SOD3-induced WNT pathway activation in endothelial cells provides a permissive signal for tumor infiltration by T cells

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Supplementary Information index:

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SUPPLEMENTARY METHODS

Human samples

Samples from 95 patients with stage II infiltrating large bowel carcinoma were selected from the surgical pathology database of the Hospital Fundación Jiménez Díaz (Madrid, Spain), and a tissue microarray was constructed using 1 mm cores from the tumor blocks. Two pathologists independently reviewed the slides from the surgical resection specimen of the large bowel tumor, selected the most representative areas, and reviewed histopathological features.

For mRNA analyses, we used freshly frozen stage I–IV tumor samples (cohort 1) and formalin-fixed paraffin-embedded tumor samples from stage III CRC patients (cohort 2), both from the Hospital Clínico San Carlos Tumor Bank (Madrid, Spain) [1]. For these two cohorts, non-tumor samples correspond to colon tissue obtained >10 cm from the primary tumor in the same patients; samples in which the pathologist suspected or observed minimal alterations in colon mucosa were discarded. Appropriate informed consent was obtained from all patients and no personal data were registered. The Hospital Fundación Jiménez Díaz and the Hospital Clínico San Carlos Ethical Review Boards approved this study.

Animals

C57BL/6J, Tg(TcraTcrb)1100Mjb/J (OT-I), Tg(TcraTcrb)425Cbn/J (OT-II) and B6.Cg-Tg(Tek-cre)1Ywa/J (Tie2-Cre) mice were from The Jackson Laboratory (Bar Harbor, ME). $SOD3^{-/-}$ mice were kindly provided by Tim D. Oury (University of Pittsburgh, Pittsburgh, PA). $SOD3^{EC\text{-}Tg}$ mice were generated by crossing loxP-SOD3KI ($SOD3^{f/f}$) mice [1] with Tie2-Cre transgenic mice. HIF-$2\alpha^{EC\text{-}KO}$ mice were as described [1]. Cre^{+} and Cre^{-} littermates were used for tumorigenesis studies involving $SOD3^{EC\text{-}Tg}$ and HIF-$2\alpha^{EC\text{-}KO}$ mice. Mouse experiments were approved by the Comunidad de Madrid (PROEX 399/15) and the CNB Ethics Committees in strict accordance with the Spanish and European Union laws and regulations concerning care and use of laboratory animals.

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Cell lines, overexpression and silencing

The Lewis lung carcinoma (LLC; ATCC), the EG7-SOD3 thymoma, and the murine microvascular 1G11-mock and 1G11-SOD3 cell lines were cultured as described [1, 2]. The OVA-expressing EG7-SOD3 thymoma (and the control EG7-mock) were generated by retroviral transduction with pRV-sod3-IRES-gfp and pRVIRES-gfp, respectively, and selected by cell sorting (MoFlo XDP, Beckman Coulter) using GFP fluorescent emission. All cell lines were tested regularly for mycoplasma, and 1G11 endothelial origin was verified regularly by VE-cadherin staining.

HIF-2α was overexpressed and silenced in 1G11 cells as described [1], using pcDNA3-mHIF-2α cDNA and pGIPZ-mEPAS1 shRNA (#481932; Dharmacon GE Healthcare) or pGIPZ Non-Target shRNA (shNT), respectively, and Lipofectamine 3000 (Thermo Fisher Scientific) as transfection reagent. Stable transfectants were obtained after puromycin selection (4 μg/ml, Clontech). For overexpression of the stable β-catenin mutant Δ90βcat, 1G11 cells were transfected with the pCAG-Δ90-GFP construct (Addgene 26645; a gift of Anjen Chenn) and cells selected by cell sorting (MoFlo XDP; Beckman Coulter). For FoxM1 overexpression, 1G11 cells were transfected with pCMV6-Entry/FoxM1-Myc-DDK (Origene, MR210493), and clones selected by limiting dilution with neomycin (750 μg/ml; Apollo Scientific). For *LAMA4* silencing, 1G11-SOD3 cells were transfected with esiRNA targeting mouse Lama4 (esiRNA1; Sigma-Aldrich; EMU083481) or a medium GC content siRNA duplex (Stealth RNAi siRNA Negative Control Med GC, 12,935,300, Invitrogen), using Lipofectamine 3000. For SOD3 overexpression *in vivo*, high-titer stocks of adenovirus expressing mouse SOD3 (Ad-SOD3) or β-galactosidase (Ad-C) were prepared by the Unidad de Producción de Vectores (Centro de Biotecnología Animal y Terapia Génica, Barcelona, Spain) as described [1].

Syngeneic tumors and adoptive transfer

For tumor models, 2- to 5-month-old female mice were used. Tumors were measured blind to treatment/genotype, and tumor volume calculated using the formula V = (width^2 × length)/2. For Kaplan-Meier curves, mice were considered dead when tumor volume >700 mm^3.

For SOD3 overexpression experiments, subcutaneous tumors were implanted by injection of exponentially growing LLC cells [5 x 10^5/100 µl phosphate-buffered saline (PBS)] into the right flank of WT or SOD3$^{-/-}$ mice. At day 7 post-inoculation (mean tumor size, 100 mm^3), mice were randomized for treatment by a technician blind to treatments. Mice received intratumor Ad-mSOD3 or Ad-C viruses (10^9 pfu/50 µl) injections on days 7, 9, 11, and 15 post-implantation, and Vhcl or Doxo (2.5 mg/kg, i.p.; Farmitalia Carlo Erba, Italy) on days 7, 11, and 15. For adoptive transfer, EG7-mock and EG7-SOD3 cells (5 x 10^6/100 µl) were implanted s.c. in the indicated mouse strains. At day 3 post-inoculation, mice were randomized and received injections of OT-I cells (10^7 cells/mouse, i.v.) purified by negative selection (Dynabeads Untouched Mouse CD8 (11417D) Kit; Thermo Fisher). In experiments involving HIF-2α^{EC-KO} mice, tamoxifen (Sigma-Aldrich) was diluted in ethanol and corn oil, heated (100ºC) and injected (1 mg/mouse, i.p.) on days 3, 4 and 5 post-EG7-SOD3 inoculation. E*x vivo*-activated OT-1 cells (see below) were transferred to HIF-2α^{EC-KO} mice on day 10. Tumorogenesis experiments involving Doxo treatment in HIF-2α^{EC-KO} mice have been described [1]. In the experiment involving WNT pathway inhibition, EG7-SOD3 tumors were implanted as above and then treated with XAV-939 (5 mg/Kg, i.t; Selleckchem) on days 5, 8, 11, 12 and 13; *ex vivo*-activated OT-1 cells were adoptively transferred on day 13, and mice sacrificed 2 days later. Lovastatin and Doxo were co-administered to C57BL/6J and SOD3$^{-/-}$ mice bearing LLC tumors as reported [1].

