Supplemental Information

Chromosome 8p tumor suppressor genes SH2D4A and SORBS3 cooperate to inhibit interleukin-6 signaling in hepatocellular carcinoma

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Supplemental Experimental Procedures

Immunohistochemistry

From the paraffin-embedded TMA block 4 µm thin sections were cut, deparaffinized and rehydrated. For heat-induced epitope retrieval Ultra CC1 (Cell Conditioning Solution, Ventana Medical Systems, Tucson, AZ, USA) was used. After blocking of endogenous peroxidase primary antibodies against SH2D4A, CD3, CD4, CD8, CD20, CD68 or FOXP3 were incubated (Table S2). For further steps the biotin-free system OptiView DAB IHC Detection Kit (OptiView) was used including OptiView Universal Linker, OptiView HRP Multimer and DAB-Chromogen. Granulocytes were stained with LEUCOGNOST® NASDCL (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Finally, the slides were counterstained with hematoxylin. SH2D4A staining was scored as 0: negative, 1: low expression, 2: moderate expression or 3: high expression. CD3-, CD4-, CD8- CD20-, CD68- and FOXP3-positive immune cells were counted in each core. The mean of the two cores for each case was calculated.

Cell lines

Nine liver cancer cell lines (HuH1, HuH6, HuH7, SNU182, SNU387, HepG2, Hep3B, HLE, HLF), the normal liver cell line THLE2 which was derived by transformation with SV40 large T antigen and HEK293T cells were used in this study. Cell lines were obtained from ATCC (SNU182, SNU387, HepG2, Hep3B, THLE2 and HEK293T) or JCRB (HuH1, HuH6, HuH7 HLE and HLF). HuH1, HuH6, HuH7, HLF, HLE and HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum and 1% Penicillin-streptomycin (100 IU/ml and 100 g/ml, respectively). Hep3B cells were cultured in Minimum Essential Medium (MEM) and HepG2, SNU182 and SNU387 in RPMI-1640 medium, each supplemented with 10% fetal calf serum and 1% Penicillin-streptomycin. All media and supplements were obtained from Life Technologies (Darmstadt, Germany). For the cultivation of THLE-2 cells, Bronchial Epithelial Basal Medium (BEBM) was

supplemented with epidermal growth factor (5 ng/ml), phosphoethanolamine (70 ng/ml), 10% fetal calf serum and BEGM SingleQuot Kit, except for Gentamycin/Amphotericin and Epinephrine. BEBM and BEGM SingleQuot Kit were purchased from Lonza (Walkersville, MD, USA). All cell lines were incubated at 37°C with 5% CO_2. Cell lines were transfected using Lipofectamine 2000 transfection reagent (Life Technologies), polyethylenimine (PEI; Polysciences, Warrington, PA, USA) or the Amaxa Nucleofector Kit T (Lonza, Cologne, Germany) according to the manufacturer's instructions.

RNA extraction, cDNA synthesis and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. cDNA was synthesized from 0.5-1 µg total RNA depending on isolated total RNA concentration using RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies). Samples were analyzed in triplicates using GoTaq PCR Master Mix (Promega, Mannheim, Germany) on a StepOnePlus real-time PCR instrument (Applied Biosystems, Darmstadt, Germany) or TaqMan assays by Applied Biosystems 7500 Sequence Detection System. The reference gene serine/arginine-rich splicing factor 4 (SRSF4) was used as an internal control. Relative mRNA expression values were calculated using the comparative Ct method. Primers were obtained from ThermoFisher Scientific (Offenbach, Germany) and Apara-Bioscience (Denzlingen, Germany) and are listed in Table S3.

Construction of expression vectors

SORBS3α from pCMV6-Entry-SORBS3 (RC210669) and SH2D4A from pCMV6-Entry-SH2D4A (RC210260) (both myc-DDK (Flag) tagged; from OriGene Technologies, Rockville, MD, USA) were subcloned into the mammalian expression vector pDEST via Gateway Technology (Life Technologies) using primers attB1-SORBS3 or attB1-SH2D4A and attB2-

Flag according to the manufacturer's protocol (see Table S4 for primer sequences). The SORBS3β N-terminal sequence was PCR amplified from HLF complementary DNA (cDNA) using sense oligonucleotide 5'-GCACCATGGCTGATGGAGGAAGCCCCTTC-3' and antisense 5'-GAAGGTGTACTGGGCCACAGCCTCTC-3'. PCR-amplified fragments were cloned into NcoI and CsiI sites of pDONR-SORBS3α followed by a recombinant reaction to obtain the expression plasmid pDest-SORBS3α. All constructs are C-terminally myc-Flag tagged and were verified by sequencing.

For cloning STAT3 plasmids pMXs-STAT3-C (#13373, Addgene, Cambridge, MA, USA (1)) expressing constitutively active STAT3 (STAT3-CA) was used as template. STAT3-CA was subcloned into pDONR201 via Gateway Technology, thus pDONR201-STAT3-CA served as template for cloning the constructs STAT3wt, STAT3 Y705F, STAT3 S727A and STAT3 Y705F/S727A (STAT3 YF/SA) via site-directed mutagenesis. Primers are listed in Table S4. STAT1α was synthesized and cloned into pDONR221 by GeneArt Gene Synthesis (Life Technologies). Finally, all constructs were subcloned into expression vector pDest-HA-N (N-terminal HA-tag) via Gateway Technology.

Knockdown by shRNA

Knockdown was performed by lentiviral transduction. Lentiviral particles were produced by transfecting HEK293T cells with psPAX2, pMD2.G and pLKO.1 shRNA vectors (Table S5). As a control shRNA targeting eGFP was used. shRNA was transduced into cell lines by lentiviral infection followed by selection with 1 µg/ml Puromycin for one week.

Cell viability assay

HepG2 cells were seeded in 6cm dishes and transiently transfected after 24 h. After another 24 h cells were selected with G418 for 1-2 days. HuH1 cells were transfected using Amaxa Nucleofector Kit T (Lonza). 3000 HepG2 cells or 2000 HuH1 cells, respectively, were seeded

in a 96-well plate in sextuplicates and viability was measured on days 1, 3, 5 and 7 with CellTiter-Blue Cell Viability Assay (Promega, Mannheim, Germany). Twelve microliters of reaction solution diluted 1:2 in PBS and 60 µl medium was added to the cells and incubated for 1 h at 37°C and measured ($560_{Ex}/590_{Em}$) with an Omega FLUOstar Microplate Reader (BMG LABTECH, Ortenberg, Germany).

Colony formation assay

Cells were seeded after transfection and selection in 6-well plates and cultured for 10–14 days (4000 HepG2 cells per well; 10000 HuH1 cells per well). Then cells were washed twice with ice-cold PBS and stained with 0.5 % crystal violet solution in 25% methanol for 20-30 min at room temperature. All experiments were conducted at least three times in triplicates.

Western Blot

Total protein was extracted from cell lines with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA pH 8.0) supplemented with PhosStop and protease inhibitor Complete Mini EDTA-free (Roche Diagnostics, Mannheim, Germany). The protein concentration of each sample was determined by Bradford assay (Sigma-Aldrich, Taufkirchen, Germany). 20 µg of protein lysates were separated on 8% or 10% Bis/Tris-polyacrylamide gel and then transferred to an equilibrated polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% milk in TBST and immunoblotted with the indicated antibodies overnight at 4°C. Proteins were detected with IRDye secondary antibodies using an Odyssey Sa Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany). Protein abundance was quantified using Image Studio v3.1.4 (LI-COR Biosciences).

