Dll4/Notch/TGF-β signaling links tumor-infiltrating myeloid cells to tumor progression

Supplemental Data

Files in this Data Supplement (provided as a unique PDF) include:

1. Extended Experimental procedures. This section provides details of the experimental procedures such as times and temperature, antibody source and concentrations used, instrumentation settings, primer derivation and catalogue numbers; animal breeding and genotyping.
2. Supplementary Figure S1 with legend: shows the kinetics of EL4, LLC1 and B16F10 tumor growth in WT and Gfi1-KO mice
3. Supplemental Figure S2 with legend: analysis of EL4 and LLC1 tumor vascularization (A); distribution and typical profiles of CD11b+Ly6C+Ly6G- and CD11b+Ly6C+Ly6G+ in spleen and bone marrow of naïve and tumor-bearing mice (B-F).
4. Supplementary Figure S3 with legend: typical sorting profiles of LLC1 tumor-infiltrating cell populations (A); expression profiles of Notch ligands and receptors in tumor cells and tumor-infiltrating populations (B); cartoon representation of interacting Notch ligands and receptors.
5. Supplementary Figure S4 with legend: kinetic analysis of tumor growth with or without DAPT
6. Supplementary Table 1: Extensive gene expression profiling by qPCR.

Extended Experimental Procedures

Cells and reagents

The murine cell lines EL4 (gift of Dr. L. Wolf, NCI), LLC1 and B16F10 (both from ATCC) were maintained in RPMI (EL4) or DMEM (LLC1 and B16F10) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100IU penicillin and 100ng/ml streptomycin. Primary cells from bone marrow (femurs and tibias) and spleen were derived by standard techniques (De La Luz Sierra et al., 2007). Tumors were dissected and cut in small pieces; single cell suspensions were obtained by mechanical dissociation or by using 0.5% collagenase Type II (Worthington Biochemical) in PBS; the resulting single cell suspensions were washed in PBS containing 2% FBS and filtered through 40mM nylon strainers (BD Falcon). Subsets of cells from bone marrow, spleen and tumor tissues were obtained through cell sorting.

In vitro assays

Transmigration assays were performed with primary CD11b+Gr1+ ($2x10^6$ cells/well) sorted from bone marrow, 5mM pore size Transwell (Corning), incubation medium consisting of DMEM with 1% bovine serum albumin and 50mM 2-ME, and incubation time of 4 hours at 37°C. Mouse CCL2 (R&D Systems) was used at 50ng/ml.

Tgfb was measured by mouse Tgfb1 quantikine ELISA kit (R&D Systems) in the culture medium and culture supernatant of bone marrow CD11b+Gr1+ cells incubated for 3 days in medium only (DMEM with 10% FBS) or in medium containing mouse M-CSF (20 ng/ml) or GM-CSF (40 ng/ml); both from R&D Systems. Mouse CCL2, M-CSF and GM-CSF were measured by quantikine ELISA kits (R&D Systems) in the complete medium and culture supernatant of EL4, LLC1 and B16F10 cell lines seeded at 70%

confluence and incubated for 72 hours.

Cell proliferation in triplicate cultures was measured by ^{3}H thymidine uptake over 6-72 hours incubation at 37°C. Exponentially-growing EL4, LLC1 and B16F10 cells were washed extensively, and re-plated at various cell densities in culture medium (EL4: RPMI; LLC1 and B16F10: DMEM) supplemented with 1%BSA, 2mM L-glutamine, 100IU penicillin and 100ng/ml streptomycin and 50μM 2ME. Cultures were supplemented with various concentrations of human Tgfb1 (R&D Systems), DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine tert-butyl ester; Sigma-Aldrich), DBZ ((2S)-2-[2-(3,5-difluorophenyl)-acetylamino]-*N*-(5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[b,d]azepin-7-yl)-propionamide; Millipore) and appropriate diluent controls. To determine the effects of immobilized Dll4 on EL4 and LLC1 cell proliferation, 96-well plates were coated (50 μL/well) with recombinant mouse his-tagged Dll4 (R&D Systems; 1 μg/mL PBS mL) or his control (Millipore). Wells were washed with PBS before 4×10^3 cells per well were seeded in triplicate in medium only or in medium supplemented with Tgfb1, DAPT and/or DBZ. In co-culture experiments, tumor cells (10-15 $x10^3$ cells/well) and CD11b+Ly6C+Ly6G- bone marrow cells (1.0-1.5 $x10^3$) were incubated in DMEM with mouse Tgfb (0-5ng/ml) with or without DAPT (1μM).

