding complex using a *hSIE* probe. For antibody supershift, 1 μg Stat3-specific antibody was pre-incubated (15 min) with protein prior to the addition of radiolabeled probe.

Cell co-culturing. MB49 tumor cells were transfected with either control or *S1PR1* plasmids. Tumor cells were mixed with splenocytes from wild-type mice at a 1 to10 ratio. After 24h, CD11b^{+} cells were isolated from the cell mixture using specific antibodies in combination with EasySep magnetic nanoparticles (StemCell Technologies), then lysed with modified RIPA buffer for protein detection.

Flow cytometry. For extracellular staining of immune markers, we prepared single-cell suspensions from B16 tumors grown in mice with *Stat3*$^{+/+}$ and *Stat3*$^{-/-}$ myeloid compartments. Cells were resuspended in flow buffer (2% fetal calf serum and 0.1% (w/v) sodium azide in PBS), fixed with paraformaldehyde then permeabilized with methanol. Then cells were subjected to intracellular staining with anti-phospho-Stat3 antibody (BD Biosciences). Fluorescence data were collected on a FACSCalibur system

(Beckton Dickinson) and analyzed using FlowJo software (Tree Star). Tumor cells were gated as $CD11b^{-}$, FSC^{high}, SSC^{high} (due to high content of melanin granules).