The ratio of STAT1 to STAT3 expression is a determinant of colorectal cancer growth

SUPPLEMENTARY DATA

Staining for receptors of the GP130 family cytokines on colon carcinoma cell lines

Cells were harvested with Accutase and cell number was determined. For each antibody staining $1x10^6$ cells were resuspended in 100 µl 5% FCS/PBS. The primary antibody (see list below) was added in the indicated concentration and cells were incubated for 30 minutes at 4°C in the dark. After washing with PBS, anti-mouse IgG FITC conjugate (Sigma) was diluted 1:100 in 100 µl 5% FCS/PBS and cells were incubated with the secondary antibody for 30 minutes at 4°C in the dark. After a final wash with PBS, cells were resuspended in 500 µl 5% FCS/PBS and analysed on a FACScan.

Antibody	Amount	Details	Positive Control
CNTFRa	5 µl	AN-B2, Santa Cruz sc-9993	Cos7
GP130	10 µl	BR-3, Diaclone	293T HEK cells
IL-6R	10 µl	BR-6, Diaclone	293T HEK cells
LIFR	10 µl	Gift from Prof. Müller-Newen	STA-ET-7.2
OSMRb	5 µl	AN-A2, Santa Cruz sc-9992	STA-ET-7.2

mRNA expression levels of STAT1/STAT3 targets

1 µg of total RNA from shcontrol and shSTAT3 xenografts (n=4) was reverse-transcribed into complementary DNA using the Revert Aid cDNA synthesis kit (Fermentas, Burlington, Canada). qRT-PCR was performed using the SYBR green method. mRNA levels were normalized for beta-2 microglobulin (*B2m*), and relative abundance was calculated using the $2^{-\Delta\Delta Ct}$ method (gene-specific expression level relative to that of an endogenous housekeeping gene). Each reaction was performed in duplicate. Statistical significance is denoted as - *p<0.05, **p<0.01, ***p<0.001, Student's *t* test.

Primer sequences are listed below.

Gene Name	Gene Symbol	Primers
Interferon alpha-inducible protein 27	IFI27	FP:GCCTCTGCTCTCACCTCATC RP:CCACAACTCCTCCAATCACA
Bone marrow stromal cell antigen 2	BST2	FP:CAGAAGGGCTTTCAGGATGT RP:TGATCTCTCCCTCAAGCTCC
Interferon-induced protein 44	IFI44	FP:CCTGTGCAGGGATGACATATT RP:AGCGATGGGGAATCAATGTA
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	FP:CCTCCTTGGGTTCGTCTACA RP:AGTGGCTGATATCTGGGTGC
Suppressor of cytokine signaling 3	SOCS3	FP:CCAAGGACGGAGACTTCGATTC RP:GGAGTATTCCGGGAACCTGG
B-cell CLL/lymphoma 2	BCL2	FP:TCCGCATCAGGAAGGCTAGA RP:AGGACCAGGCCTCCAAGCT
Myeloid cell leukemia 1	MCL1	FP:GGACATCAAAAACGAAGACG RP:GCAGCTTTCTTGGTTTATGG
Hypoxia inducible factor 1 alpha subunit	HIF1A	FP:CGTTCCTTCGATCAGTTGTC RP:TCAGTGGTGGCAGTGGTAGT
Beta-2 microglobulin	B2M	FP:GTGCTCGCGCTACTCTCTCT RP:TTCAATGTCGGATGGATGAA

Specificity of STAT1 and STAT3 antibodies

The STAT1 antibody (Santa Cruz - sc-592) is derived against the C-terminal end of human STAT1. The STAT3 antibody (Cell Signaling #4904) is derived against the C-terminal end of mouse STAT3. Since the exact epitope is not disclosed, we performed an alignment of the last 250 amino acids of human and mouse STAT1 and STAT3 proteins using the online SIM alignment tool - http://web.expasy.org/sim/.

The following alignment parameters were used.