Analyses of immune infiltrate in tumors

For experiments involving adoptive transfer, tumors were excised and cell suspensions prepared as indicated [2]. OT-I cell number was estimated by FACS (Gallios; Beckman

Coulter) after staining with anti-Vα2-biotin (clone B20.1; eBioscience), -Vβ5-PE (clone MR9-4; BD-Pharmingen), -CD45-BViolet570 (clone 30-F11; eBioscience) and -CD8-eFluor450 (clone 53-6.7, eBioscience) antibodies, followed by streptavidin-APC (Beckman Coulter).

In the other tumor models, immune infiltration was determined by immunohistochemistry (IHC). Tumor sections (10 μm) from snap-frozen samples (Tissue-Tek, Sakura) were acetone-fixed and stained with anti-CD11b-biotin (clone M1/70, Beckman Coulter), followed by streptavidin-Cy3 (Jackson ImmunoResearch), anti-CD3 (A0452, Dako), -CD8 (clone 53-6.7, BioLegend), -FoxP3 (clone 1054C, R&D) and -CD4 (clone EPR19514, Abcam) antibodies. Specific binding was developed using appropriate Alexa 488- and Alexa 594-conjugated secondary antibodies (Thermo Fisher). Sections were mounted with DAPI Fluoromount G (Southern Biotech) and examined in a Leica (DM RB) microscope equipped with an Olympus DP70 camera, at x200 total magnification (X20 objective) or an Olympus FluoView 1000 confocal microscope with a ×60 1.4 oil plan-Apo objective. We recorded at least two fields per mouse analyzed, covering the center and the border of the tumor.

TMA analyses

For human samples, deparaffinized TMA slips were treated with pre-warmed (95ºC) citrate buffer (pH 6.0, 20 min) and then incubated with anti-SOD3 (LS-C91837, LSBio), anti-CD8 (clone C8/144B, Dako), anti-LAMA4 (HPA015693, Sigma-Aldrich) or anti β-catenin (prediluted, Dako) antibodies, followed by appropriate peroxidase-labeled secondary antibodies. The reaction was developed with IHC chromogen substrate (AEC for SOD3; Thermo Fisher) or diaminobenzidine, and hematoxylin counterstained. In all cases, appropriate tissues were used as positive and negative controls. Staining was evaluated in a Leica DM500 optical microscope by a single pathologist (MJF-A) blinded to experimental data. Cells stained with anti-CD8 antibody were counted in 1 mm^2. For SOD3 and LAMA4, a z-score was calculated (range 0-300) as the product of intensity of the staining (1-3) and the percentage of

stained cells. β-catenin was measured as the percentage of stained EC per high-power field; it was usually intense in epithelium and weak in endothelium, with no significant differences between the cases.

Adhesion and migration assays

All adhesion and migration assays were performed with primary cells. CD3 and CD11b cells were isolated from splenocytes by negative selection using anti-CD19 (clone 1D3) and -CD11b (clone M1/70), or -CD3 (clone 17A2, all from BD Pharmingen) and -CD19, respectively, followed by sheep anti-rat Ig-coated magnetic beads (Dynabeads Invitrogen). $CD4^+$ and $CD8^+$ T cells were isolated from the spleen of OT-II and OT-I mice, respectively, by negative selection with Dynabeads Untouched Mouse CD4 (11415D) or CD8 (11417D) Kits (Thermo Fisher). When indicated, these cells were activated *ex vivo* by incubation (48 h) of the splenocytes with the peptides OVA-323-339 (1 μM; OT-II) and OVA-257-264 (10 pM; OT-I). In these conditions, 85% of recovered OT-II or OT-I cells were activated, as determined by flow cytometry after staining with an anti-CD69 antibody (clone H1.2F3, eBioscience).

Adhesion assays were performed with 8-chamber glass slides (Lab-Tek II Chamber Slide System, Nunc) coated with 1G11-mock or 1G11-SOD3 cells (1×10^5) the day before the experiment. CellTracker Orange CMTMR-labeled $CD3^+$ and $CD11b^+$ cells (1×10^6 cells, 30 min, 1 μM; Invitrogen) were seeded onto the EC-coated chambers. After the indicated incubation times, non-adhered cells were removed and, after gentle washing with PBS, attached cells were fixed with paraformaldehyde (4%; PFA). Slides were mounted with Vectashield plus DAPI (Vector Laboratories, Burlingame, CA) and CMTMR-labeled cells counted (total magnification X200). Four fields per condition were acquired in the periphery and the central area of each well; images were processed with NIH-ImageJ software to quantify cell numbers.

Migration assays were performed in p24 transwells (6.5 mm insert, pore size 3 μm for lymphocytes, 5 μm for CD11b cells; Corning Costar) coated with the indicated 1G11

transfectants (5 x 10^4) the day before the assay. In these conditions, 1G11 cells formed a monolayer covering almost 100% of the insert area. For some experiments, 1G11 cells were seeded onto laminin 411- or laminin 511-coated inserts (5 mg/ml, 2 h, 37ºC; BioLamina), or were incubated (48 h) with WISP2 (250 ng/ml, Peprotech), XAV-939 (20 μM, Selleckchem) or the corresponding vehicles before the assay. Purified naïve or activated CD3, CD4, CD8 or CD11b cells (0.5 x 10^6) were added to the transwell, and the unit was placed carefully into wells filled (600 μl) with CCL19 (250 ng/ml; naïve T cells), CXCL10 (500 ng/ml; activated T cells; all chemokines from Peprotech), medium supplemented with 10% FCS, or basal medium (RPMI+0.5% BSA). After incubation (3-4 h, 37ºC), the medium in the lower chamber was recovered and migrating cells were counted by flow cytometry using a fixed acquisition time (300 s). The migration rate was calculated as the ratio between the number of cells detected in the chemokine and the basal medium wells for each condition.

mRNA quantification

Total RNA was extracted from mouse tumors and cell lines using TRI-Reagent (Sigma Aldrich) or RNeasy Mini or Micro Kit (Qiagen). RNA from human samples was extracted using TRIzol (Invitrogen) or RNeasy FFPE Kit (Qiagen) as described [1]. RNA was treated with DNase (RNeasy Micro Kit, Qiagen), and first cDNA strand was synthesized from 0.5-2 μg total RNA (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) using random primers. mRNA levels were quantified by qPCR in an ABI PRISM 7900HT System (Applied Biosystems), using a SYBR EvaGreen-based reaction mix (5X PyroTaq EvaGreen qPCR Mix Plus ROX, Cmb-Bioline), with the primers listed in Supplementary Table S1. β-actin or CD31 (mouse samples) and RLP10A genes (human samples) were used for normalization. Values for each gene are expressed as relative quantity (Rq), calculated as $2^{-\Delta\Delta Ct}$ relative to the sample with the lowest expression or the control sample. Each data point represents the mean of a technical triplicate in an independent experiment.