Gene expression analysis

Total RNA from cell lines, primary cells and mouse tumor tissue was extracted using TRIzol (Invitrogen), RiboPure^{TM} Blood kit (Invitrogen), RNeasy Mini kit (Qiagen) or Absolutely RNA Nanoprep Kit (Stratagene). Total RNA (1-5 μg) was reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) or Superscript III (Life Technologies) with oligo (dT) 20 primer. Expression of

mouse Ccl2, Ccl3, Cxcl10, Cxcl11, Cxcl12, Cxcl15, Cxcr4, Il2, Il4, Il6, Il10, Il12a, Il12b, Il17, Il18, Csf1, Csf2, Tnf, Il18, Ccr2, Ccr3, Cxcr2, Cxcr7, Il2ra, Il6st, Dll4, Hey1, Hey2, Hes1, Hes5, Ifng, S100a9, Lcn2, Mmp9, Vegfa, Prok2, Nos2 and Gapdh was measured by real-time PCR using TaqMan Gene expression assays (Life Technologies, Invitrogen). Expression of mouse Tgif1, Tgif2, Cxcl1, Il1b, Tgfbr1, Tgfbr2, Il12rb1, Il12rb2, Csf3r, Dll1, Dll4, Jag1, Jag2, Notch1 Notch2, Notch3, Notch4, Arg1 and Foxp3 was measured by real-time PCR using QuantiTect primer assays for SYBR green-based analysis (SA Bioscience, Qiagen). Expression of the following mouse genes was evaluated with the indicated primers: Hey1 forward 5'-tcagcgtggggaatcttaac-3' and reverse 5'-aacacctcggtccatcaaag-3'; SMAD3 forward 5'-gagcgagttggggagacatt-3' and reverse 5'-tgtaagttccacggctgcat-3', using SYBR green-based quantitative analysis. Expression of mouse Hey2 (ID 7305158c3), Tgfbr1 (ID 40254607c3), Smad5 (ID 255708404c3), cMyc (ID 293629266c2) and Gapdh (ID 6679937a1) were measured with primers from PrimerBank (MGH) using SYBR green-based quantitative analysis. PCR reactions were performed with 1 μL cDNA, Taqman PCR Universal Master Mix (Life Technologies, Invitrogen). Fluorescence signals were monitored after each PCR cycle with ABI Prism 7900 sequence detection system (Applied Biosystems). Relative differences of PCR products were measured by the comparative cycle threshold method. The experiments were independently repeated at least 3 times, each performed in triplicate. mRNA levels were expressed as relative units.

Tumor models

All mouse studies were approved by the NCI-Bethesda Animal Care and Use Committee and conducted per protocol. Gfi1-/-, Gfi1+/- and Gfi+/+ mice (Zhu et al., 2006) (De La

Luz Sierra et al., 2007) were housed and bread in the animal facilities at the National Cancer Institute; genotyping was performed as described (Zhu et al., 2006) (De La Luz Sierra et al., 2007). The murine cell lines EL4, LLC1 and B16F10 (all from C57BL6) were implanted subcutaneously ($10x10^6$ cells/mouse) in the left abdominal quadrant of male and female 6- to10-week-old Gfi1-/-, Gfi1+/- and Gfi+/+ (backcrossed to C57BL mice for 15-20 generations). In adoptive transfer experiments, EL4 or LLC1 cells were inoculated subcutaneously either alone ($1x10^6$ cells/mouse) or mixed with primary cells freshly obtained from Gfi1-/-, Gf11+/- or Gfi1+/+ mice. The primary cells included: unfractionated or Ly6G+ cell-depleted (negative sorting) splenocytes ($2x10^6$) from EL4 tumor-bearing mice (2 weeks after EL4 subcutaneous injection); and CD11b+Ly6C+Ly6G- cells ($0.5x10^6$) sorted from pooled bone marrows of LLC1-bearing mice (2 weeks after LLC1 subcutaneous injection). For experiments involving the Notch signaling inhibitor DAPT, the mice (Gfi1-/-; Gfi1 +/- and Gfi+/+) were first injected subcutaneously with LLC1 cells ($10x10^6$/mouse) and 5 days later (at this time all mice have developed a measurable subcutaneous mass) the tumor-bearing mice were injected intra-peritoneally with DAPT (10 mg/kg) or vehicle control 5 days/week (total number of injections no=7). The experiment was stopped on day 15 after tumor cell injection. Tumor volume was measured with the formula: Pi/6 x L x W x W where L is the longest tumor diameter and W is the diameter perpendicular to L, as previously described (Shojaei et al., 2007). Tumors were harvested, weighed, and processed for immunoblotting, histology and gene expression studies.

