Supplemental Information

Supplemental Figures

Supplemental Figure 1

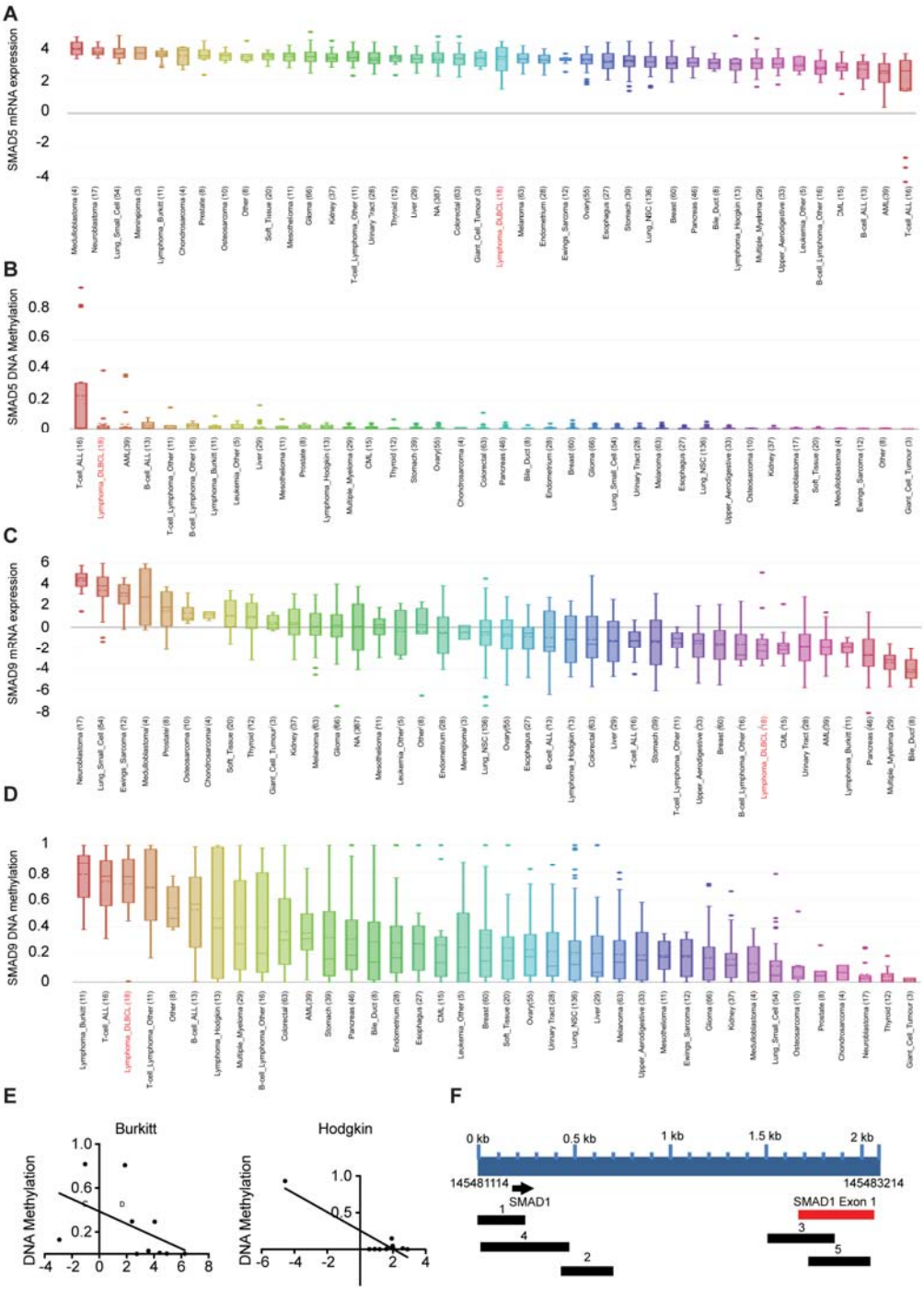

Figure S1. SMAD1 is silenced by DNA methylation of its promoter region in the majority of DLBCL cell lines. (A-D) Analysis of cell lines of different origins that are publicly available through the CCLE database with respect to SMAD5 (A-B) and SMAD9 (C-D) gene expression (A,C) and gene methylation (B,D). DLBCL cell lines are shown in red. (E) Correlation between SMAD1 expression and gene methylation in Burkitt and Hodgkin's lymphoma cell lines in the CCLE database. (F) Graphic representation of the *SMAD1* promoter region with bisulfite sequencing primers indicated in black and Exon 1 of SMAD1 in red.