Immunoblot

1G11 cell transfectants or total lung tissue (positive control for SOD3) were lysed with protease inhibitor-supplemented RIPA buffer, proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with mouse anti-SOD3 (A-11, sc-271170, Santa Cruz Biotechnology), rabbit anti-β-catenin (D10A8, #8480, Cell Signaling Technology), mouse anti-FoxM1 (G-5, sc-376471, Santa Cruz Biotechnology), or rabbit anti-HIF-2α (ab109616, Abcam); mouse anti-β-actin (AC-15, A5441, Sigma-Aldrich) was used as loading control. For CHX chase assays, 1G11-mock and -SOD3 cells were treated at the indicated times with CHX (100 μg/ml, Sigma-Aldrich) and β-catenin levels analyzed as above; the β-catenin/β-actin ratio was calculated from densitometric values (NIH-ImageJ), and normalized to that of time 0 in 1G11-mock cells.

For Wnt/β-catenin signaling pathway activation/inhibition experiments, 1G11-mock or -SOD3 cells were treated respectively (96 h) with recombinant WISP2 (250 ng/ml) or with the tankyrase inhibitor XAV-939 (48 h, 20 μM; Selleckchem); the agonist and the inhibitor in the culture were refreshed daily. The equivalent concentration of PBS or DMSO were used as vehicle. Cell extracts were processed as above and immunoblotted with the indicated antibodies.

Immunofluorescence

1G11 cell transfectants were seeded in 1% gelatin-coated Nunc Lab-Tek chamber slides, fixed with ethanol:acetic acid (95:5, 1 min, -20°C; for HIF-2α staining) or with 4% PFA (15 min, 20ºC; for FoxM1 and β-catenin), permeabilized with 0.3% Triton X-100 (5 min, 20°C), and stained with rabbit anti-HIF-2α (NB100-122, Novus Biologicals), mouse anti-FoxM1 (clone G-5, sc-376471, Santa Cruz Biotechnology) and mouse anti-β-catenin (clone 12F7; ab22656 Abcam), followed by Alexa488- or Alexa594-labeled secondary antibodies (Thermo Fisher). For LAMA staining, cells were fixed with methanol (10 min, -20ºC) and stained with

goat anti-LAMA4 (N20; sc-16589, Santa Cruz Biotechnology), followed by Alexa488-labeled secondary antibody. Sections (10 μm) from snap-frozen (OCT; Sakura) tumors were fixed with cold acetone and used directly for immunostaining with anti-CD31 (MEC13.3; BD Biosciences Pharmingen), -SOD3 (M106, Santa Cruz; PAA117Mu01, CloudClone) or -LAMA4, followed by appropriate fluorescently labeled secondary antibodies (see above).

In all cases, samples were mounted with 4,6-diamidino-2-phenylindole (DAPI)-containing Fluoromount-G (SouthernBiotech; fluorescence analyses). Images were analyzed with an Olympus FluoView 1000 confocal microscope with a X60 1.4 oil plan-apo objective (for LAMA4 in tumors and β-catenin), or a Leica DM RB equipped with a Olympus DP70 camera (tumor infiltrate, HIF-2α, FOXM1, and LAMA4). Nuclear HIF-2α and FoxM1 were quantified as described [1]. For LAMA4 quantification in mouse tumor sections, images were transformed to 8-bit with ImageJ and, after threshold adjustment, the Image calculator tool was used to select CD31- and LAMA4-stained coincident regions. The ratio between LAMA4- and CD31-stained areas was calculated and expressed as a percentage. In other cases, mean fluorescence intensity was quantified using the ImageJ tool.

ELISA and protein array

1G11-mock and -SOD3 cells (4 x 10^5 cells/well) were plated; after 48 or 72 h, culture supernatants were harvested, centrifuged, and analyzed using a mouse-specific WISP2 ELISA (mouse WISP2 ELISA Kit; LS-F12969; LSBio) and the Proteome Profiler Mouse Cytokine Array Kit (Panel A; ARY006, R&D Systems) according to manufacturer's protocols.

ChIP assays

ChIP assays were performed with the Magna ChIP G – Chromatin Immunoprecipitation Kit (17-611, Merck Millipore) according to the protocol provided. Briefly, 1G11-mock and -SOD3 cells were fixed (10 min, 20ºC) with 1% PFA in culture medium and quenched (5 min, 20ºC) with 125 mM glycine. After medium removal, cells were snap-frozen in liquid nitrogen,

harvested in cell lysis buffer (2 x 10^7 cells/ml, 15 min, 4ºC), and nuclear extracts were prepared in the same volume of nuclear lysis buffer (15 min, 4ºC). Chromatin was sheared by sonication (10 cycles, 30 s on/30 s off; Bioruptor Pico, Diagenode) in 0.2 ml aliquots, and 1% of each sonicated nuclear lysate was stored as input reference. Sheared chromatin was immunoprecipitated (4ºC, 14 h with rotation) with rabbit anti-HIF-2α (NB100-122, Novus Biologicals, 10 µg) and purified rabbit IgG as control. Immune complexes were captured using Protein G Magnetic Beads, retained with a magnet, and washed sequentially with low-salt buffer, high-salt buffer, lithium chloride buffer, and Tris-EDTA buffer (25 mM Tris-HCl pH 7.2, 150 mM NaCl, 1 mM EDTA) to minimize nonspecific binding. Chromatin immune complexes were eluted from beads and protein/DNA links reversed by incubation with elution buffer (100 mM $NaHCO_3$, 1% SDS) supplemented with proteinase K (100 µg/ml, 2 h, 62ºC). DNA was purified using spin columns (mi-PCR Purification Kit, Metabion). WISP2 gene promoter sequences were analyzed by RT-qPCR using the primers 5'-TAGTCTCGATAAAAC ATAGAA3', 5'CGAGTCACATACAGACTCTG3' (amplicon: 160 bp), 5'CCAGATCTGAT GGCAGTCAA3', 5'CTTTTGTAACGGCGGACATT3' (amplicon: 180 bp) for proximal and distal promoter regions, respectively. Dissociation curves indicated a single PCR product.

The relative quantity of amplified product in the input and the ChIP samples was determined as follows: the Ct values determined in ChIP (antibody and control IgG) and input samples were used to calculate the signal relative to input as $2^{((Ct_{input} - 6.64) - Ct_{ChIP})} \times 100$ of each sample. After subtraction of the IgG ChIP value, the signal relative to input of each sample was obtained. Each data point indicates the average of a technical triplicate in an independent experiment.

Bioinformatic analyses

RSEM-normalized RNAseq expression data from 382 CRC tumor samples were obtained from TCGA through the Firebrowse repository (http://firebrowse.org/). A T cell inflammatory

signature was computed as the average of the log expression values of 1346 genes [3]. Samples with a T cell average above the median were classified as T cell inflamed, whereas samples below this median value were classified as T cell non-inflamed. Correlation between SOD3 expression values and T cell average was estimated based on the Pearson correlation coefficient.