Tumor immunohistochemistry

Tumors dissected from the mice were fixed in 4% PFA and cryopreserved. Tissue

sections were stained with rat anti-mouse CD31 monoclonal antibody (1:100; BD Pharmingen) followed by Alexa Fluor647 goat anti-rat IgG (1:1000; Invitrogen); with rabbit anti-PCNA antibody (1:500; Abcam) followed by Alexa Fluor488 goat anti–rabbit IgG (1:500; Invitrogen); rabbit monoclonal anticleaved caspase 3 (1:500; Cell Signaling Technology) followed by Alexa Fluor 488-conjugated anti-rabbit (1:2000 Invitrogen); rabbit anti-pSMAD3 (Epitomics) followed by Alexa Fluor 488-conjugated anti-rabbit (1:2000 Invitrogen); nuclei were visualized with DAPI (1:2000; Invitrogen).

Sections were imaged through an Axiovert 200 fluorescence microscope (Carl Zeiss, Thornwood, NY) or a Zeiss LSM710 confocal microscope. The pseudocolored images were then converted into .tif files, exported to Adobe (San Jose, CA) Photoshop, and overlaid as individual layers to create multicolored merged composites.

Images from fluorescent PCNA, Caspase3, CD31 and DAPI staining were quantified using ImageJ Version 1.46 NIH software (http://rsb.info.nih.gov/ij/download.html). Pixel values for PCNA, Caspase3, CD31 and DAPI fluorescence were from the entire tissue section. Ratios of PCNA/DAPI, Caspase3/DAPI and CD31/DAPI pixel counts were calculated from each section to derive a relative mean PCNA, Caspase3 and CD31 pixel count. Relative mean pixel counts from groups of tissue sections were averaged.

Western blotting

Protein extracts (RIPA lysis buffer with protease inhibitor cocktail setIII (Calbiochem), 50mM NaF, and 1mM Na_3VO_4) were resolved in NuPAGE 4%-12% Bis-Tris Gel (Invitrogen) and dry-transferred with iBlot system (Invitrogen). Membranes were probed with: rabbit mAb to phospho-SMAD3 (pS423/425; Epitomics), rabbit IgG to phospho-SMAD2 (Ser465/467; Cell Signaling Technology); rabbit IgG to SMAD2/3 (Cell

Signaling Technology); rabbit mAb to SMAD3 (Epitomics); rabbit mAb to c-Myc (Epitomics); and goat anti-actin (Santa Cruz Biotechnology). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG was from Amersham Pharmacia Biotech. Bound secondary antibodies were visualized by Immobilon Western chemioluminescent HRP substrate (Millipore). Band intensities were measured with ImageJ software.

Flow cytometry and cell sorting

The following antibodies directed at murine antigens were used for cell-surface staining: CD16/CD32 (block, clone 2.4G2, BD Pharmingen); APC-CD11b mAb (clone M1/70; BD Pharmingen and Biolegend); PE-conjugated Ly6C (clone AL21, BD Pharmingen; clone HK1.4 Biolegend); FITC-conjugated Ly6G (clone 1A08, BD Pharmingen); PE-Cy7, APC, PE, FITC-conjugated Gr1 (clone RB6-8C5; eBioscience and BD Pharmingen); PE-conjugated CD3 complex (clone 17A2, BD Pharmingen); APC-conjugated CD3e (clone 145-2C11; BD Pharmingen); FITC-conjugated CD4 (clone GK1.5; BD Pharmingen); PE-CD8a (clone 53-6.7; BD Pharmingen); PE-CD25 (clone 3C7, BD pharmingen); APC-CD45/B220 (clone RA3-6B2; BD Pharmingen); F4/80 (clone BM8; eBioscience); F4/80-like receptor (clone 6F12, BD Pharmingen); CD80 (B7-1, clone 16-10A1; eBioscience); CD86 (B7-2, clone GL1, eBioscience); γδ TCR (clone GL3; eBioscience); CD25 (clone PC61.5; eBioscience); PE-I-A/I-E (clone M5/114.15.2; BD Pharmingen); SMA (clone 8/SMN; BD Biosciences); PE-CD49b (clone Dx5; BD Pharmingen). Cell viability was assessed by DAPI staining viability due eFluor450 (eBioscience). Flow cytometry data were collected using a MoFlo Astrios cell sorter equipped with Summit6.1acquisition software and the resulting data analyzed using Kaluza 1.2 software (all from Beckman Coulter) or were collected with a

FACSAria™ III (Becton Dickinson) and the data analyzed using FlowJo 9.2 (Industrynet). Cell sorting was carried out with either MoFlo Astrios or FACSAria™ III cell sorters.

ELISA

Levels of mouse Tgfb1, CCL2/MCP-1, GM-CSF and M-CSF were measured by using Quantikine ELISA kits (R&D Systems) following the manufacturer's instructions.