Comparison matrix: BLOSUM62

Number of alignments computed: 50

Gap open penalty: 12

Gap extension penalty: 4

Mouse STAT1versus human STAT1: 94.4% sequence identity

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94.4% identity in 250 residues overlap; Score: 1240.0; Gap frequency: 0.4%

Human           1 VLSWQFSSVTKRGLNVDQLNMLGEKLLGPNASPDGLIPWTRFCKENINDKNFPFWLWIES
Mouse           2 VLSWQFSSVTKRGLNADQLSMLGEKLLGPNAGPDGLIPWTRFCKENINDKNFSFWPWIDT
                  *************** *** *********** ******************** ** **

Human          61 ILELIKKHLLPLWNDGCIMGFISKERERALLKDQQPGTFLLRFSESSREGAITFTWVERS
Mouse          62 ILELIKKHLLCLWNDGCIMGFISKERERALLKDQQPGTFLLRFSESSREGAITFTWVERS
                  ********** **************************************************

Human         121 QNGGEPDFHAVEPYTKKELSAVTFPDIIRNYKVMAAENIPENPLKYLYPNIDKDHAFGKY
Mouse         122 QNGGEPDFHAVEPYTKKELSAVTFPDIIRNYKVMAAENIPENPLKYLYPNIDKDHAFGKY
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Human         181 YSRPKEAPEPMELDGPKGTGYIKTELISVSEVHPSRLQTTDNLLPMSPEEFDEVSRIVGS
Mouse         182 YSRPKEAPEPMELDDPKRTGYIKTELISVSEVHPSRLQTTDNLLPMSPEEFDEMSRIVGP
                  ************** ** ********************************** *****

Human         241 VEFDSMMNTV
Mouse         242 -EFDSMMSTV
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Mouse STAT3 versus human STAT3: 99.6% sequence identity

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99.6% identity in 250 residues overlap; Score: 1323.0; Gap frequency: 0.0%

Human           1 SIEQLTTLAEKLLGPGVNYSGCQITWAKFCKENMAGKGFSFWVWLDNIIDLVKKYILALW
Mouse           1 SIEQLTTLAEKLLGPGVNYSGCQITWAKFCKENMAGKGFSFWVWLDNIIDLVKKYILALW
                  ************************************************************

Human          61 NEGYIMGFISKERERAILSTKPPGTFLLRFSESSKEGGVTFTWVEKDISGKTQIQSVEPY
Mouse          61 NEGYIMGFISKERERAILSTKPPGTFLLRFSESSKEGGVTFTWVEKDISGKTQIQSVEPY
                  ************************************************************

Human         121 TKQQLNNMSFAEIIMGYKIMDATNILVSPLVYLYPDIPKEEAFGKYCRPESQEHPEADPG
Mouse         121 TKQQLNNMSFAEIIMGYKIMDATNILVSPLVYLYPDIPKEEAFGKYCRPESQEHPEADPG
                  ************************************************************

Human         181 SAAPYLKTKFICVTPTTCSNTIDLPMSPRTLDSLMQFGNNGEGAEPSAGGQFESLTFDME
Mouse         181 SAAPYLKTKFICVTPTTCSNTIDLPMSPRTLDSLMQFGNNGEGAEPSAGGQFESLTFDMD
                  ************************************************************

Human         241 LTSECATSPM
Mouse         241 LTSECATSPM
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Since there is such high similarity between the human and mouse STAT1 and STAT3, we conclude that the epitope recognition in human and mouse STAT3 will be close to 100% or identical and the one in STAT1 has a high probability >94% to be also shared in these species. Both antisera are polyclonal rabbit sera and we tested the specificity of the STAT1 and STAT3 antibodies used for the TMA analysis by performing immune-histochemical analysis on mouse livers deficient either in STAT1 [1] or STAT3 [2]. Furthermore, we used positive controls for STAT1 and STAT3 staining of consecutive liver sections from mice lacking STAT5 [3] that were injected with 2 mg/kg recombinant human growth hormone (Immunotools, Friesoythe; Germany) as described [4] (Figure S1).