Dual-Luciferase Reporter Assay

HepG2 cells were co-transfected with control vector, SH2D4A, SORBS3α or both, together with Firefly Luciferase reporter vector pGL4.47 [luc2P/SIE/Hygro] and pRL-TK (Renilla Luciferase control reporter vector) (both Promega) using Lipofectamine 2000 transfection reagent. HuH1 cells were additionally transfected with control vector, STAT3wt, STAT3 Y705F or STAT3 S727A. Cells were starved 30 h post-transfection in serum-free media overnight and harvested 2 h after stimulation with IL-6 (20 ng/ml). For STAT1 luciferase reporter assays, pGL4 [luc2P/GAS-RE/Hygro] (Promega) and pRL-TK were co-transfected with control vector, SORBS3α, SORBS3β, SH2D4A alone or in combinations into HuH7 and HepG2 cells. For IL-6 stimulation cells were starved in serum-free media overnight. Cells were either stimulated with 20 ng/ml IL-6 for 2 h or with 500 U/ml IFNγ (Sigma-Aldrich) for 8 h. For ERα reporter assays, 3X ERE TATA luc reporter plasmid (#11354, Addgene (2)), TK-Renilla, ERα, SORBS3α, SORBS3β or SH2D4A were transfected with Lipofectamine 2000 into HuH7 cells and incubated for 48 h in phenol red-free media containing 10% charcoal-stripped serum with or without 10 nM β-estradiol (Sigma-Aldrich). Luciferase activity was analyzed by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol using an Omega FLUOstar Microplate Reader.

Proximity Ligation Assay (PLA)

Cells were seeded on glass cover slips before coated with poly-l-lysine for 1 h at 37°C and transfected 24 h later. Forty eight hours post-transfection cells were fixed in 4% PFA and permeabilized with 0.2% Triton X-100/PBS. PLA was performed with DUOLINK assay (Sigma-Aldrich) according to the manufacturer's protocol. Cells were incubated with anti-Vinexin (D-18, goat, Santa Cruz), anti-ERα (D-12, mouse, Santa Cruz), anti-SH2D4A (S-16, goat, Santa Cruz) or anti-STAT3 (124H6, mouse, Cell Signaling) as indicated overnight at 4°C. To detect the protein-protein interactions the Duolink In Situ Detection Kit Orange was

used. For staining F-actin cells were incubated with 50 µl CytoPainter Phalloidin-iFluor 488 Reagent (ab176753, Abnova) for 30 min at room temperature according to the manufacturer's protocol. Cover slips were air dried and mounted with Duolink In Situ Mounting Medium with DAPI (DUO82040, Sigma-Aldrich). Cells were examined with a fluorescence microscope OLYMPUS IX81 (Olympus, Hamburg, Germany) and a confocal microscope ZEISS LSM 710 ConfoCor 3 (Carl Zeiss Microscopy, Oberkochen, Germany). The dots per cell indicating protein-protein interaction were manually quantified using Fiji/ImageJ (3).

Co-immunoprecipitation (Co-IP)

To identify protein-protein interactions co-immunoprecipitation (co-IP) using Dynabeads Protein G (Life Technologies) was performed. HEK293T cells were transfected with PEI and after 48 h harvested in non-denaturing lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with 1 mM PMSF, 1 mM DTT, PhosStop and protease inhibitor Complete Mini EDTA-free. Dynabeads were incubated with 2 µg Flag M2 antibody (Sigma-Aldrich) or negative control mouse IgG1 (Dako) diluted in PBST for more than 4 h at 4°C while rotating. After gentle washing with PBST 1 mg of protein lysate was added and incubated overnight at 4°C while rotating. After three washing steps immunoprecipitated proteins were eluted by incubating beads in 2x sample buffer (125 mM Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 50 mM DTT) at room temperature for 20 min while shaking. The supernatants were boiled for 8 min at 95°C and separated by SDS-PAGE. Detection was carried out with anti-SH2D4A (Santa Cruz), anti-STAT3 (Cell Signaling), anti-HA (Santa Cruz), anti-ERα (Santa Cruz) or anti-SORBS3 (Abnova) antibodies. ImmunoCruz secondary antibodies (Santa Cruz) were used to avoid detection of heavy and light chains of the IP antibody.

Cell fractionation

HEK293T cells were seeded on 10cm plastic dishes. After 24 h cells were transfected with empty vector and HA-STAT3wt or SH2D4A-Flag and HA-STAT3wt, respectively with PEI. The next day, cells were starved in serum-free medium overnight and 72 h after seeding, cells were treated with 20 ng/ml IL-6 (Biomol, Hamburg, Germany) for 30 min at 37°C. Cells were immediately trypsinized, washed with PBS at 4°C and cell fractionation was performed by applying the NE-PER Nuclear and Cytoplasmic Extraction Kit (Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol. Poly (ADP-ribose) polymerase 1 (PARP) and beta-Tubulin antibodies were used as markers for the nuclear and cytoplasmatic fraction, respectively to assess the fractionation efficiency.

Blue native polyacrylamide gel electrophoresis (BN-PAGE)

HEK293T cells were seeded in 6-well plates and transfected the following day with N-terminally GFP-tagged STAT3wt and Flag-tagged SH2D4A. The cells were stimulated with 20 ng/ml IL-6 for 1 h or left untreated, harvested 48 h post-transfection and stored at -80°C till analyzing. Cells were resuspended in 50 µl lysis buffer (1x NativePAGE Sample Buffer, 1% DDM, PhosStop and protease inhibitor Complete Mini EDTA-free) and centrifuged for 30 min at 20.000 x g and 4°C. Five µl of the supernatants were mixed with 1x NativePAGE Sample Buffer and 0.25% G-250 Sample Additive, and the total 10 µl were separated on a NativePAGE 4-16% Bis-Tris protein gel according to the manufacturer's protocol (Life technologies)(4). Proteins were transferred to PVDF membranes via semi-dry blotting and fixed in 8% acetic acid for 15 min. After destaining in 62.5 mM Tris/HCl pH 6.7, 2% SDS for 5 min and washing with H2O the membranes were blocked with 5% milk powder in TBST for 1 h at room temperature. Proteins were detected with anti-STAT3 and HRP-conjugated horse anti-mouse (Cell Signaling) using the FluorChem SP Imaging System (Alpha Innotech, San Leandro, CA, USA).

Electrophoretic Mobility Shift Assay (EMSA)

HepG2 cells were transfected with empty vector control or C-terminally Flag-tagged SH2D4A with Lipofectamine 2000. The next day cells were starved overnight in serum-free medium. Before performing cell fractionation with the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scienctific), cells were treated with 20 ng/ml IL-6 for 2 h. Protein concentrations were measured using NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Nuclear extracts (1.5 µg, 3 µg and 5 µg) were incubated for 20 min in gel shift incubation buffer with 66 fmol double-stranded mutated SIE (sis-inducible element)-oligonucleotide (m76SIE) (5). Biotinylated probes were purchased from Integrated DNA Technologies (Leuven, Belgium) with the following sequences: Forward: 5'-Biotin-GATCCGGGAGGGATTTACGGGAAATGCTA-3', Reverse: 5'-Biotin-TAGCATTTCCCGTAAATCCCTCCCGGATC-3'. EMSA was performed by using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer's protocol.

Supplemental Figure Legends

Supplemental Figure S1: Expression of SORBS3 and SH2D4A in normal liver and HCC cell lines. (**A**) Schematic diagram of the transcript variants and protein isoforms of SH2D4A (upper panel) and SORBS3 (lower panel) obtained from NCBI (www.ncbi.nlm.nih.gov) and UniProt (www.uniprot.org). Open reading frames of SH2D4A isoform a (454 aa), SH2D4A isoform b (409 aa), SORBS3α (671 aa) and SORBS3β (329 aa) are shown by boxes. SH2D4A isoforms possess one SH2 domain (gray boxes) and SORBS3 three SH3 domains (red boxes). SH2D4A isoforms as well as SORBS3 isoforms differ in their N-terminal regions. (**B**) Western blot analysis of SORBS3 and SH2D4A protein expression of the SV40 large T antigen transformed normal liver cell line THLE2 and nine HCC cell lines. (**C**) Relative SORBS3 (left panel) and SH2D4A (right panel) mRNA expression in normal liver and HCC cell lines measured by semi-quantitative real time RT-PCR. (**D**) Correlation of SORBS3 and SH2D4A protein (left) and mRNA (right) expression by Pearson correlation. Protein expression levels represent total amount of all protein isoforms normalized to actin.

Supplemental Figure S2: SH2D4A and SORBS3 inhibit tumor cell proliferation and colony formation in HuH1 cells. (**A**) Cell proliferation assay showing the relative viability of HuH1 cells transfected with empty vector control, SORBS3α, SH2D4A alone or together as indicated. (**B**) Cell proliferation assay showing the relative cell number of HepG2 cells transfected with empty vector control, SORBS3β, SH2D4A alone or together. Data represent mean ± SEM normalized to transfection control at each time point of three independent experiments. * $p < 0.05$ of two-tailed Mann-Whitney U test of control vs. overexpression groups. (**C**) Representative images of colony formation assay of HuH1 cells transfected with empty vector control, SORBS3α, SORBS3β, SH2D4A alone or together as indicated (left panel). The right panel shows the statistical representation of the number of colonies in three replicates. One out of three independent experiments is shown. Data represent mean ± SD; * $p < 0.05$ of two-sided Mann-Whitney U test.