Statistical analyses

Qualitative data are represented with percentages and absolute numbers, quantitative data as mean ± SEM, unless otherwise indicated; individual values are also shown and the number of replicates is given in figure legends. For mouse studies, sample size was estimated using the power method and values corrected for 20% attrition. For human samples, cases were divided into negative and positive for SOD3 or LAMA4 staining using a receiver operating characteristic (ROC) curve approach. For data with a gaussian distribution and homogeneity of variances, statistical significance was calculated with a two-tailed Student's t-test, with the Welch correction when the number of replicas was low, for comparison of two independent groups, and one- or two-way ANOVA with Dunnett's, Tukey's, or Bonferroni's post-hoc test for multiple comparisons. When these requirements were not fulfilled, data were analyzed using non-parametric tests. The potential associations between qualitative variables were analyzed with the χ^2 test. Correlation analyses were performed using the non-parametric Spearman's rank correlation coefficient. The outcome measure used for the human stage II cohort study was disease-free survival, defined as the time elapsed between surgical resection of the tumor with a curative intent and recurrence of disease in months. In the univariate analysis, Kaplan-Meier survival curves were compared with log-rank tests. SOD3 expression values were compared between T cell inflamed and non-inflamed TCGA signatures using Student's *t*-test. Differences were considered significant when $p < 0.05$. Statistical analyses were performed using the statistical package SPSS 20.0 for Windows, and Prism software. All statistical tests were two-sided.

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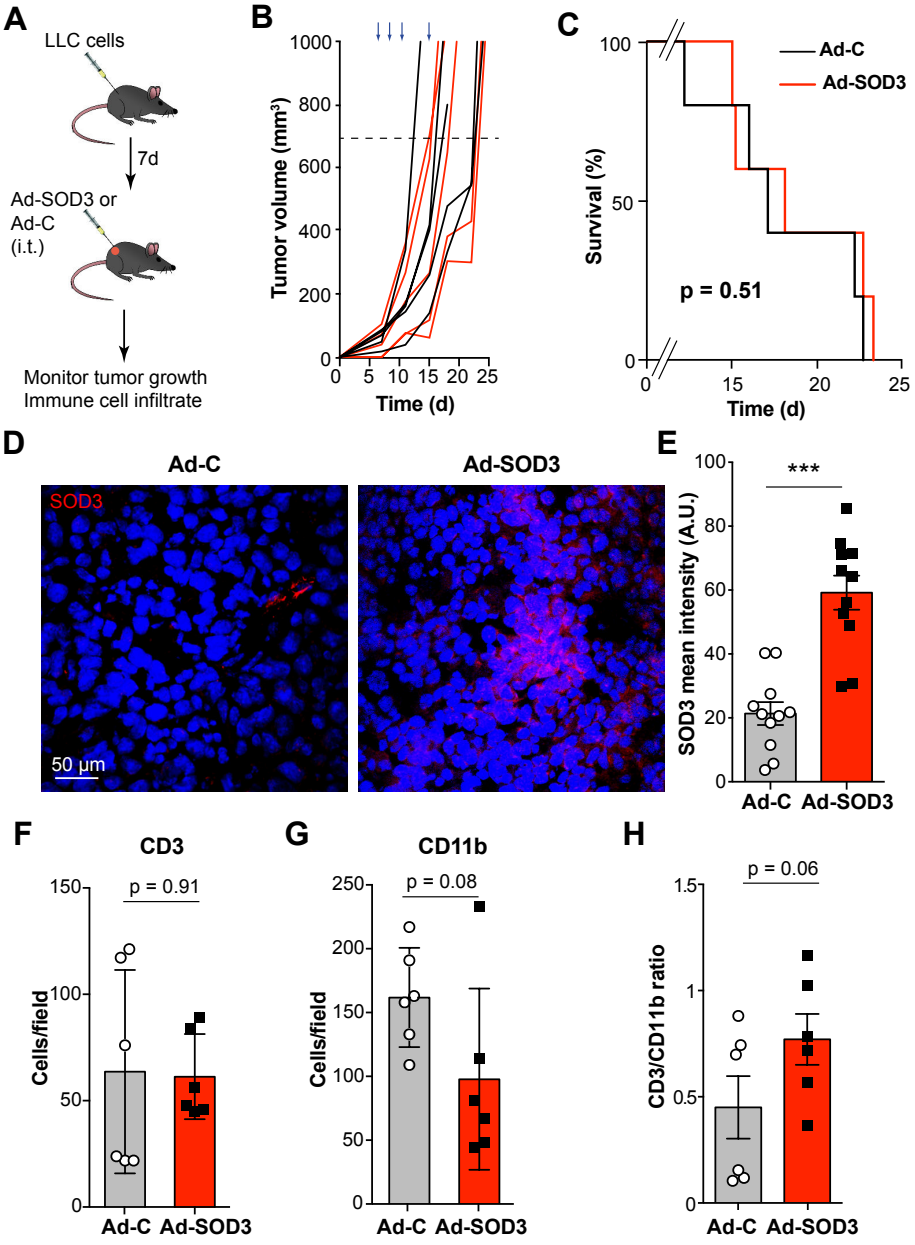
Supplementary Table S1. Primers used for RT-qPCR