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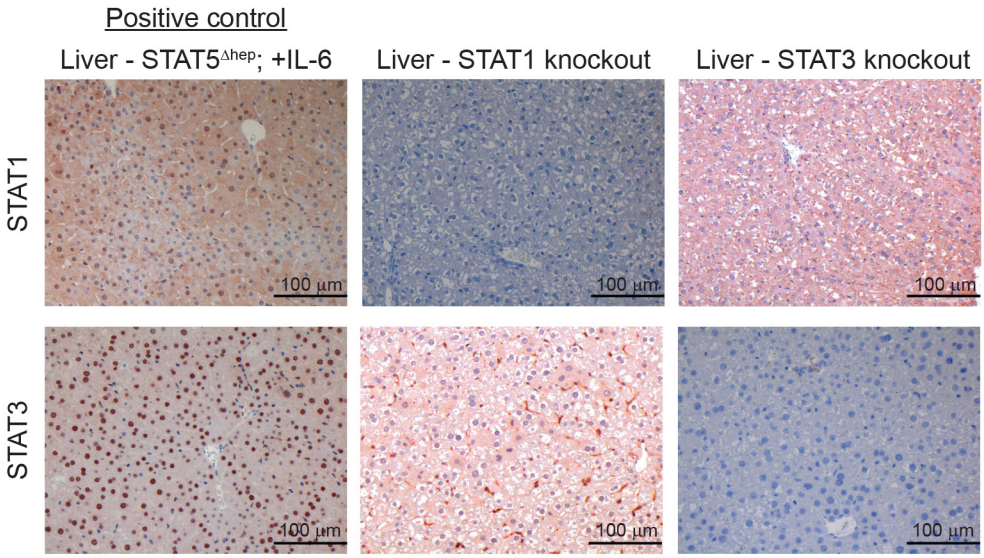
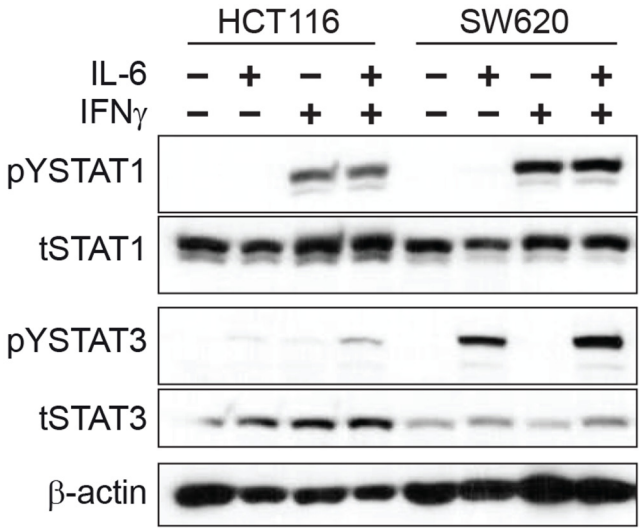
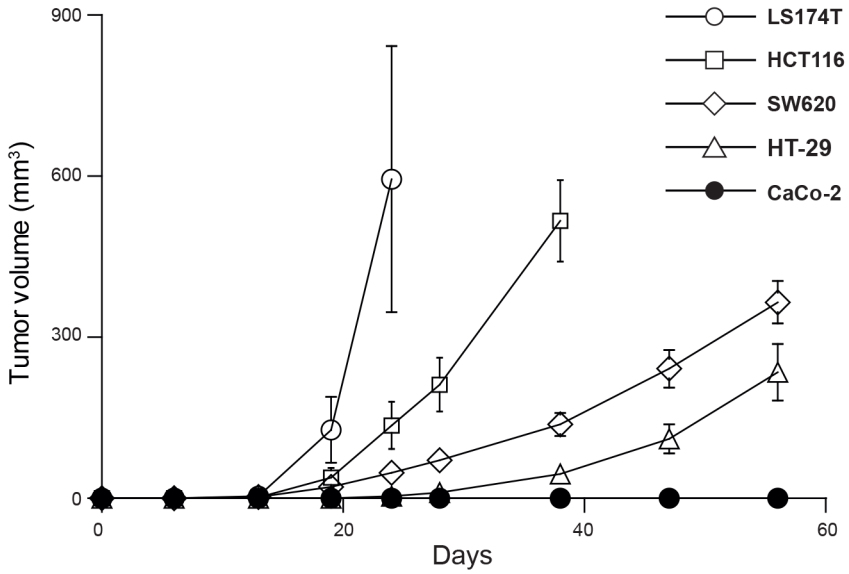