Bioinformatic and statistical analysis

Human tumor data sets are from The Cancer Genome Atlas (TCGA) found at http://cancergenome.nih.gov/); data was downloaded directly from TCGA data matrix. For Head and Neck squamous cell carcinoma, the data set includes 303 cancers and 37 normal tissue control; 37 samples are paired tumor and control tissues from individual patients. For Lung Squamous Cell carcinoma the data set includes 369 cancers and 50 normal tissue control; 50 samples are paired tumor and control tissues from individual patients. The data was analyzed using the R system (2.14.1) for statistical computation and graphics to calculate Mann-Whitney U-test (wilcox.test) and graph box plots (boxplot). Group means, standard deviations (SD) and standard error of the mean (SEM) were calculated by standard methods; group differences were evaluated by two-tailed Student's t test with equal variance assumption; with small sample size differences were evaluated with Fisher's exact test. P values ≤ 0.05 were considered significant.

References

De La Luz Sierra, M., Gasperini, P., McCormick, P. J., Zhu, J., and Tosato, G. (2007). Transcription factor Gfi-1 induced by G-CSF is a negative regulator of CXCR4 in myeloid cells. Blood *110*, 2276-2285.

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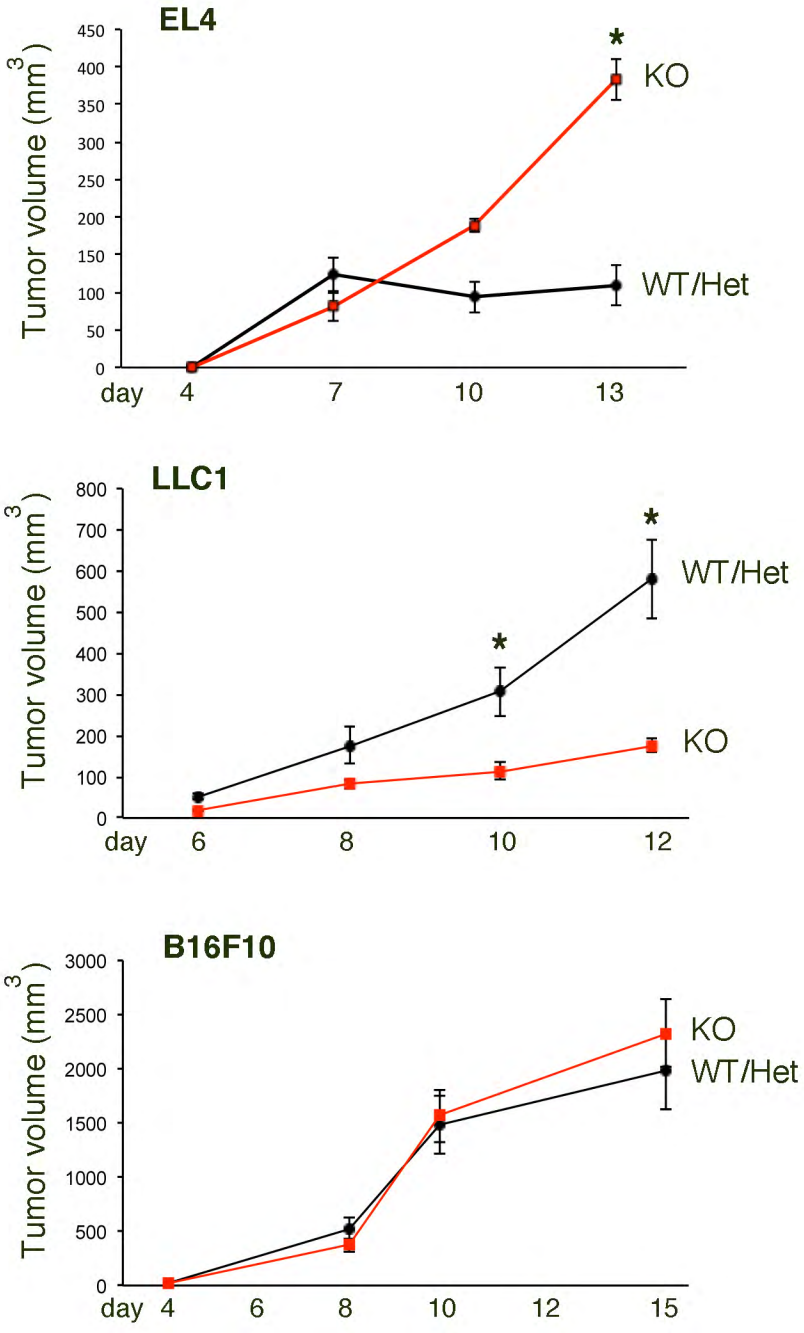

Figure S1. Growth kinetics of tumors from control (WT/Het Gfi1) and Gfi1 KO (Gfi1-/-) mice analyzed over 12-15 days post subcutaneous injection of EL4, LLC1 and B16F10 tumor lines. Tumor volume was calculated using the formula Pi/6 x L x W x W with L as the longest diameter and W the diameter perpendicular to L. Data are averages ± SEM. EL4 tumors WT/Het n=12; KO n=10; LLC1 tumors WT/Het n=15, KO n=12; and B16F10 tumors WT/Het n=10, KO n=8; *denotes statistical difference at the indicated time-point; p values from Student's t test.