Supplemental Figure S3: Overexpression of SORBS3α, SORBS3β and SH2D4A in HepG2 cells and knockdown of SORBS and SH2D4A in HuH7 cells. (**A**) Western blot analysis of HepG2 cells transfected with respective plasmid DNA constructs. (**B**) Relative mRNA expression (left panel) and immunoblot (right panel) of SORBS3 and SH2D4A upon lentiviral knockdown using shRNAs against SORBS3 and SH2D4A, respectively.

Supplemental Figure S4: Western blot of IL-6 signaling pathway proteins of HepG2 cells overexpressing SORBS3 and SH2D4A. HepG2 cells were transfected with control vector, SORBS3α, SORBS3β or SH2D4A and stimulated with 20 ng/ml IL-6 for 20 min or left untreated. Western blot shows protein expression levels of phosphorylated JAK1, JAK2, STAT1 and STAT3 as indicated.

Supplemental Figure S5: Proximity ligation assay (PLA) of SH2D4A and STAT3 upon IL-6 stimulation. HEK293T cells were transfected with empty control vector or SH2D4A-Flag and HA-STAT3wt. Cells were starved overnight and left untreated or treated with 20 ng/ml IL-6 for 30 min and 60 min before performing PLA. Images were taken with the fluorescence microscope OLYMPUS IX81 and a 60x oil immersion objective.

Supplemental Figure S6: SORBS3α and SH2D4A directly interact with STAT1. (**A**) Co-IP assays of HEK293T transfected with Flag-tagged SORBS3α or SORBSβ and HA-tagged STAT1. Proteins were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-HA and anti-SORBS3. (**B**) HEK293T cells were transiently co-transfected with SH2D4A-Flag together with STAT1. After anti-Flag immunoprecipitation, samples were separated by SDS–PAGE and subjected to Western blot analysis with anti-HA-reactive and anti-SH2D4A antibodies. (**C**) Luciferase assay analyzing STAT1 signaling activity using a GAS-luciferase

reporter construct in HuH7 cells expressing control vector, SORBS3α, SORBS3β or SH2D4A as indicated. Prior harvesting, cells were stimulated with or without 20 ng/ml IL-6 for 2 h. Data represent mean ± SD of three independent biological experiments normalized to stimulated control. (**D**) Luciferase assay analyzing STAT1 signaling activity using a GAS-luciferase reporter construct in HuH7 cells expressing control vector, SORBS3α, SORBS3β or SH2D4A alone and in combination as indicated. Prior harvesting cells were stimulated with or without 500 U/ml IFNγ for 8 h. Data represent mean ± SD of three independent biological experiments normalized to stimulated control. * $p < 0.001$; ** $p < 0.0001$ vs. control of two-tailed Mann-Whitney U test.

Supplemental Figure S7: Expression of immune cell markers in HCC patient groups with good or poor outcome. (**A**) Expression of CD3D, CD3E, CD3G, CD8A, CD8B and CD4 obtained by Affymetrix gene expression profiling of HCC tumor tissues of patients in the poor or good prognosis patient subgroups. (**B**) Pearson correlation analysis of SH2D4A and FOXP3 in the good prognosis or (**C**) in the poor prognosis HCC patient group.

Supplemental Figure S8: Immunohistochemical analysis of immune cell infiltration in HCC tumor tissues of a tissue microarray (TMA). (**A**) Quantitative analysis of CD68-positive macrophages, (**B**) NASDCL-positive granulocytes and, (**C**) CD20-positive B cells in HCC patients groups without SH2D4A (Score 0), with low (Score 1), moderate (Score 2) or high (Score 3) SH2D4A expression. Data represent mean ± SEM; p-value obtained by ANOVA analysis of variance.

Supplemental Figure S9: YAP signaling upon overexpression of SORBS3 and SH2D4A. (**A**) Relative mRNA expression of SORBS3, SH2D4A and YAP signaling target genes CTGF and FOXM1. HuH1 cells were transfected with control vector or SORBS3α, SORBS3β and SH2D4A individually or in combinations. Data represent mean ± SD of two

independent experiments. (**B**) Relative mRNA expression of SORBS3, SH2D4A and YAP signaling target genes CTGF and FOXM1 upon overexpression of SORBS3 and SH2D4A in HepG2 cells. Data represent mean ± SD of two independent experiments. (**C**) Immunoblot of HepG2 cells transfected with control vector, SORBS3α, SORBS3β or SH2D4A showing total and phosphorylated YAP protein levels as indicated.

References

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Supplemental Tables:

Table S1: Expression of IL-6 target genes in HCC subgroup poor versus good prognosis

Rank	p-value	Fold difference	UG cluster	Gene symbol	Map Location
1	**0.0001**	**0.43**	**Hs.427202**	**TTR**	**18q12.1**
2	0.0118	1.54	Hs.127799	BIRC3	11q22
3	0.0237	0.88	Hs.81134	IL1RN	2q14.2
4	**0.0377**	**2.17**	**Hs.407856**	**SPINK1**	**5q32**
5	**0.0395**	**2.04**	**In multiple clusters**	**CRP**	
6	0.0831	1.09	Hs.656213	JAK2	9p24
7	0.0959	1.05	Hs.50640	SOCS1	16p13.13
8	0.1244	0.78	Hs.160562	IGF1	12q22-q23
9	0.1582	0.83	Hs.300774	FGB	4q28
10	0.3552	0.88	Hs.28988	GLRX	5q14
11	0.3623	1.02	Hs.527973	SOCS3	17q25.3
12	0.3672	0.83	Hs.154078	LBP	20q11.23-q12
13	0.4528	0.93	Hs.463059	STAT3	17q21.31
14	0.5872	0.86	Hs.1955	SAA2	11p15.1-p14
15	0.6005	0.84	Hs.466804	PLA2G2A	1p35
16	0.6854	1.14	Hs.517070	SLPI	20q12
17	0.7752	0.96	Hs.534847	C4A	6p21.3

Table S2: Primary and secondary antibodies

Antigen	Species	Product/Company	Experiments*
Actin	Mouse	691001/ MP Biomedicals	WB
Anti-mouse (HRP)	Horse	7076/ Cell Signaling	WB
CD20	Mouse	L26/ DAKO	IHC
CD3	Rabbit	SP7/ Zytomed	IHC
CD4	Rabbit	SP35/ Ventana	IHC
CD68	Mouse	PG-M1/ DAKO	IHC
CD8	Mouse	C8/144B/ DAKO	IHC
ERα (D-12)	Mouse	sc-8005/ Santa Cruz	PLA, co-IP
FLAG M2	Mouse	F1804/ Sigma-Aldrich	co-IP
FOXP3 (236A/E7)	Mouse	ab20034/ abcam	IHC
HA (Y-11)	Rabbit	sc-805/ Santa Cruz	co-IP
phospho-JAK1 (Tyr 1022/1023)	Rabbit	3331/ Cell Signaling	WB
phospho-JAK2 (Tyr 1007/1008) (C80C3)	Rabbit	3776/ Cell Signaling	WB
PARP	Rabbit	9542/ Cell Signaling	WB
phospho-STAT1 (Ser727) (D3B7)	Rabbit	8826/ Cell Signaling	WB
phospho-STAT1 (Tyr701) (D4A7)	Rabbit	7649/ Cell Signaling	WB
phospho-STAT3 (Ser 727)-R	Rabbit	sc-8001-R/ Santa Cruz	WB
phospho-STAT3 (Tyr705) (D3A7) XP	Rabbit	9145/ Cell Signaling	WB
phospho-YAP (S127) (D9W2I)	Rabbit	13008/ Cell Signaling	WB
SH2D4A (63-J)	Mouse	sc-100288/ Santa Cruz	IHC
SH2D4A (S-16)	Goat	sc-98126/ Santa Cruz	PLA, WB
SORBS3	Mouse	H00010174-B01P/ Abnova	WB
SORBS3/Vinexin (D-18)	Goat	sc-14145/ Santa Cruz	PLA
STAT1 (D1K9Y)	Rabbit	14994/ Cell Signaling	WB
STAT3 (124H6)	Mouse	9139S/ Cell Signaling	PLA, WB
β-Tubulin	Mouse	556321/ BD Biosciences	WB
YAP	Rabbit	4912/ Cell Signaling	WB
IRDye 680LT anti-mouse IgG	Donkey	926-68022/ LI-COR Biosciences	WB
IRDye 800CW anti-mouse IgG	Donkey	926-32212/ LI-COR Biosciences	WB
IRDye 800CW anti-rabbit IgG	Donkey	926-32213/ LI-COR Biosciences	WB
IRDye 800CW anti-goat IgG	Donkey	926-32214/ LI-COR Biosciences	WB
anti-goat from ImmunoCruz IP/WB Optima A System		sc-45038/ Santa Cruz	WB
anti-mouse from ImmunoCruz IP/WB Optima E System		sc-45042/ Santa Cruz	WB