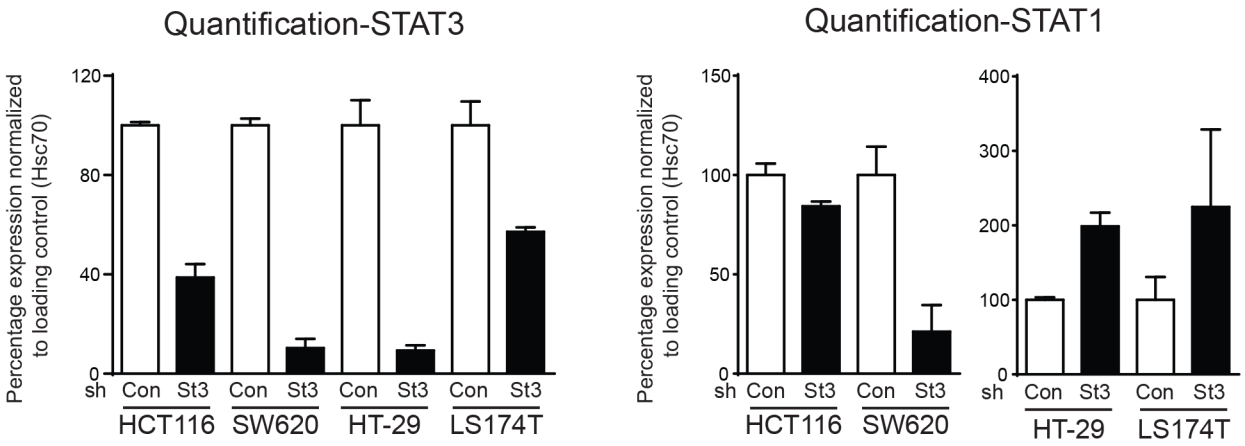
Supplementary Figure S1: Specificity of STAT1 and STAT3 antibodies. Paraffin embedded fixed liver samples from mice with respective genotypes were immunohistochemically stained with anti-STAT1 and STAT3 antibodies.



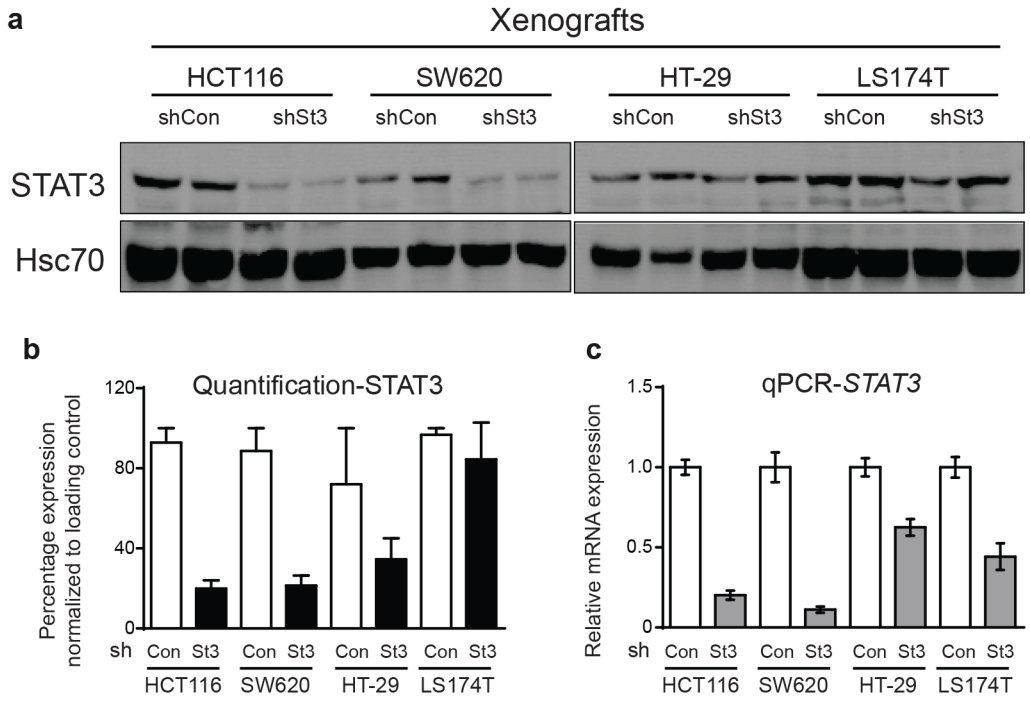
Supplementary Figure S2: STAT1 and STAT3 activation by IL-6 and IFNγ. Western blot analysis of HCT116 and SW620 cell lines stimulated IL-6, IFNγ and both, with anti-(pY)STAT1 and anti-(pY)STAT3 antibodies. B-actin was used as loading control.