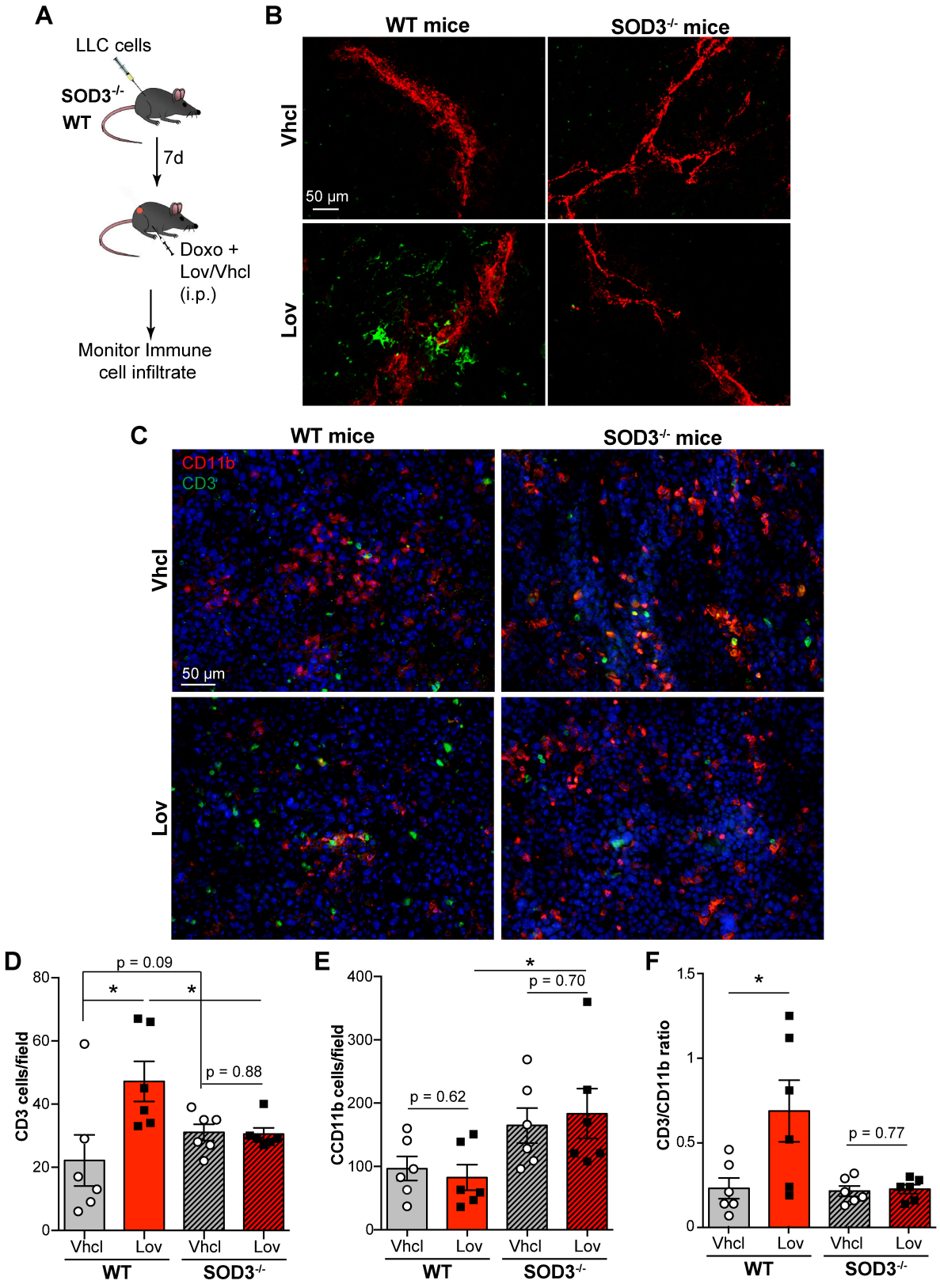
Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Mouse *ctnnb1*	TGCAGATCTTGGACTGGACAT	AAGAACGGTAGCTGGGATCA
Mouse *axin2*	AGGAACCACTCGGCTGCT	CAGTTTCTTTGGCTCTTTGTGA
Mouse *ccnd1*	TTTCTTTCCAGAGTCATCAAGTGT	TGACTCCAGAAGGGCTTCAA
Mouse *c-myc*	CCTAGTGCTGCATGAGGAGA	TCCACAGACACCACATCAATTT
Mouse *vegfa*	GGTTCCCGAAACCCTGAG	GCAGCTTGAGTTAAACGAACG
Mouse *lama4*	CACGTGACCGACATGAACTC	TTCTCTTTCTGACAGCCTTGTTC
Mouse *epas1*	CTCCAGGAGCTCAAAAGGTG	CAGGTAAGGCTCGAACGATG
Mouse *foxM1*	AGCTAAGGGTGTGCCTGTTC	CTGTTGTCCAGCGTGCAG
Mouse *efna1*	CAGGAGAAGAGACTCCAAGCA	AGGAGCAATACTGCCCAGAC
Mouse *ccn1*	GGATCTGTGAAGTGCGTCCT	CTGCATTTCTTGCCCTTTTT
Mouse *ccn2*	TGACCTGGAGGAAAACATTAAGA	AGCCCTGTATGTCTTCACACTG
Mouse *ccn3*	TGCGAAAAGTGGACCTGTG	TCAACTCCTACGGTGGCTTC
Mouse *ccn4*	GTGGACATCCAACTACACATCAA	CCTGCGAGAGTGAAGTTCGT
Mouse *ccn5*	GTGTGTGACCAGGCAGTGAT	AAGGGCAGAAAGTTGGTGTC
Mouse *ccn6*	CTGCAGCAGGAACACATCAC	GGAGTTGGAAAGTGGGTTGA
Mouse *wnt1*	ACAGTAGTGGCCGATGGTG	CTTGGAATCCGTCAACAGGT
Mouse *wnt2*	CCTGATGAACCTTCACAACAAC	TCTTGTTTCAAGAAGCGCTTTAC
Mouse *wnt2b*	CGGGACCACACTGTCTTTG	GCTGACGAGATAGCATAGACGAA
Mouse *wnt3*	CTCGCTGGCTACCCAATTT	GAGGCCAGAGATGTGTACTGC
Mouse *wnt3a*	GGAGTGCCAGCACCAGTT	GCATGGACAAAGGCTGACTC
Mouse *wnt4*	ACTGGACTCCCTCCCTGTCT	TGCCCTTGTCACTGCAAA
Mouse *wnt5a*	ACGCTTCGCTTGAATTCCT	CCCGGGCTTAATATTCCAA
Mouse *wnt5b*	AGCACCGTGGACAACACAT	AAGGCAGTCTCTCGGCTACC
Mouse *wnt7a*	CGCTGGGAGAGCGTACTG	CGATAATCGCATAGGTGAAGG
Mouse *wnt10b*	AATGCGGATCCACAACAAC	CTCCAACAGGTCTTGAATTGG
Mouse *ccl2*	TCTGGGCCTGCTGTTCACA	TTGGGATCATCTTGCTGGTG
Mouse *cxcl1*	CCTTGACCCTGAAGCTCCCT	CGGTGCCATCAGAGCAGTCT
Mouse *cxcl9*	TCTGCCATGAAGTCCGCTG	CAGGAGCATCGTGCATTCCT
Mouse *cxcl10*	TGCTGGGTCTGAGTGGGACT	CCCTATGGCCCTCATTCTCAC
Mouse *cxcl11*	GGCTGCTGAGATGAACAGGAA	AAAGACAGCGCCCCTGTTT
Mouse *cd31*	AGGCTTGCATAGAGCTCCAG	TTCTTGGTTTCCAGCTATGG
Mouse *vcam1*	TCTTACCTGTGCGCTGTGAC	ACTGGATCTTCAGGGAATGAGT
Mouse *actb*	GAGAAACGGCTACCACATCC	GGGTCGGGAGTGGGTAAT
Human *lama4*	GGATGCCGAAGACATGAAC	TTCCCTCACTCTTTCCTGTTGT
Human *sod3*	GGTGCAGCTCTCTTTTCAGG	AACACAGTAGCGCCAGCAT
Human *rpl10a*	CTTCCCTGCTCACACACAAC	CCAACAGCTACAGCCAGACA