Figure S1

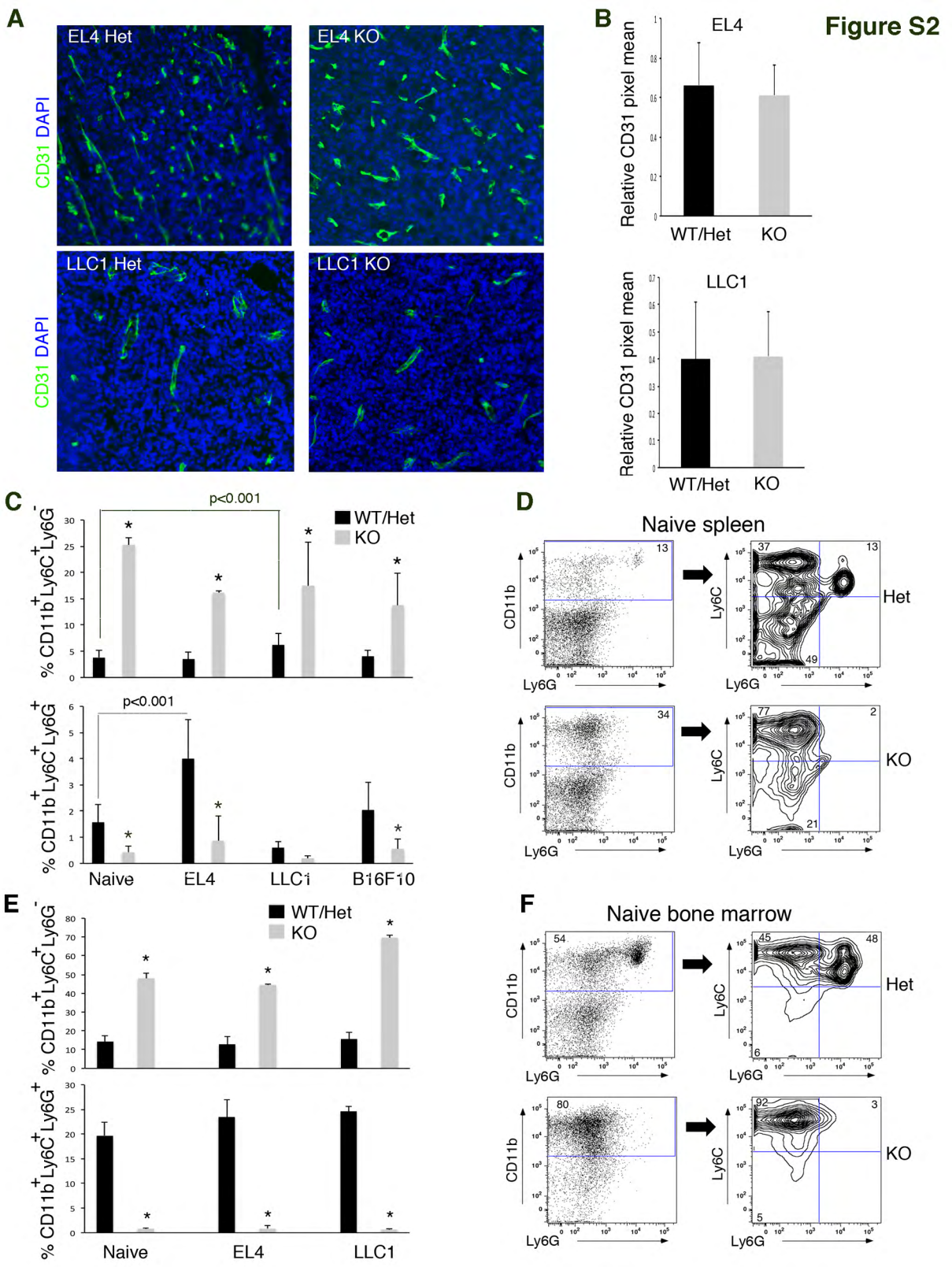

Figure S2. Vascularization of EL4 and LLC1 tumors evaluated by CD31 immunohistochemistry **(A, B)**. Typical appearance (A) and quantitative analysis (B) of tumor vessels from Gfi1-KO and control heterozygous mice is shown; EL4: n=5/group; LLC1: n=7-9/group. Distribution of monocytes and granulocytes in spleens and bone marrows from naïve and tumor-bearing control (WT/Het) and Gfi1-KO mice is shown **(C-F)**. In the bar graphs, flow cytometry data are expressed as average percentage of total cells from spleen (C) and bone marrow (E) ± SD; EL4: n=5; LLC1: n=5; B16F10 n=3. In the representative profiles from flow cytometry, the numerical values indicate the cells in the quadrant expressed as percentages of total CD11b+ leukocytes in the spleen (D) and bone marrow (F); p values from Student's t test.