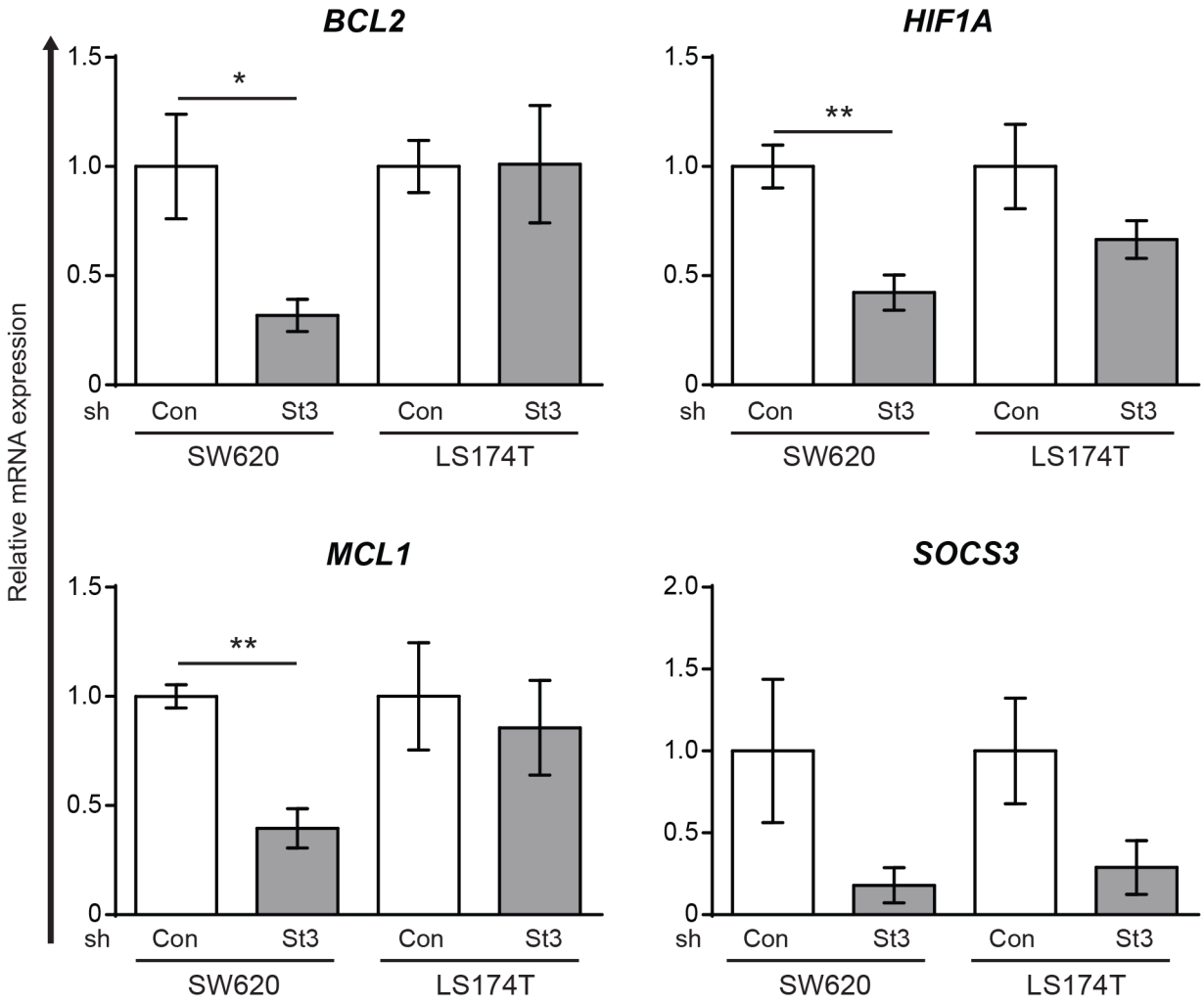
Supplementary Figure S3: Tumor growth in xenografts by colon carcinoma cell lines. 1 million cells of the indicated cell line were injected sub-cutaneously in the hind flanks of SCID mice and the development of tumor volume was followed for up to 60 days. Mean values are shown and error bars are SEM.