* WB, Western blot; PLA, proximity ligation assay; co-IP, co-immunoprecipitation, IHC, immunohistochemistry

Table S3: Primers used for qRT-PCR

Gene	Accession	Sequence 5'-3'	Amplicon
CTGF	NM_001901.2	Forward: CCAAGGACCAAACCGTGG Reverse: CTGCAGGAGGCGTTGTCAT	181 bp
FOXM1	NM_202002.2/ NM_021953.3/ NM_202003.2/ NM_001243088.1/ NM_001243089.1	Forward: ATAGCAAGCGAGTCCGCATT Reverse: TTCCTCCCCAGGCTGGATTT	284/ 170 bp
SH2D4A	NM_022071.3/ NM_001174159.1/ NM_001174160.1	Forward: GTATGGGTGATGGGCGAACA Reverse: CGGGCCCTCTCAGCAATAAT	77 bp
SORBS3	NM_005775.4/ NM_001018003.2	Forward: AAGAACTGGCTGGAGGGAGA Reverse: TTGAAGGTGTACTGGGCCAC	155 bp
SRSF4	NM_005626.4	Forward: TGCAGCTGGCAAGACCTAAA Reverse: TTTTTGCGTCCCTTGTGAGC	80 bp
SPINK1	NM_003122.3	Hs00162154_m1 (Thermo Fisher Scientific)	85 bp
TTR	NM_000371.3	Hs00174914_m1 (Thermo Fisher Scientific)	56 bp
CRP	NM_000567.2	Hs00265044_m1 (Thermo Fisher Scientific)	72 bp

Table S4: Primers used for Gateway (GW) cloning and site-directed mutagenesis (SDM)

Primer name	Primer sequences 5'-3'
attB1 SORBS3 (GW)	Fwd: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCG CAGGGCCCACCCCGCAGCCT
attB1 SH2D4A (GW)	Fwd: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCG CTGAAACAGATACTGTCGGA
attB2 Flag (GW)	Rev: GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAACCTTAT CGTCGTCAT
STAT3 wt (SDM)	Fwd: GCTATAAGATCATGGATGCGACCAACATCCTGGTGTCTCC Rev: GGAGACACCAGGATGTTGGTCGCATCCATGATCTTATAGC
STAT3 Y705F (SDM)	Fwd: GTGCTGCCCCGTTCCTGAAGACCAAG Rev: CTTGGTCTTCAGGAACGGGGCAGCAC
STAT3 S727A (SDM)	Fwd: GACCTGCCGATGGCCCCCCGCACTTTAG Rev: CTAAAGTGCGGGGGGCCATCGGCAGGTC

Table S5: shRNAs used for lentivirus-mediated knockdown

Gene	Clone ID	Company	Target Sequence 5'-3'
SH2D4A	TRCN0000145225	OpenBiosystems	GACTAAAGACATCTGGAAGAA
SORBS3	TRCN0000123148	OpenBiosystems	CGGAACGTTCCCTGGAAATTA

Figure S1

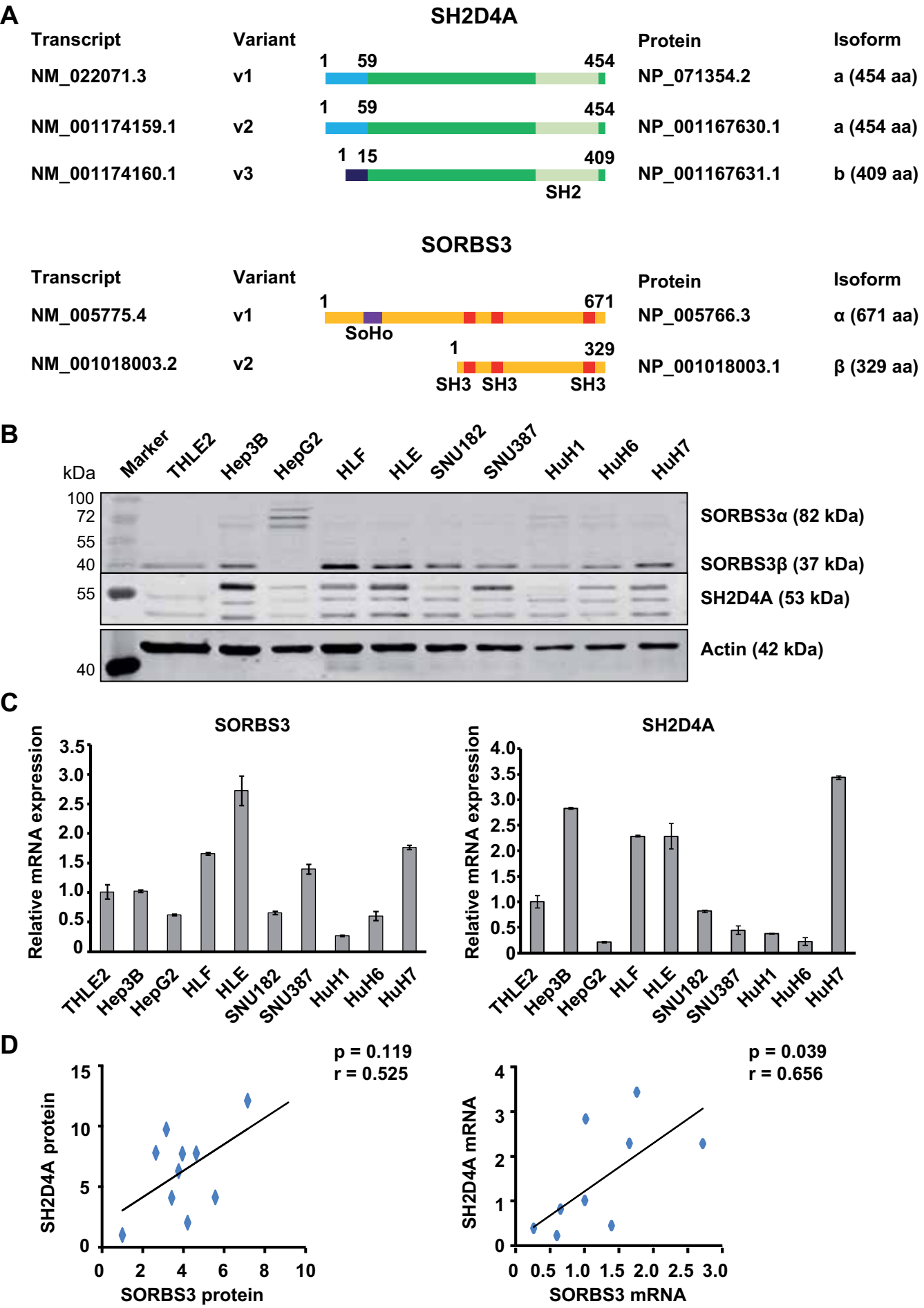

A
SH2D4A
Transcript
Variant
Protein
Isoform
NM_022071.3
v1
NP_071354.2
a (454 aa)
NM_001174159.1
v2
NP_001167630.1
a (454 aa)
NM_001174160.1
v3
NP_001167631.1
b (409 aa)
SH2
SORBS3
NM_005775.4
v1
NP_005766.3
α (671 aa)
SoHo
NM_001018003.2
v2
NP_001018003.1
β (329 aa)
SH3 SH3 SH3
B
kDa
Marker
THLE2
Hep3B
HepG2
HLF
HLE
SNU182
SNU387
HuH1
HuH6
HuH7
SORBS3α (82 kDa)
SORBS3β (37 kDa)
SH2D4A (53 kDa)
Actin (42 kDa)
C
SORBS3
SH2D4A
Relative mRNA expression
D
p = 0.119
r = 0.525
SH2D4A protein
SORBS3 protein
p = 0.039
r = 0.656
SH2D4A mRNA
SORBS3 mRNA