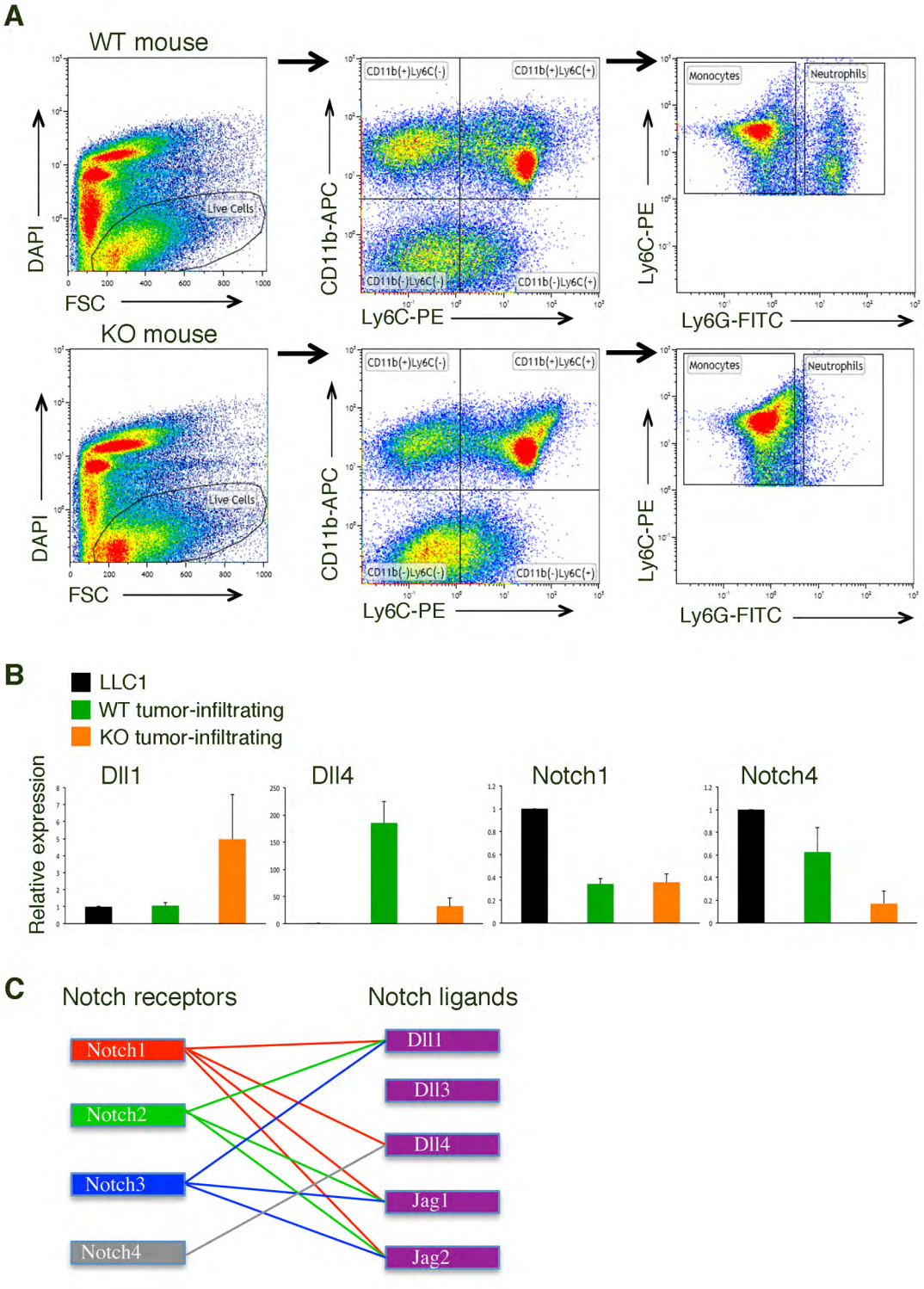

Figure S3. Sorting profiles of monocytes and granulocytes from single-cell suspensions of LLC1 tumor tissue from WT and Gfi1-KO mice **(A)**. After labeling with antibodies to CD11b, Ly6C and Ly6G, gating on live cells expressing CD11b, the Ly6C+Ly6G- monocytes and the Ly6C+Ly6G+ granulocytes were separated by cell sorting. Expression of the Notch ligands Dll1 and Dll4 and Notch 1 and Notch 2 receptors in LLC1 cells and LLC1 tumor-infiltrating Ly6C+Ly6G- monocytes from WT and KO mice (no=3). The results from qPCR are expressed as relative units (± SD) **(B)**. Cartoon representation of interacting Notch ligands and receptors **(C)**.