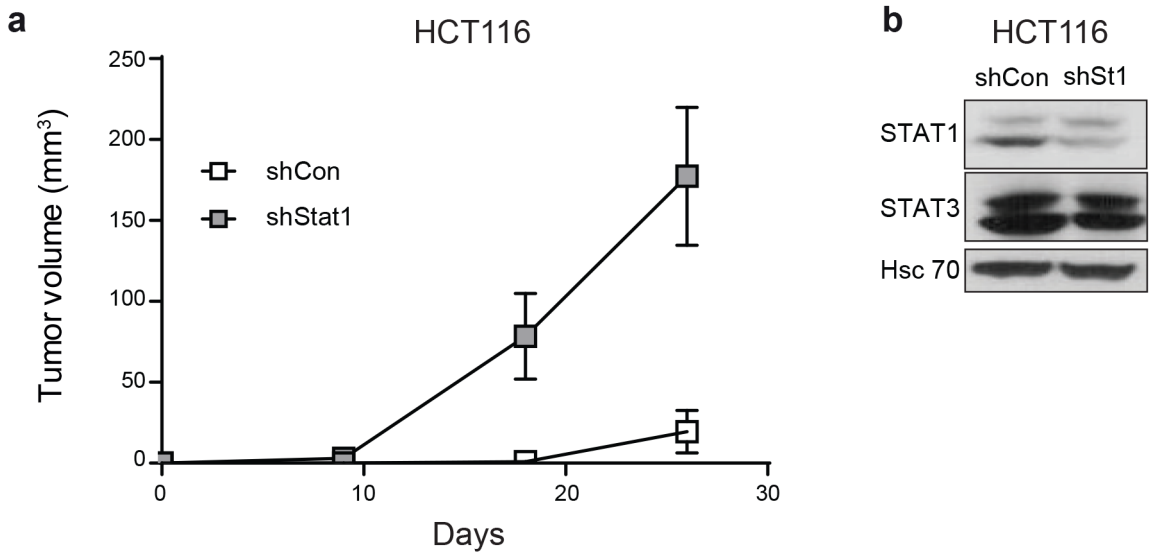
Supplementary Figure S4: Quantification of western blots. The open source software Image J program was used to quantify the intensity of the STAT3 and STAT1 bands in figures 3b and 3c. Mean values are shown and error bars show the range of expression levels.