Supplementary Table S2. Demographic and clinical characteristics

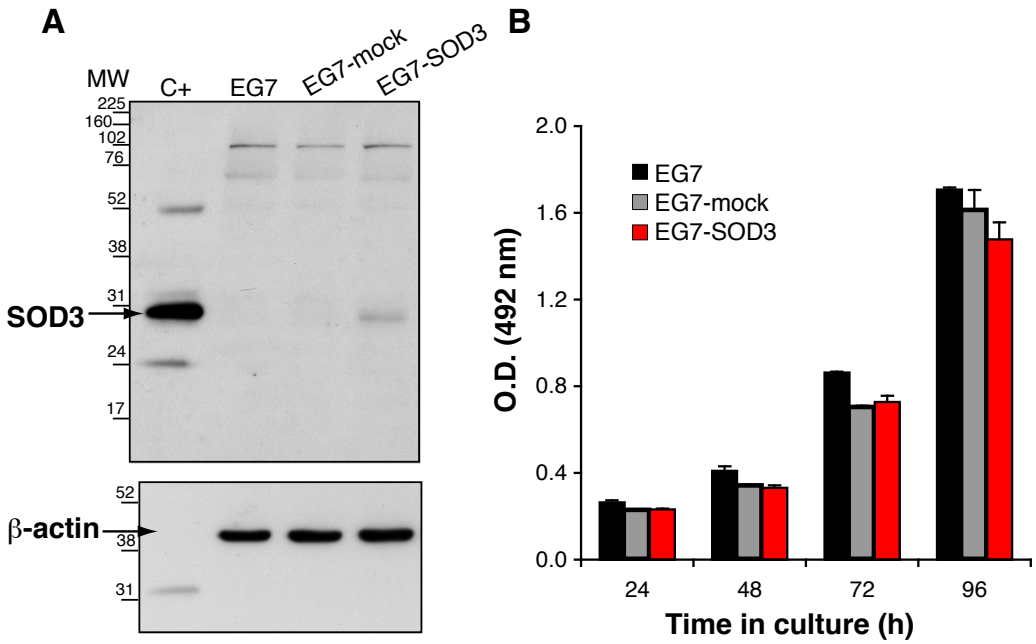
Characteristic		Value
Gender	Female	40%
	Male	60%
Age	(median, SD)	73.03 (9.6) years
Site		
	Cecum	13.7%
	Right colon	26.3%
	Transverse colon	7.4%
	Left colon	5.3%
	Sigmoid colon	25.3%
	Rectum	22.1%
Grade		
	Well differentiated	18.9%
	Moderately differentiated	72.6%
	Poorly differentiated	8.4%
T stage		
	T1	3.2%
	T2	31.6%
	T3	64.2%
	T4	1.1%
Recurrence	No	68.4%
	Yes	31.6%
Time to recurrence (median)		52 months



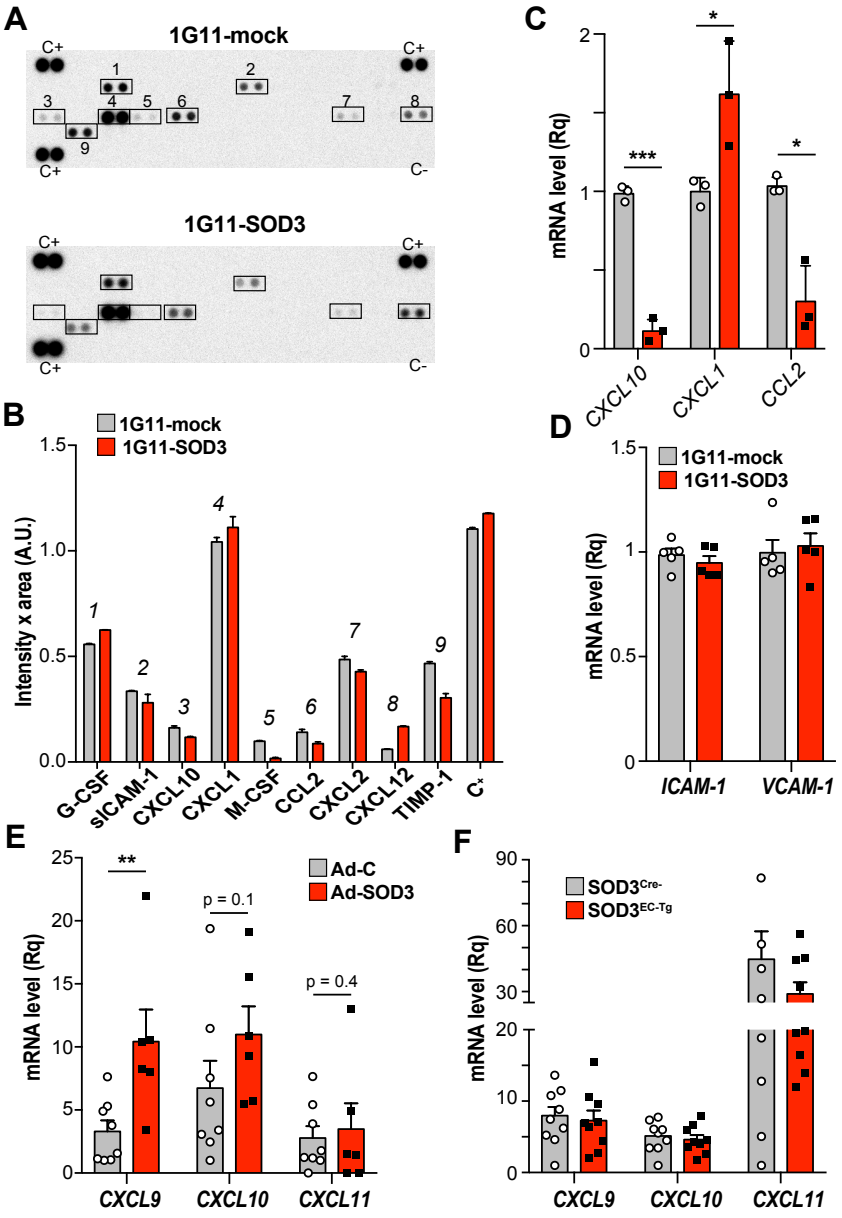
Supplementary Figure S1. Effect of Ad-SOD3 injection into untreated LLC tumors. **A.** Scheme of the tumor model used; i.t., intratumor. **B.** Growth kinetics of Ad-C- or Ad-SOD3-injected LLC tumors (n = 5 mice/group). Arrows indicate treatment schedule with adenovirus; one representative experiment of two is shown. **C.** Kaplan-Meier survival curves (cut-off tumor volume 700 mm^3) for mice in *B*. **D.** Representative images showing SOD3 staining (red) in Ad C- and Ad-SOD3-injected LLC tumors; nuclei were DAPI-stained (blue). **E.** Quantification of SOD3 mean fluorescence intensity from *D* (n = 11 fields/condition; n = 9 mice/group). **F, G.** Quantification of infiltrating $CD3^+$ (*F*) and $CD11b^+$ cells (*G*) in tissue sections from Ad-C- or Ad-SOD3-injected LLC tumors (n = 6 fields/condition). **H.** Ratio of tumor-infiltrating $CD3^+$ and $CD11b^+$ cells. Mean ± SEM and individual values are shown; ***p< 0.001 two-tailed Student's t-test.