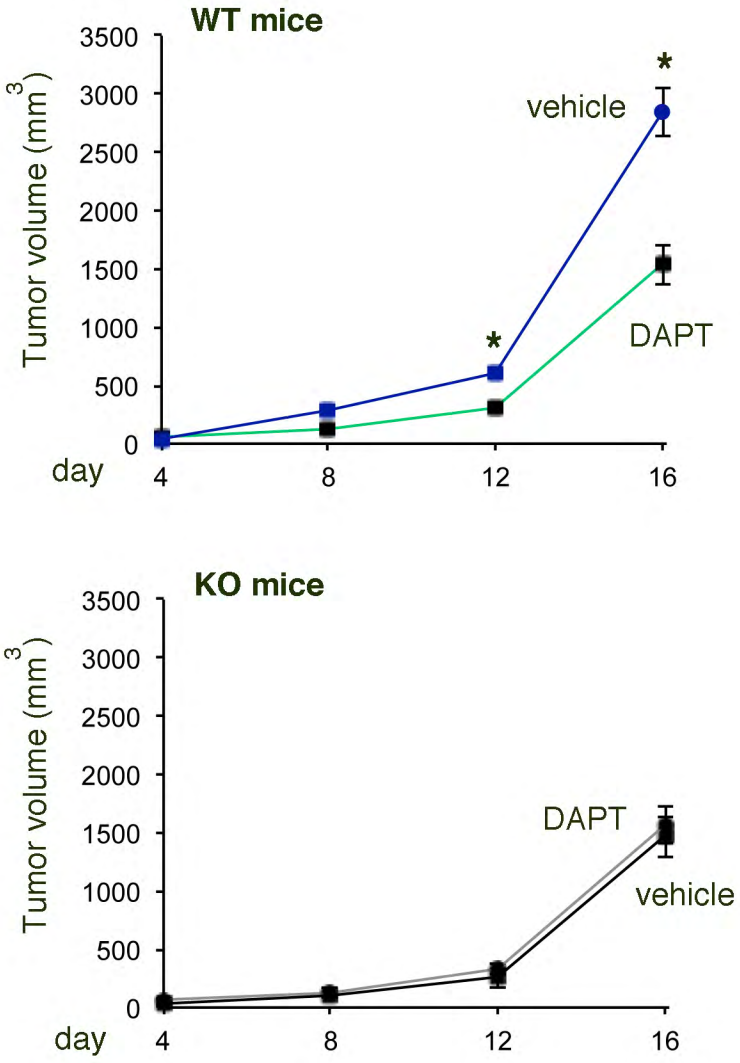

Figure S4. Kinetic analysis of LLC1 tumor growth in WT and Gfi1-KO mice treated with DAPT (10mg/kg) or vehicle alone. Treatment was started 24hr after s.c. inoculation of LLC1 cells. The results reflect the average tumor volume/group ± SEM; WT: n=9 (vehicle), n=9 (DAPT); KO: n=3 (vehicle), n=3 (DAPT). *denotes statistical difference at the indicated time-point; p values from Student's t test.