Figure S2

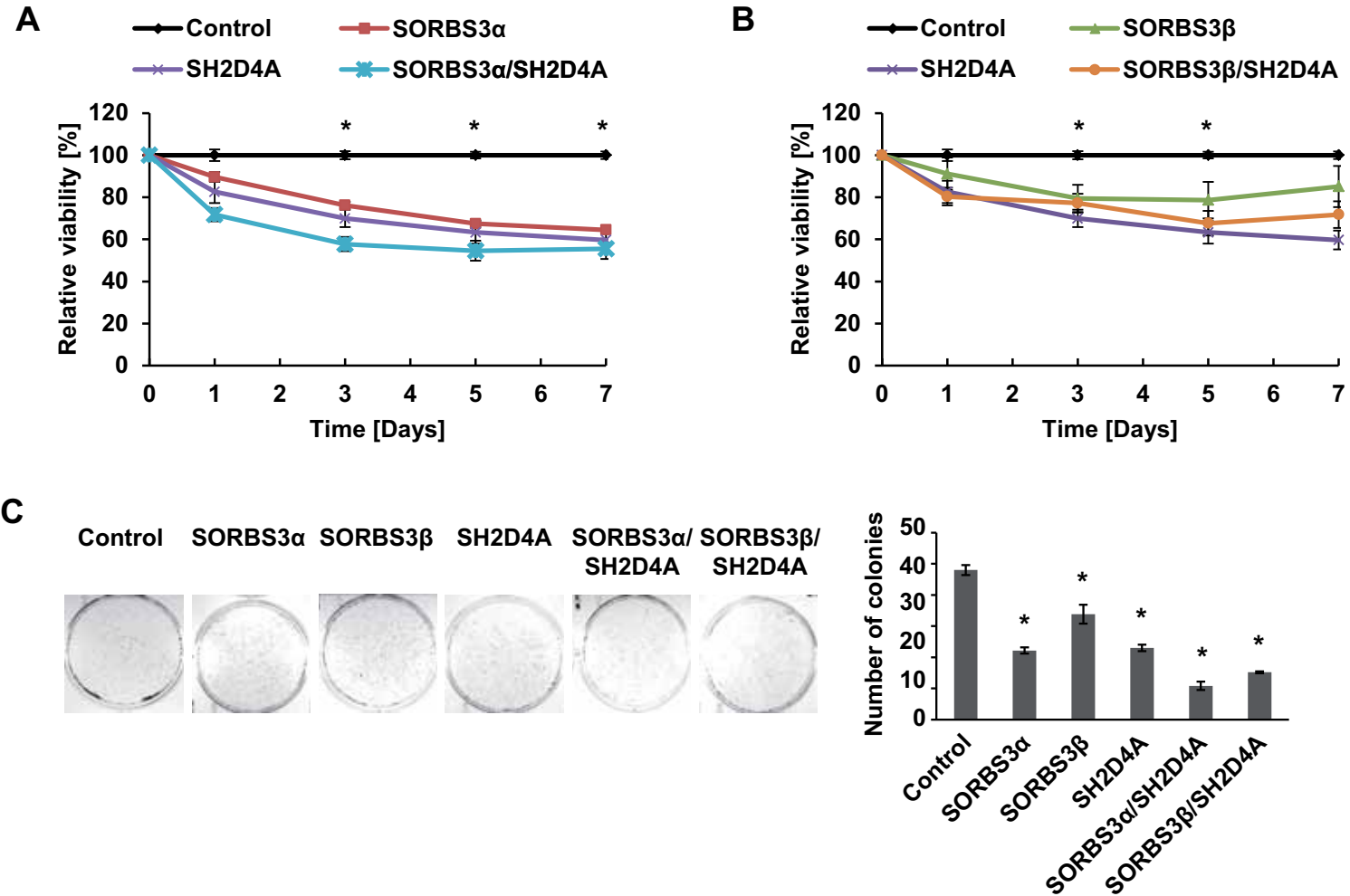

A
Control
SORBS3α
SH2D4A
SORBS3α/SH2D4A
Relative viability [%]
Time [Days]
B
Control
SORBS3β
SH2D4A
SORBS3β/SH2D4A
Relative viability [%]
Time [Days]
C
Control
SORBS3α
SORBS3β
SH2D4A
SORBS3α/ SH2D4A
SORBS3β/ SH2D4A
Number of colonies
Control
SORBS3α
SORBS3β
SH2D4A
SORBS3α/SH2D4A
SORBS3β/SH2D4A

Figure S3

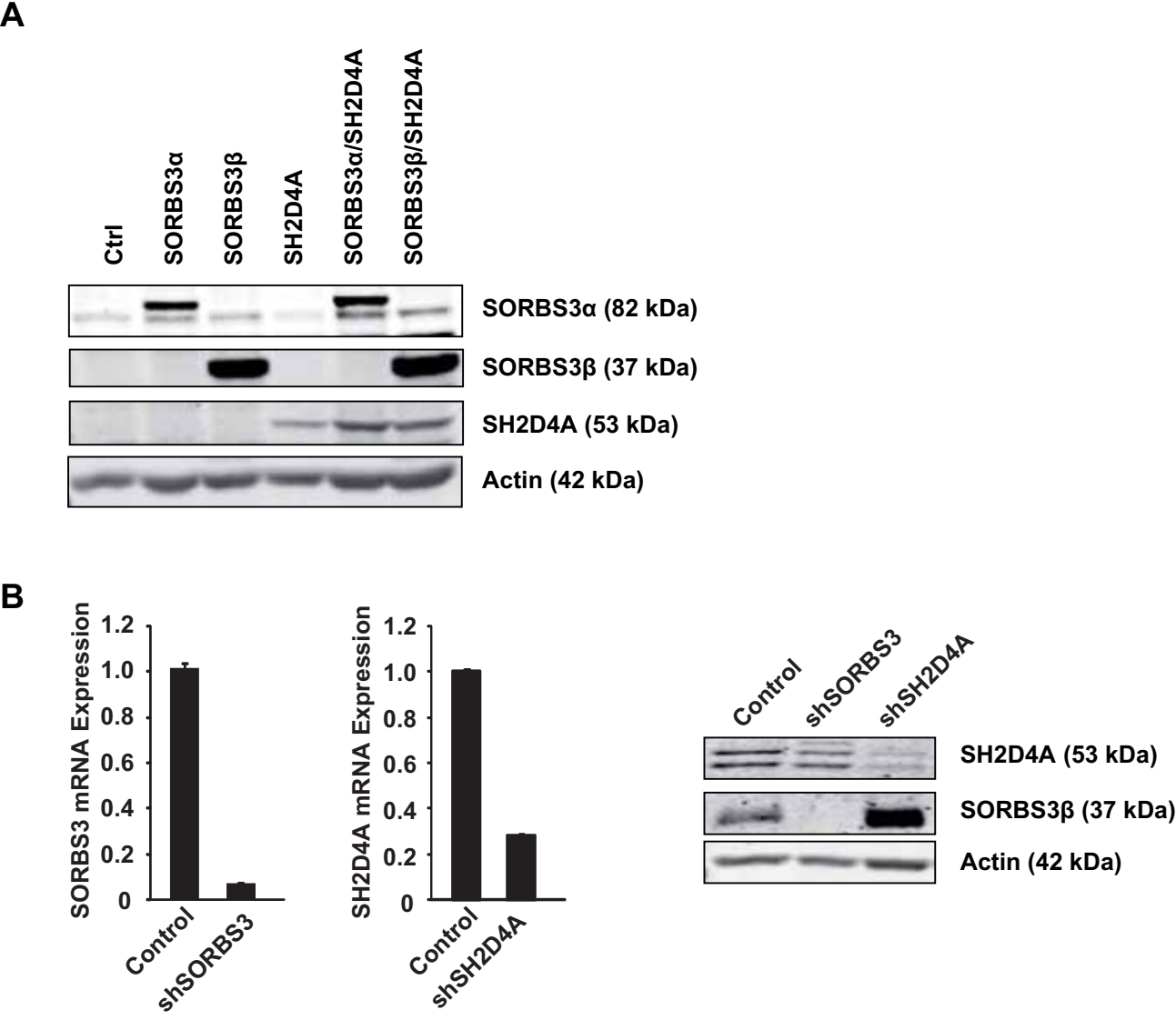

A
Ctrl
SORBS3α
SORBS3β
SH2D4A
SORBS3α/SH2D4A
SORBS3β/SH2D4A
SORBS3α (82 kDa)
SORBS3β (37 kDa)
SH2D4A (53 kDa)
Actin (42 kDa)
B
SORBS3 mRNA Expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
shSORBS3
SH2D4A mRNA Expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
shSH2D4A
Control
shSORBS3
shSH2D4A
SH2D4A (53 kDa)
SORBS3β (37 kDa)
Actin (42 kDa)