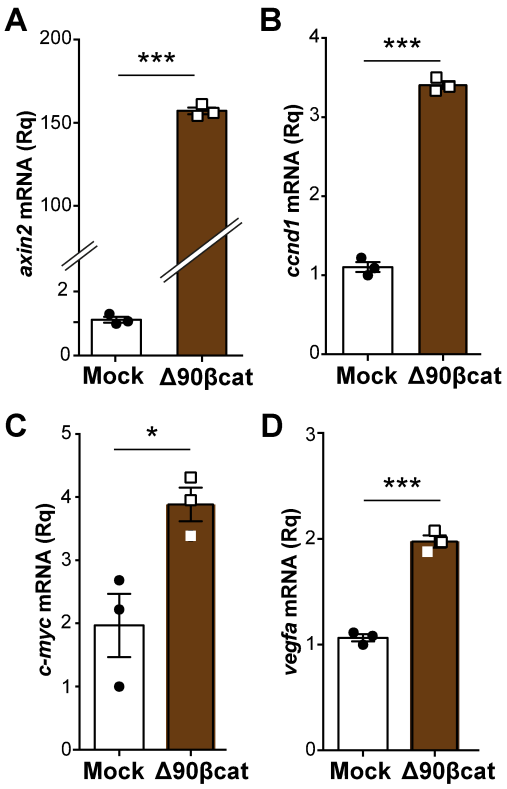
Supplementary Figure S2. Lov triggers selective T cell infiltration in a SOD3-dependent manner. A. Scheme of the tumor model used; i.p., intraperitoneal. **B.** Representative images showing SOD3 (green) and CD31 (red) staining in LLC tumors grafted in the indicated mice. **C.** CD3 (green) and CD11b (red) staining in LLC tumors grafted in the indicated mice; nuclei were DAPI-stained (blue). **D, E.** Quantification of $CD3^+$ and $CD11b^+$ cells from *B* ($n = 6$ fields/condition; $n = 3$ mice/group). **F.** Ratio of tumor-infiltrating $CD3^+$ and $CD11b^+$ cells. Mean ± SEM and individual values are shown; * $p< 0.05$, ** $p< 0.01$ two-tailed Student's *t*-test.



Supplementary Figure S3. Characterization of SOD3-expressing EG7 cells. A. Immunoblot of SOD3 and β-actin (loading control) in parental, mock- and SOD3-transduced EG7 cells. C^+, lung extract used as SOD3 reference. **B.** Cell viability assay of indicated cell types, analyzed by MTS.



Supplementary Figure S4. SOD3 effect on homing receptor expression. A. Representative protein array of 1G11-mock and -SOD3. **B.** Densitometric quantification of the protein arrays as in *A*. Data were normalized as the product of intensity x area ($n = 3$). **C.** Relative mRNA levels of the indicated chemokines in 1G11-mock and -SOD3 cells ($n = 3$). **D.** Relative mRNA levels of *ICAM-1* and *VCAM-1* in 1G11-mock and -SOD3 cells ($n = 4$). **E.** Relative mRNA levels of *CXCL9*, *CXCL10* and *CXCL11* in Ad-C and Ad-SOD3-injected tumors, as described in Figure 1E-O ($n = 7$ Ad-C; $n = 6$ Ad-SOD3, from two independent experiments). **F.** Relative mRNA levels of *CXCL9*, *CXCL10* and *CXCL11* in EG7 tumors dissected from SOD3Cre- and SOD3EC-Tg mice, as described in Figure 2H-L ($n = 9$ mice/group, from an experiment of two). For qPCR data, each point represents the average of triplicates. Mean ± SEM and individual data points are shown. *p< 0.05, ***p< 0.001, two-tailed Student's t-test.



Supplementary Figure S5. Overexpression of a nuclear β-catenin mutant triggers prototypic WNT genes in 1G11 cells. A-D. Relative expression of the prototypic WNT-regulated genes *AXIN2* (*A*), *CCND1* (*B*), *MYC* (*C*) and *VEGFA* (*D*) in mock- and Δ90βcat-transfected 1G11 cells (n = 3). *p< 0.05, ***p< 0.001, two-tailed Student's t-test.

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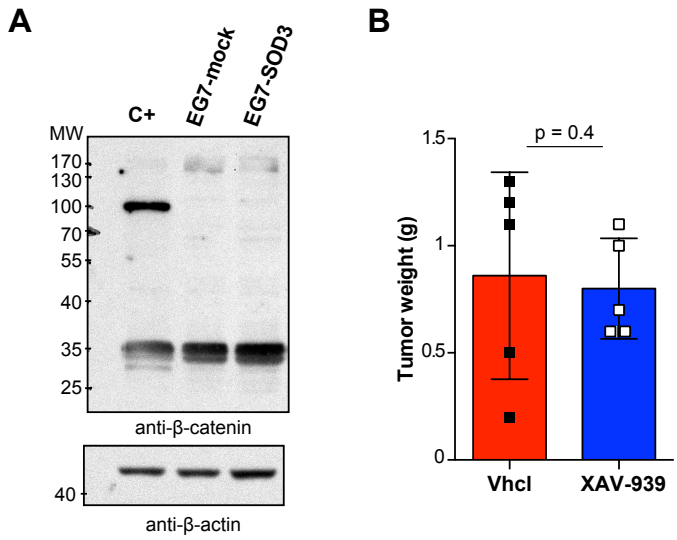
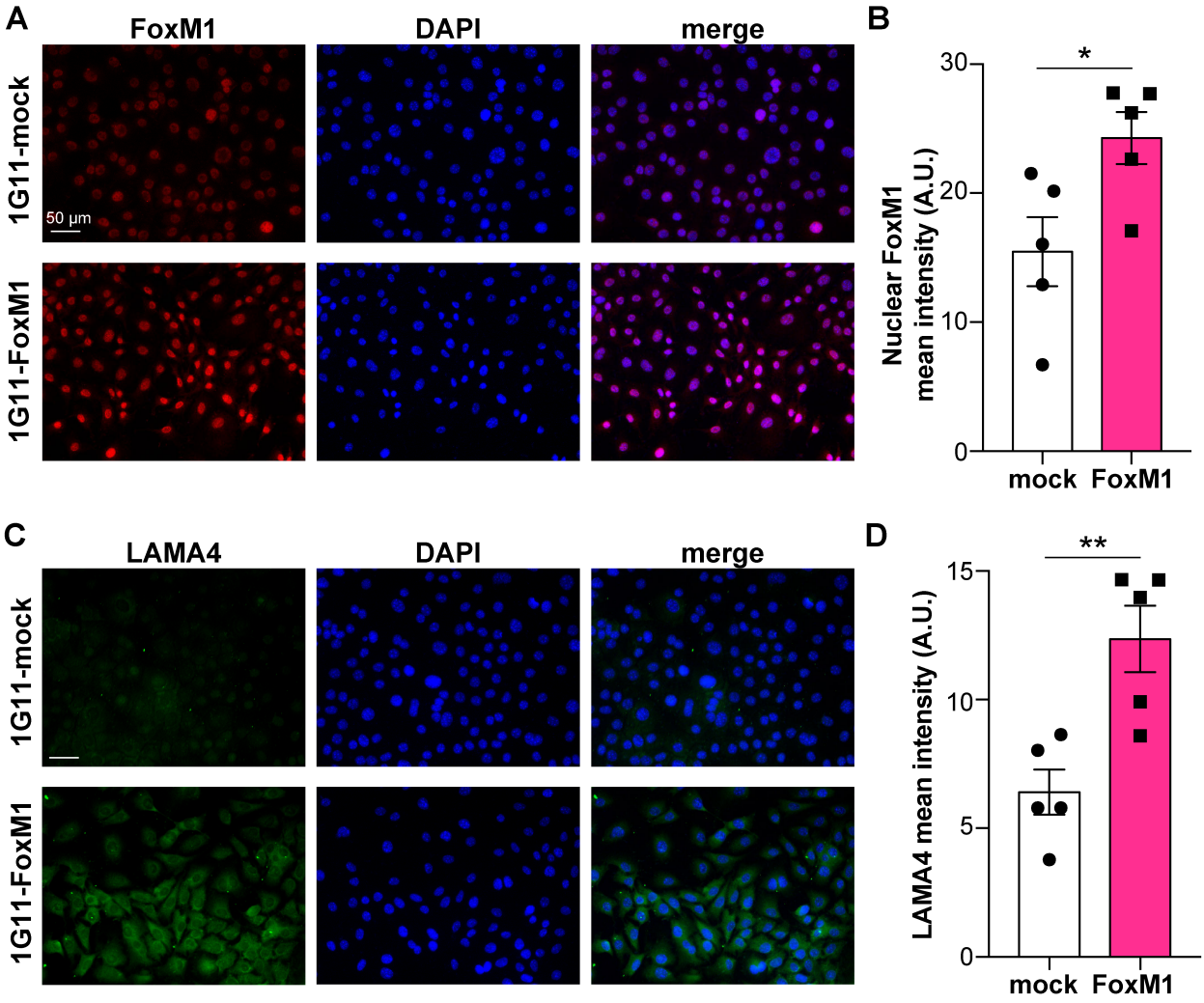
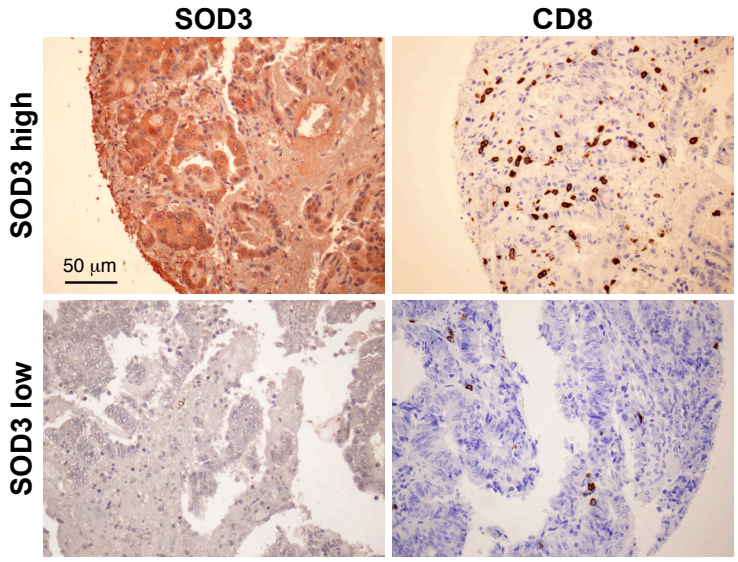