Supplemental Figure 2

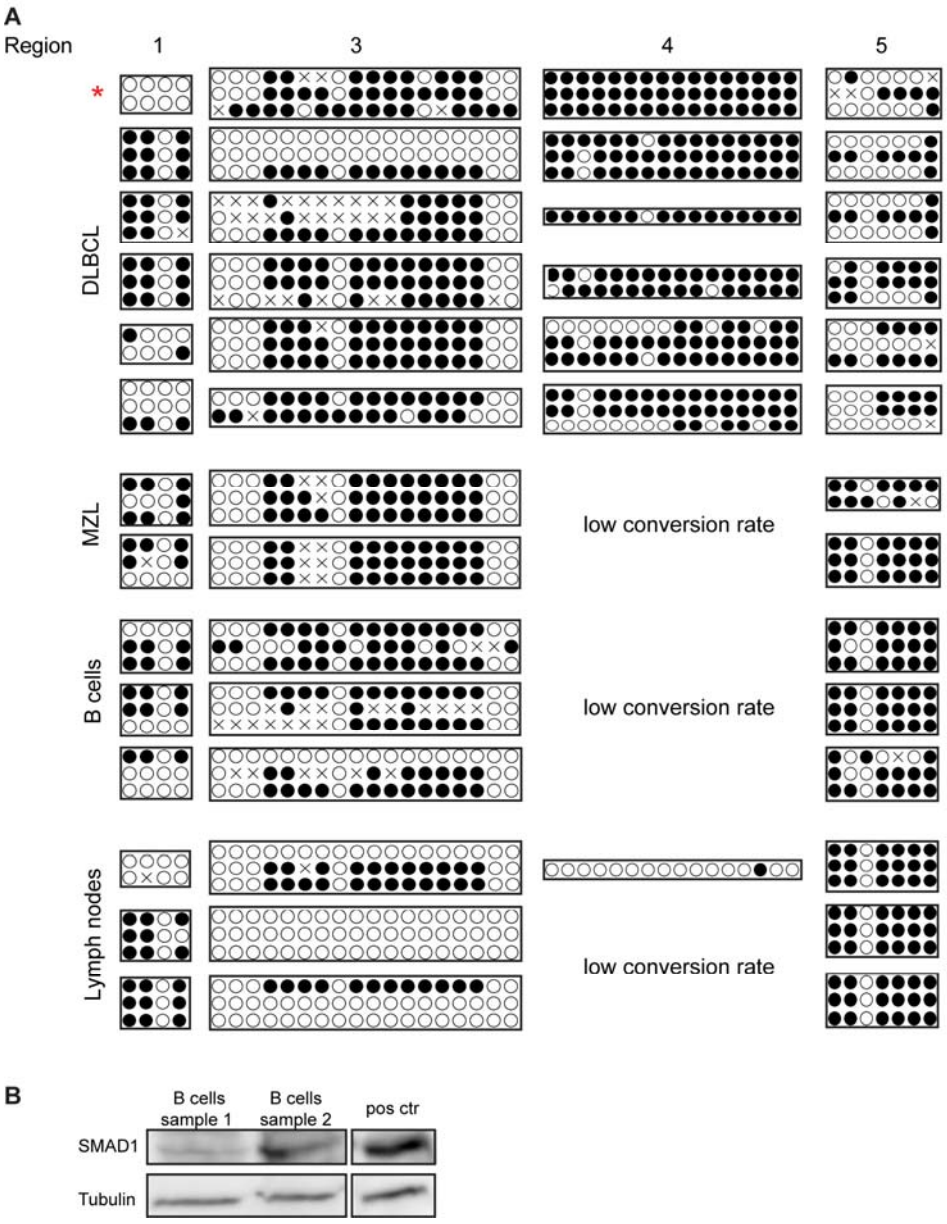

Figure S2. SMAD1 is silenced by DNA methylation of its promoter region in primary DLBCL samples. (A) Methylation analysis by bisulfite sequencing of regions 1,3,4 and 5 within the *SMAD1* promoter in DLBCL, MZL, normal B-cell and lymph node samples. Each circle represents one CG dinucleotide; black circles indicate methylated, white circles indicate unmethylated cytosines. Each line represents one clone. 2-3 clones were sequenced per sample. X indicates aligned mismatches between genomic and bisulfite sequences. Region 4 was particularly difficult to bisulfite convert and the data were not interpretable for some cases. The red asterisk marks the patient sample that was used for xenotransplantation. (B) SMAD1 expression as assessed by Western blotting of two B-cell samples that were immunomagnetically enriched from buffy coats, relative to SU-DHL-6 which served as positive control. Tubulin served as loading control.