Figure S4

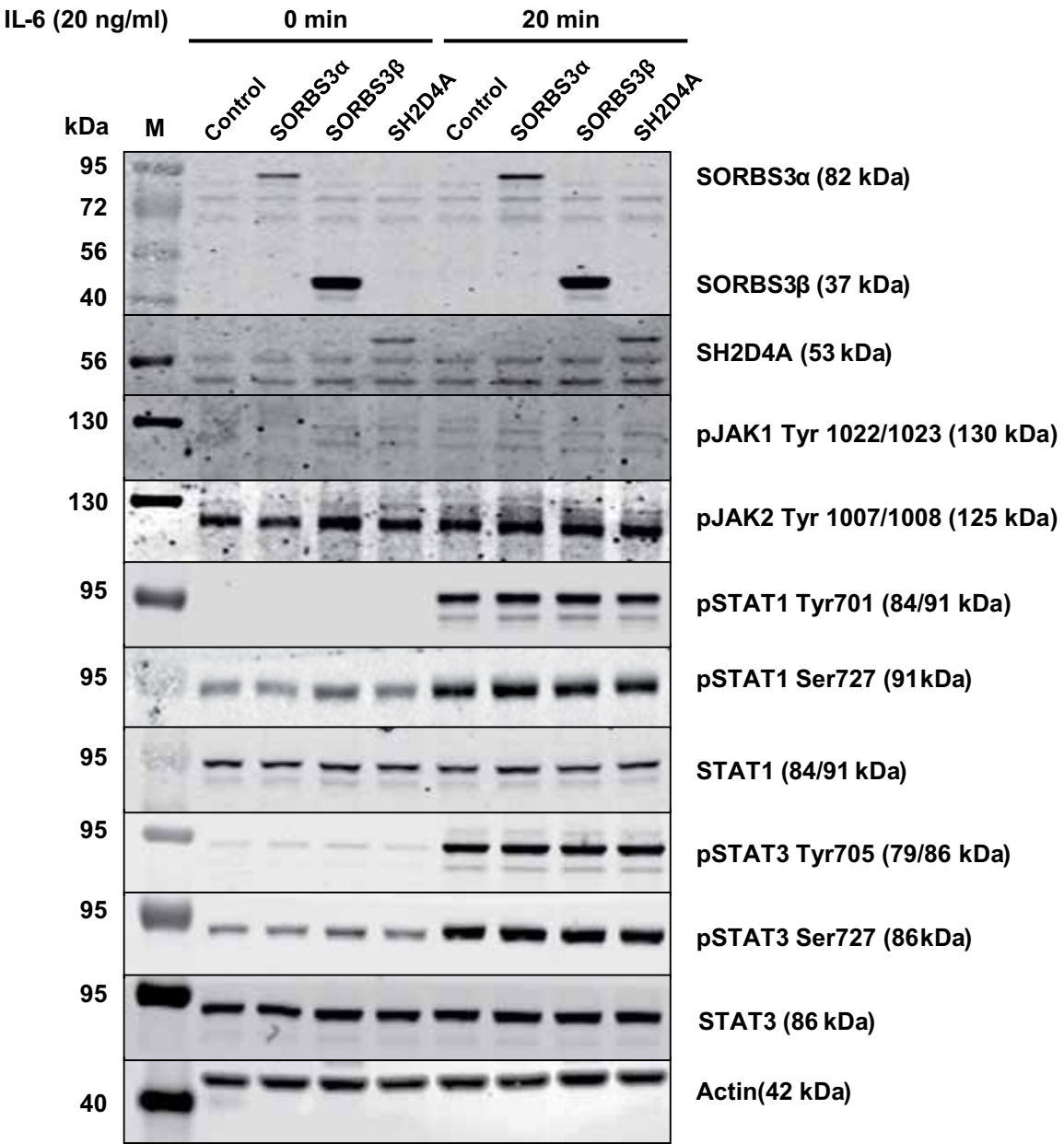

IL-6 (20 ng/ml)
0 min
20 min
kDa
M
Control
SORBS3α
SORBS3β
SH2D4A
Control
SORBS3α
SORBS3β
SH2D4A
95
72
56
40
56
130
130
95
95
95
95
95
95
40
SORBS3α (82 kDa)
SORBS3β (37 kDa)
SH2D4A (53 kDa)
pJAK1 Tyr 1022/1023 (130 kDa)
pJAK2 Tyr 1007/1008 (125 kDa)
pSTAT1 Tyr701 (84/91 kDa)
pSTAT1 Ser727 (91kDa)
STAT1 (84/91 kDa)
pSTAT3 Tyr705 (79/86 kDa)
pSTAT3 Ser727 (86kDa)
STAT3 (86 kDa)
Actin(42 kDa)