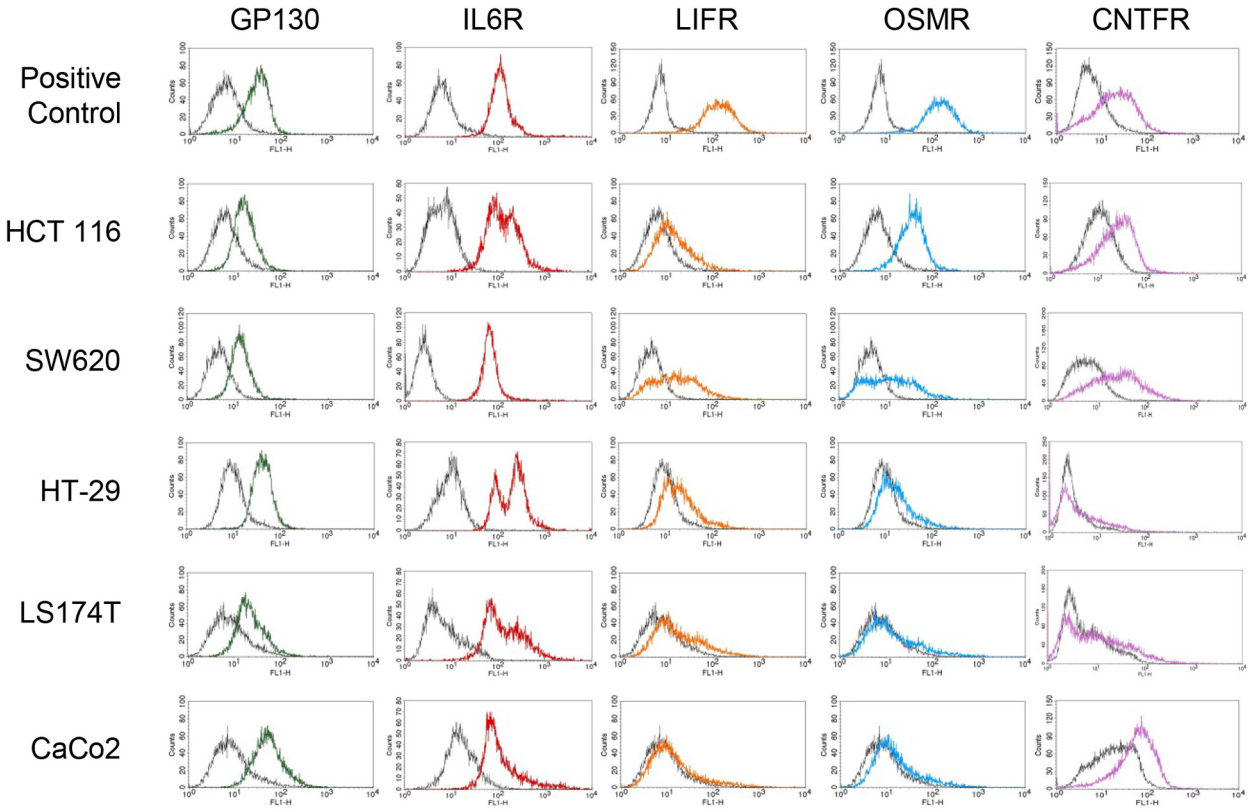
Supplementary Figure S5: STAT3 expression levels in xenografts. a. Protein lysates from xenograft samples derived from control and shSTAT3 cell lines were subjected to Western blot and probed with anti-STAT3 and anti-Hsc70 antibodies. **b.** Quantification of the Western blot (Image J), mean values are shown and error bars show the range of expression levels. **c.** mRNA expression of STAT3 (normalized to beta-2 microglobulin (*B2m*) mRNA) in the xenografts as quantified by RT-qPCR. Mean values are shown, error bars are SEM and * p<0.05, **p<0.01, ***p<0.001.



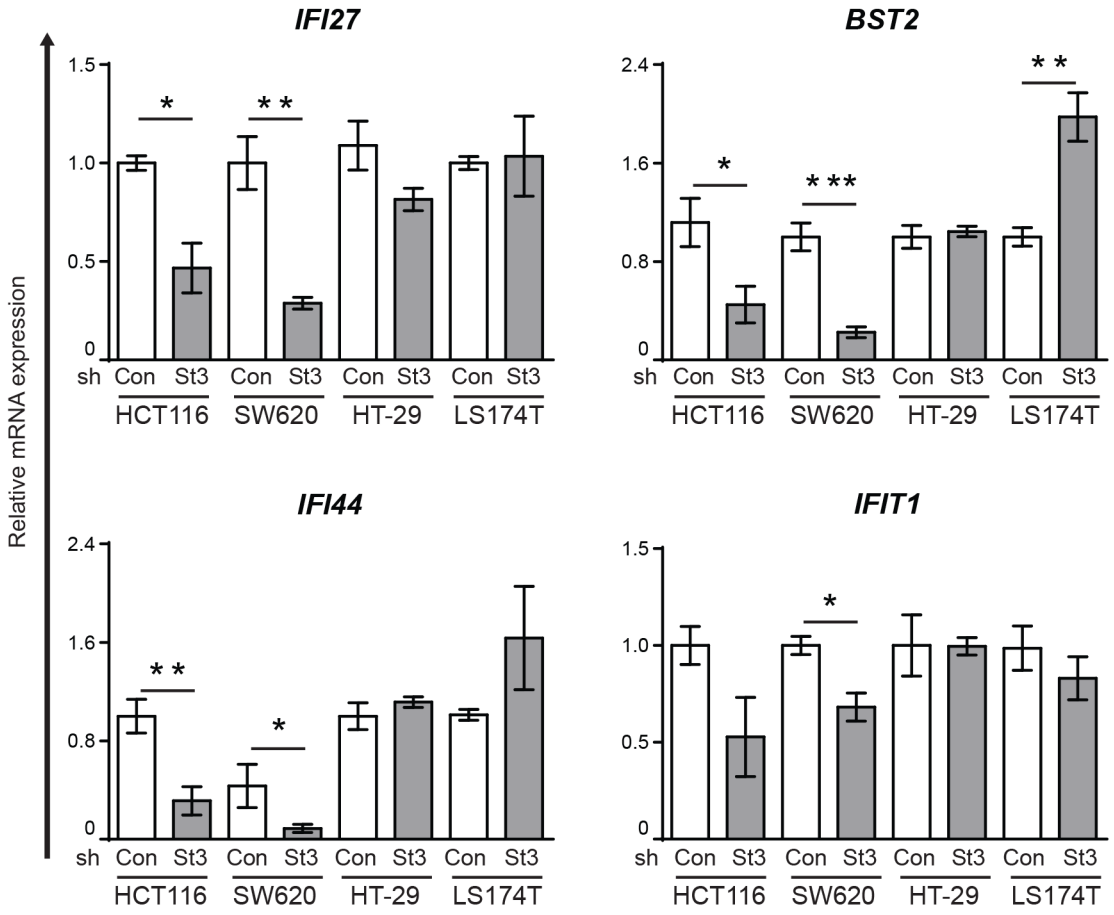
Supplementary Figure S6: Expression of STAT1 target genes in xenografts. Relative expression levels (mRNA) of the indicated STAT1 targets (normalized to beta-2 microglobulin (*B2m*) mRNA) was measured by RT-qPCR. Mean values are shown, error bars are SEM and * p<0.05, **p<0.01, ***p<0.001.