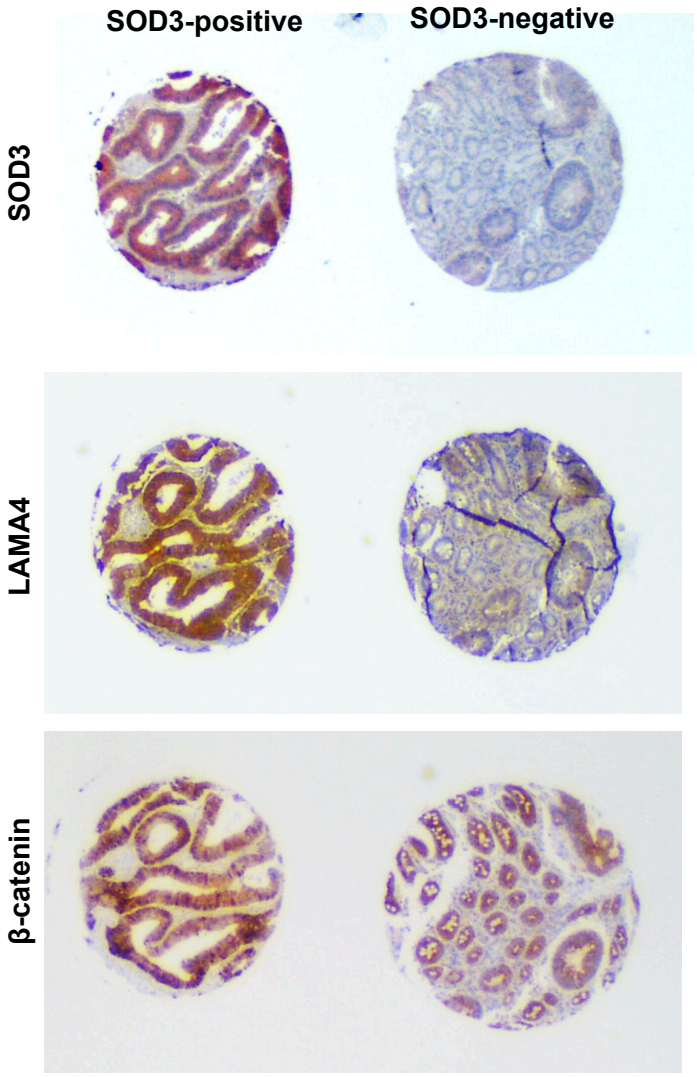
Supplementary Figure S6. SOD3 does not induce the WNT pathway in EG7 cells. A. Representative immunoblot of cell lysates from EG7-mock and EG7-SOD3 hybridized with anti-β-catenin and anti-β-actin antibodies. C^+, lysates from 1G11-SOD3 cells (n = 3). **B.** Weight of tumors grown in XAV-939- and vhcl-treated mice (n = 5 mice/condition). The p-value was calculated using a two-tailed Student's t-test.



Supplementary Figure S7. FoxM1 overexpression increases LAMA4 in 1G11 cells. A. Images show FoxM1 staining (red) in 1G11-mock and a representative FoxM1-overexpressing 1G11 cell clone; nuclei were DAPI-stained (blue; *n* = 5 fields/condition) **B.** Quantification of FoxM1 nuclear mean fluorescence intensity in images from *A*. **C.** The same FoxM1 cell clone in *A* was stained for LAMA4 (green); nuclei in blue (*n* = 5 fields/condition). **D.** Quantification of LAMA4 mean fluorescence intensity from images as in *C*. *B* and *D*, Individual values and mean ± SEM are shown; *p< 0.05, two-tailed Student's t-test.



Supplementary Figure S8. Representative TMA images stained for SOD3 and CD8. Additional images to those in Fig. 7H, showing correlation between SOD3 expression and $CD8^+$ T cell infiltration.



Supplementary Figure S9. Panoramic views of TMA samples used in Figure 7A and 7B. Low magnification images of the SOD3 positive and negative TMA samples stained with the indicated markers.