Figure S5

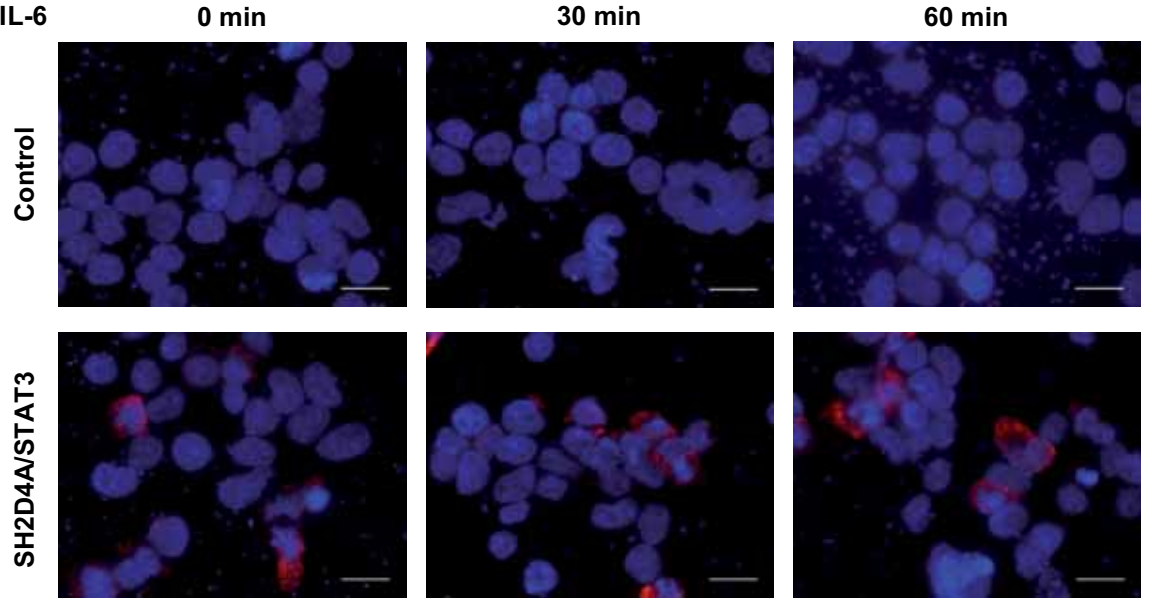


Figure S6

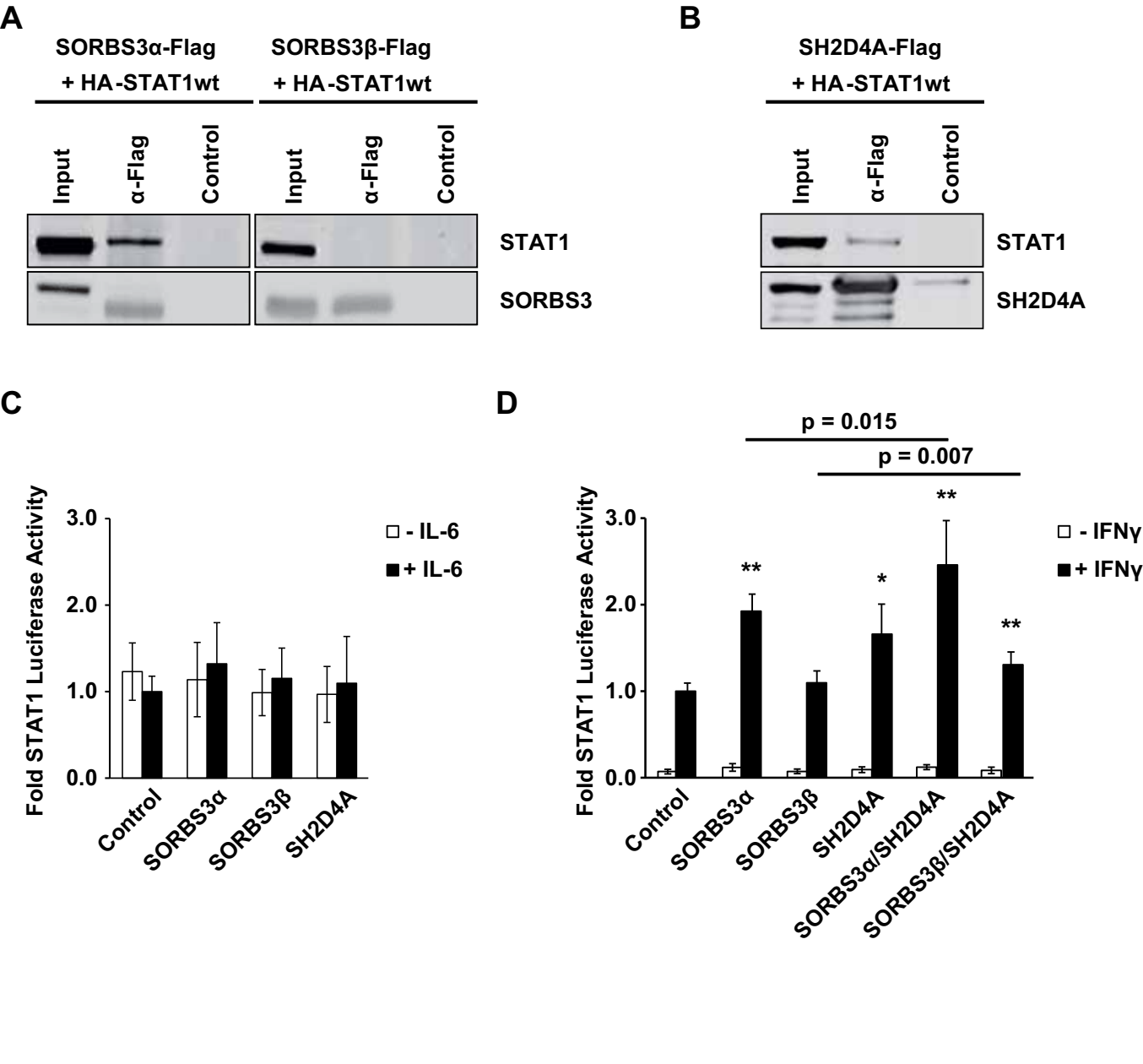

A
SORBS3α-Flag
+ HA-STAT1wt
SORBS3β-Flag
+ HA-STAT1wt
Input
α-Flag
Control
Input
α-Flag
Control
STAT1
SORBS3
B
SH2D4A-Flag
+ HA-STAT1wt
Input
α-Flag
Control
STAT1
SH2D4A
C
Fold STAT1 Luciferase Activity
3.0
2.0
1.0
0.0
- IL-6
+ IL-6
Control
SORBS3α
SORBS3β
SH2D4A
D
p = 0.015
p = 0.007
Fold STAT1 Luciferase Activity
3.0
2.0
1.0
0.0
**
*
**
**
- IFNγ
+ IFNγ
Control
SORBS3α
SORBS3β
SH2D4A
SORBS3α/SH2D4A
SORBS3β/SH2D4A

Figure S7

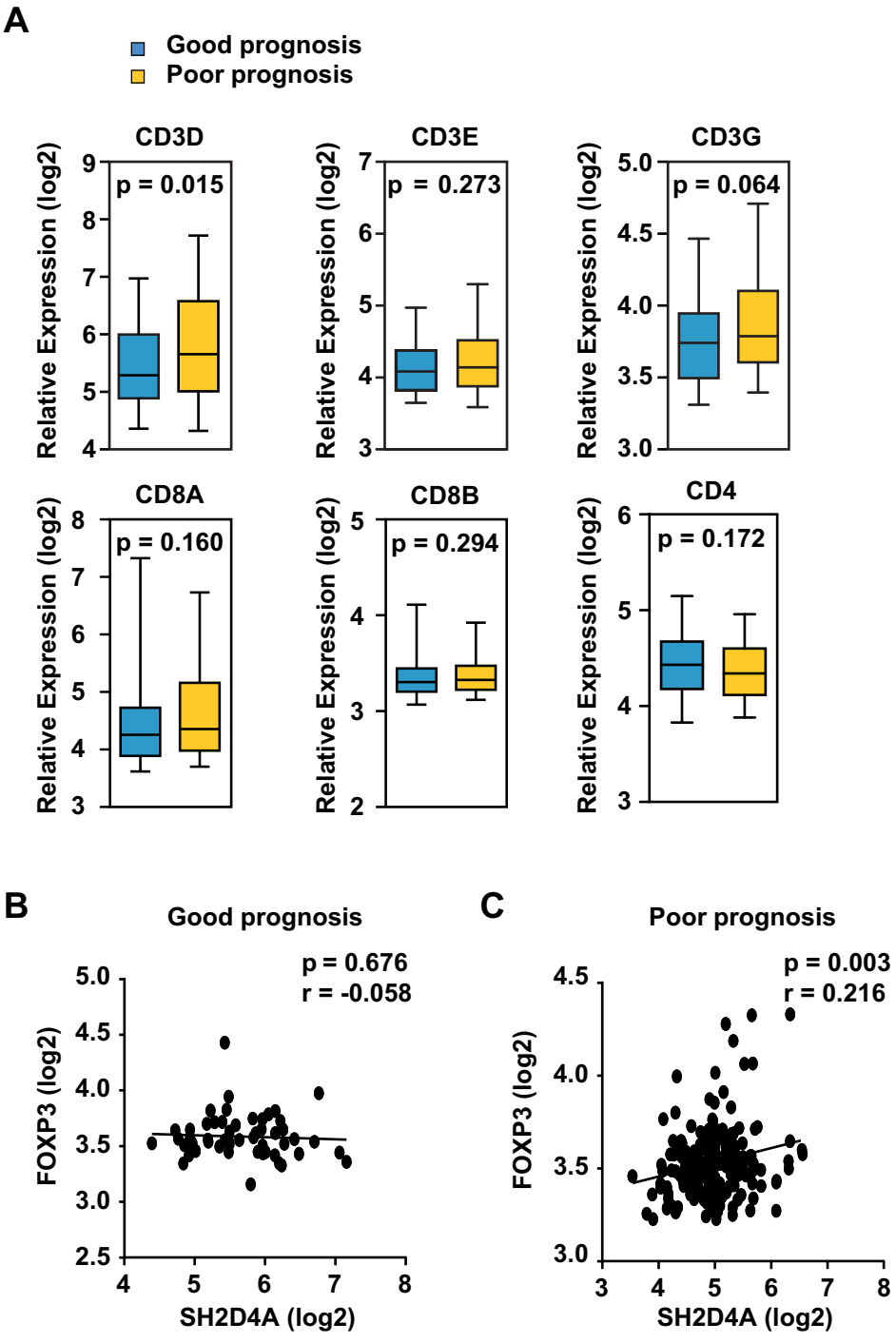
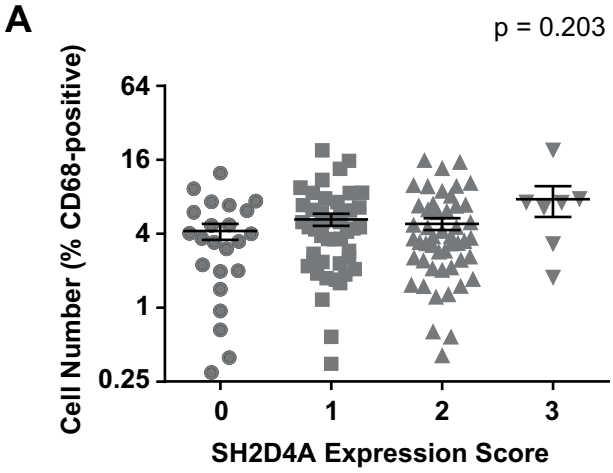
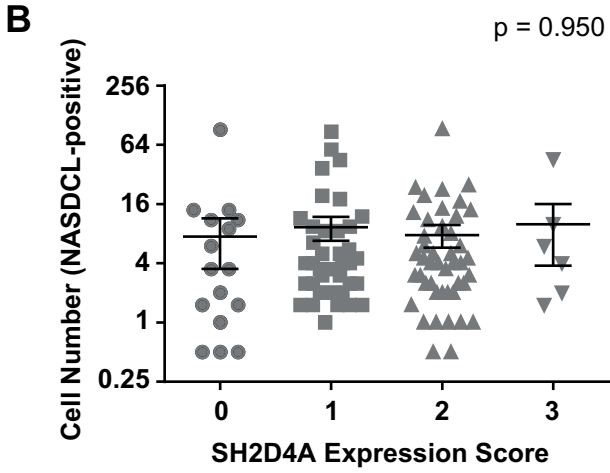