Supplementary Figure S7: Effect of STAT1 knockdown on tumor growth of HCT116 xenografts upon STAT1 knockdown. a. 1 million cells were injected subcutaneously in the hind flanks of SCID mice. Tumor volume was followed over time. Mean values are shown, error bars are SEM and * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **b.** Western blot for STAT1 and STAT3 expression in the cell lines. Hsc70 was used as loading control.



Supplementary Figure S8: Expression of GP130 and receptors of the IL-6 family cytokines on colon carcinoma cell lines. FACS analysis using specific antibodies to the indicated receptors with fluorescence labelled secondary antibodies (colored lines) vs. controls without primary antibodies (black lines).



Supplementary Figure S9: Expression of STAT1 target genes in xenografts. Relative expression levels (mRNA) of the indicated STAT1 targets (normalized to beta-2 microglobulin (*B2m*) mRNA) was measured by RT-qPCR. Mean values are shown, error bars are SEM and * p<0.05, **p<0.01, ***p<0.001.

Supplementary Table S1: Mutational status of relevant genes in the colon carcinoma cell lines

	HCT116	SW620	HT-29	LS174T	CaCo2
Gene	**Status**	**Status**	**Status**	**Status**	**Status**
APC	Wt [5, 6, 7]	p.Q1338* [5, 6, 7]	1)p.E853*, 2)p.T1556fs* 3) p.E853* [6, 7]	Wt [5, 6, 7]	p.Q1367* [5, 6, 7]
KRAS	p.G12D [7]	p.G12V [8, 9]	Wt [7]	p.G12D [10]	Wt [11]
BRAF	Wt [7]	Wt [8, 12]	p.V600E [7]	Wt [7]	Wt [13, 14, 15, 16]
CTNNB1	p.S45del [7]	Wt [17, 18]	Wt [7]	-	1) -, 2) p.G245A [17, 18, 19]
EP300	1) p.M1470fs*3 2) p.N1700fs*9 [20]	Wt [20]	p.M1470fs*3 [20]	-	Wt [20]
MLH1	p.S252* [7]	Wt [7]	Wt [7]	Wt [7]	-
CDKN2A (p16Ink4 p19ARF)	p.R24fs*20 p.G23fs p.E74fs*15 [7]	-	-	Wt [7]	-
PIK3CA	p.H1047R [7]	p.H1047R	1) p.P449T 2) – [7]	p.H1047R [7]	-
SMAD4	Wt [7]	p.? [21]	p.Q311* [7]	Wt [7]	p.D351H [21, 22, 23]
TP53	Wt [7, 24, 25]	1) p.R273H 2) p.P309S [25]	p.R273H [7, 25]	-	1) p.Glu204X 2) – [25, 26]