Figure S4

Table S1. Similarly expressed genes in tumors from Gfi1-null and control mice

Gene	Relative Expression			P value[b]	Relative Expression			P value
	EL4	WT-EL4	KO-EL4		LLC1	WT-LLC1	KO-LLC1	
Chemokine								
Ccl2	1	4.7(1.2)	4.1(0.9)	0.8	1	7.2(0.8)	6.0(0.4)	0.4
Ccl3	nd	1.6(0.4)	1.0(0.3)	0.7	nd	1.5(1.0)	0.8(0.4)	0.2
Cxcl1	nd	0.6(0.6)	0.3(0.5)	0.4	nd	nd	nd	
Cxcl10	nd	2.7(2.0)	1.5(1.4)	0.2	1	2.9(0.6)	3.8(0.5)	0.8
Cxcl11	nd	1.6(1.6)	1.1(0.6)	0.5	nd	nd	nd	
Cxcl12	nd	2.1(1.6)	1.1(0.5)	0.2	nd	1.4(0.6)	0.9(0.5)	0.2
Cxcl15	nd	nd	nd		1	nd	nd	
Cytokine								
Il1b	1	0.1(0.1)	0.2(0.2)	0.1	1	2.7(0.2)	2.1(0.2)	0.2
Il2	1	0.6(0.3)	0.1(0.02)	0.1	nd	1.4(0.5)	1.3(0.2)	0.9
Il4	1	2.1(1.8)	4.5(0.9)	0.07	nd	nd	nd	
Il6	1	5.7(0.4)	9.2(2.7)	0.1	nd	1.3(1.3)	0.9(0.1)	0.7
Il10	nd	0.7(0.4)	0.4(0.4)	0.3	nd	3.2(2.2)	1.5(1.0)	0.3
Il12a	1	2.2(0.4)	2.9(0.2)	0.3	nd	3.2(2.2)	1.5(1.0)	0.3
Il12b	nd	nd	nd		nd	nd	nd	
Il17	nd	nd	nd		nd	3.1(3.2	1.9(1.1)	0.7
Il18	nd	0.3(0.2)	0.8(0.3)	0.06	not done			
Csf3	nd	3.0(4.6)	9.2(8.6)	0.5	nd	3.6(1.6)	1.6(0.6)	0.1
Ccf1	1	2.1(1.2)	2.9(1.2)	0.5	1	1.4(0.07)	1.4(0.3)	0.9
Csf2rb	1	2.7(1.8)	1.6(1.2)	0.3	1	80(15)	58.5(20)	0.3
Tnf	1	1.3(0.8)	1.5(0.6)	0.4	nd	1.3(0.55)	1.3(1.1)	1.0
Chemokine/Cytokine Receptor								
Ccr2	not done				1	6.2 (3.6)	6.6(2.8)	0.8
Ccr3	nd	3.5(1.5)	3.0(2.4)	0.5	nd	0.7(0.3)	0.6(0.4)	0.4
Cxcr2	nd	3.3(3.1)	0.5(0.5)	0.2	nd			
Cxcr7	nd	0.6(0.5)	0.5(0.4)	0.6				
Tgfbr1	1	1.7(0.5)	1.7(0.2)	1	nd	0.6(0.4)	0.4(0.2)	0.6
Tgfbr2	1	0.8(0.6)	0.8(0.1)	0.9	1	3.2(0.4)	1.65(0.3)	0.2
Il2ra	nd	0.9(0.7)	1.1(1.0)	0.7	not done			
Il6st	1	2.1(0.4)	3.3(0.2)	0.2	1	1.1(0.4)	1.2(0.9)	0.6
Il12rb1	1	0.4(0.2)	1.1(0.6)	0.06	1	1.3(0.5)	1.3(0.2)	0.9
Il12rb2	nd	1.2(0.1)	1.3(0.4)	0.4	not done			
Csf3r	not done				nd	0.75(0.3)	0.7(0.6)	0.9
Notch-related								
Dll1	1	1.4(0.8)	1.8(0.6)	0.8	1	2.1(0.3)	1.8(0.2)	0.8
Jag1	1	1.6(0.8)	1.5(0.5)	0.7	1	10.6(0.3)	9.3(0.1)	0.8
Jag2	1	1.3(0.8)	1.6(0.7)	0.8	1	3.4(0.3)	2.6(0.1)	0.5
Notch1	nd	nd	nd		1	1.1(0.3)	0.8(0.3)	0.9
Notch2	1	1.6(0.8)	1.3(0.6)	0.7	1	3.8(0.2)	2.75(0.2)	0.5
Notch3	1	1.1(0.5)	1.4(0.7)	0.5	1	4.1(0.4)	2.1(0.1)	0.1
Notch4	1	0.8(0.3)	0.9(0.6)	0.5	1	11.7(0.6)	13.5(1.5)	0.9
Hes1	1	2.7(0.3)	3.6(2.4)	0.5	1	2.8(2.7)	3.1(1.7)	0.9
Hes5	not done				nd	nd	nd	
Other Genes								
Ifng	nd	1.5	1.5	0.9	nd	1.4(0.4)	1.2(0.1)	0.15
Mmp9	1	37.5(17.8)	16.8(8.4)	0.3	nd	1.1(0.7)	1.0(1.1)	0.8
Arg1	nd	1.1(0.1)	1.3(0.5)	0.5	nd	1.2(1.1)	0.2(0.1)	0.2
Nos2	nd	1.3 (0.6)	1.6 (0.4)	0.5	nd	1.4 (1.1)	0.8 (0.4)	0.5
Vegfa	1	16.7(1.5)	22.1(0.5)	0.8	nd	0.9(0.2)	0.8(0.3)	0.8
Foxp3	1	1.4(1.2)	1.9(0.7)	0.5	nd	1.6(1.2)	1.8(1.2)	0.6
Prok2	nd	nd	nd		nd	nd	nd	

[a]Results from qPCR analysis of RNAs from the cell lines EL4, LLC1 and tumor tissues from WT/Het or Gfi1-null (KO) mice injected with one of these cell lines (WT-EL4 and KO-EL4; WT-LLC1 and KO-LLC1). Data from tumors in WT/Het and KO mice are expressed as relative mRNA levels (±SD) compared to the injected cell line when expression was detected, or compared to each other when not detected in the injected cell line. [b]P values were determined by Student's t-test; n=4-10 tumor tissues per group. [c]not detected