A
Good prognosis
Poor prognosis
CD3D
p = 0.015
CD3E
p = 0.273
CD3G
p = 0.064
CD8A
p = 0.160
CD8B
p = 0.294
CD4
p = 0.172
Relative Expression (log2)
B
Good prognosis
p = 0.676
r = -0.058
FOXP3 (log2)
SH2D4A (log2)
C
Poor prognosis
p = 0.003
r = 0.216
FOXP3 (log2)
SH2D4A (log2)

Figure S8


A
p = 0.203
Cell Number (% CD68-positive)
64
16
4
1
0.25
0
1
2
3
SH2D4A Expression Score


B
p = 0.950
Cell Number (NASDCL-positive)
256
64
16
4
1
0.25
0
1
2
3
SH2D4A Expression Score

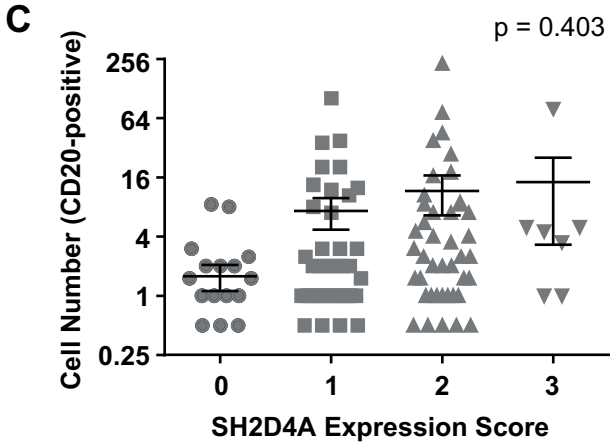
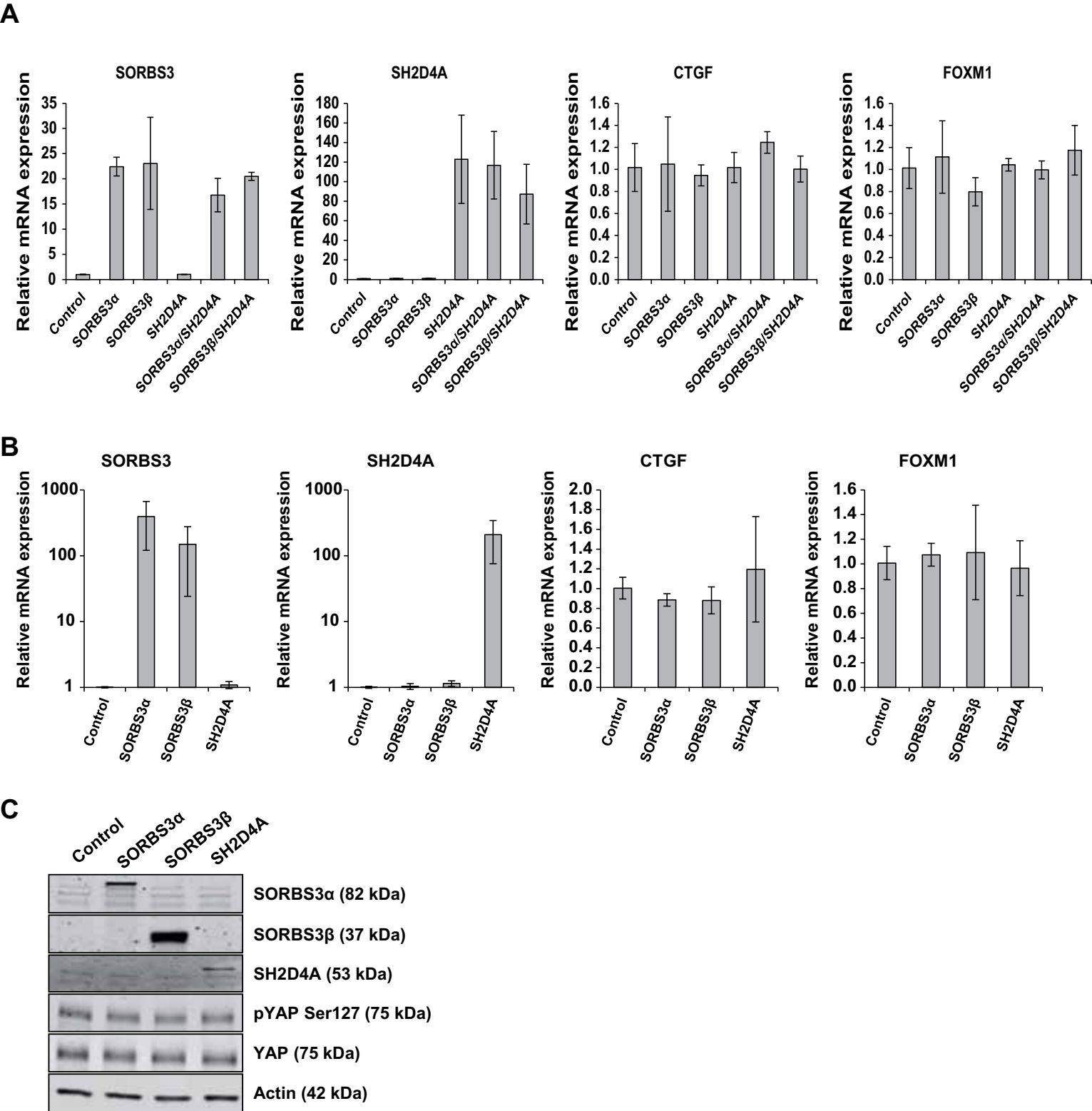

C
p = 0.403
Cell Number (CD20-positive)
256
64
16
4
1
0.25
0
1
2
3
SH2D4A Expression Score

Figure S9


A
SORBS3
SH2D4A
CTGF
FOXM1
Relative mRNA expression
Control
SORBS3α
SORBS3β
SH2D4A
SORBS3α/SH2D4A
SORBS3β/SH2D4A
B
SORBS3
SH2D4A
CTGF
FOXM1
Relative mRNA expression
Control
SORBS3α
SORBS3β
SH2D4A
C
Control
SORBS3α
SORBS3β
SH2D4A
SORBS3α (82 kDa)
SORBS3β (37 kDa)
SH2D4A (53 kDa)
pYAP Ser127 (75 kDa)
YAP (75 kDa)
Actin (42 kDa)