Supplemental Figure 3

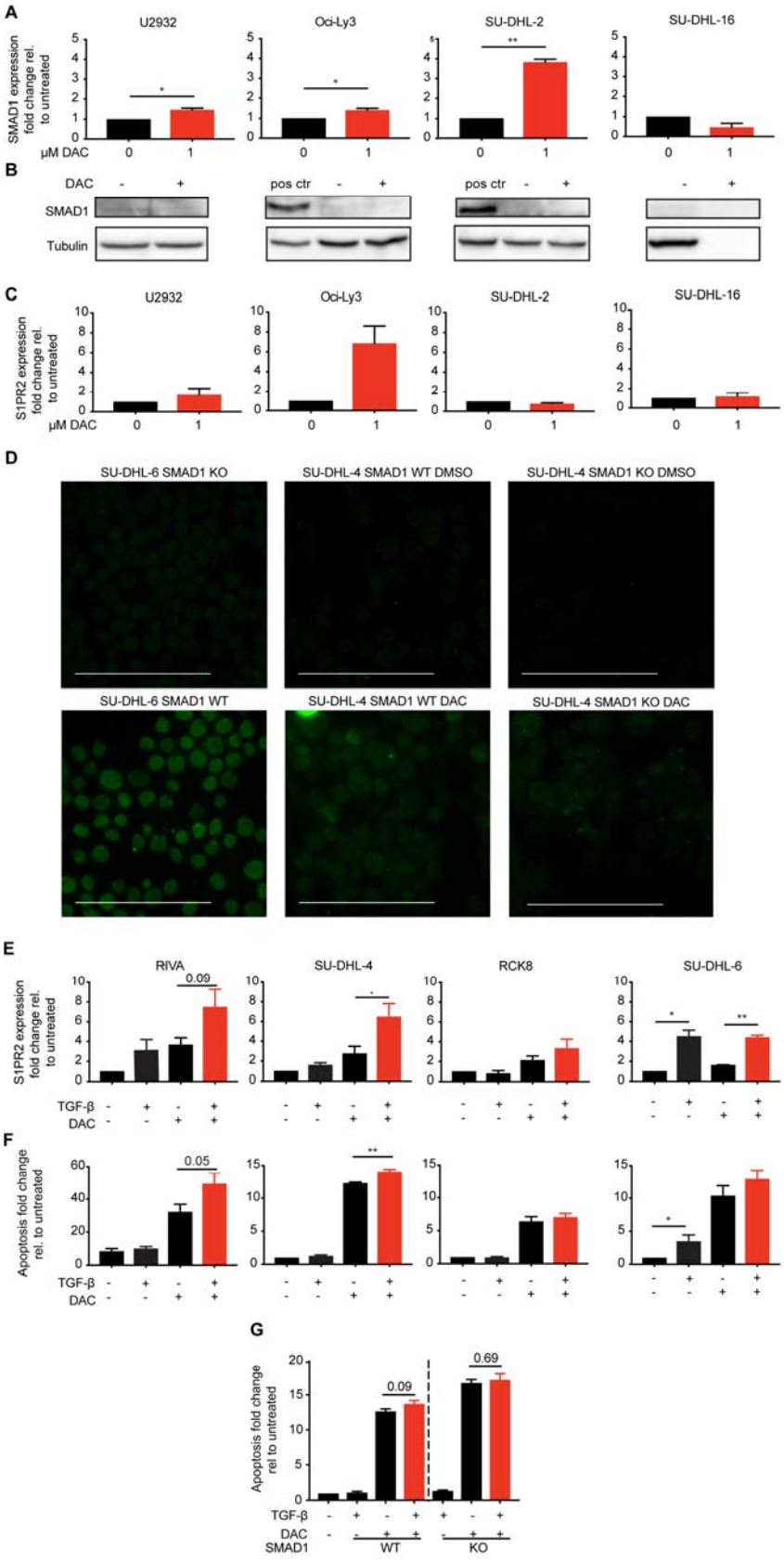

Figure S3. S1PR2 expression and susceptibility to TGF-β-induced apoptosis in DLBCL cell lines. (A,B) SMAD1 expression at the transcript (A) and protein (B) level after 1μM DAC treatment for 96 hours, of four

DLBCL cell lines as determined by qRT-PCR and Western blotting. Results from three independent experiments are pooled in A. (C) S1PR2 expression as determined by qRT-PCR after 1μM DAC treatment for 96 hours, of the cell lines shown in A. Pooled results from three independent experiments are shown. (D) SMAD1 expression as determined by immunofluorescence microscopy, of $SMAD1^{WT}$ and $SMAD1^{KO}$ SU-DHL-6 cells, and of $SMAD1^{WT}$ and $SMAD1^{KO}$ SU-DHL-4 cells after 96h 1μM DAC or vehicle treatment. SU-DHL-6 WT cells and ko derivatives are shown as positive and negative control for the SMAD1 antibody. Scale bar, 100 μm. (E) S1PR2 expression as determined by qRT-PCR of the indicated cell lines, after 96h 1μM DAC and 24h 2ng/ml TGFβ treatment. (F) Apoptosis as determined by AnnexinV staining of the cell lines shown in D, after 96h 1μM DAC and 24h 2ng/ml TGFβ treatment. (G) Apoptosis as determined by AnnexinV staining of $SMAD1^{WT}$ and $SMAD1^{KO}$ SU-DHL-4 cells after 96h 1μM DAC and 24h 2ng/ml TGFβ treatment. Pooled results from three independent experiments are shown. Graphs represent mean +SEM throughout. p-values were calculated using the Student t-test. *$p<0.05$; **$p<0.01$.

Supplemental Figure 4

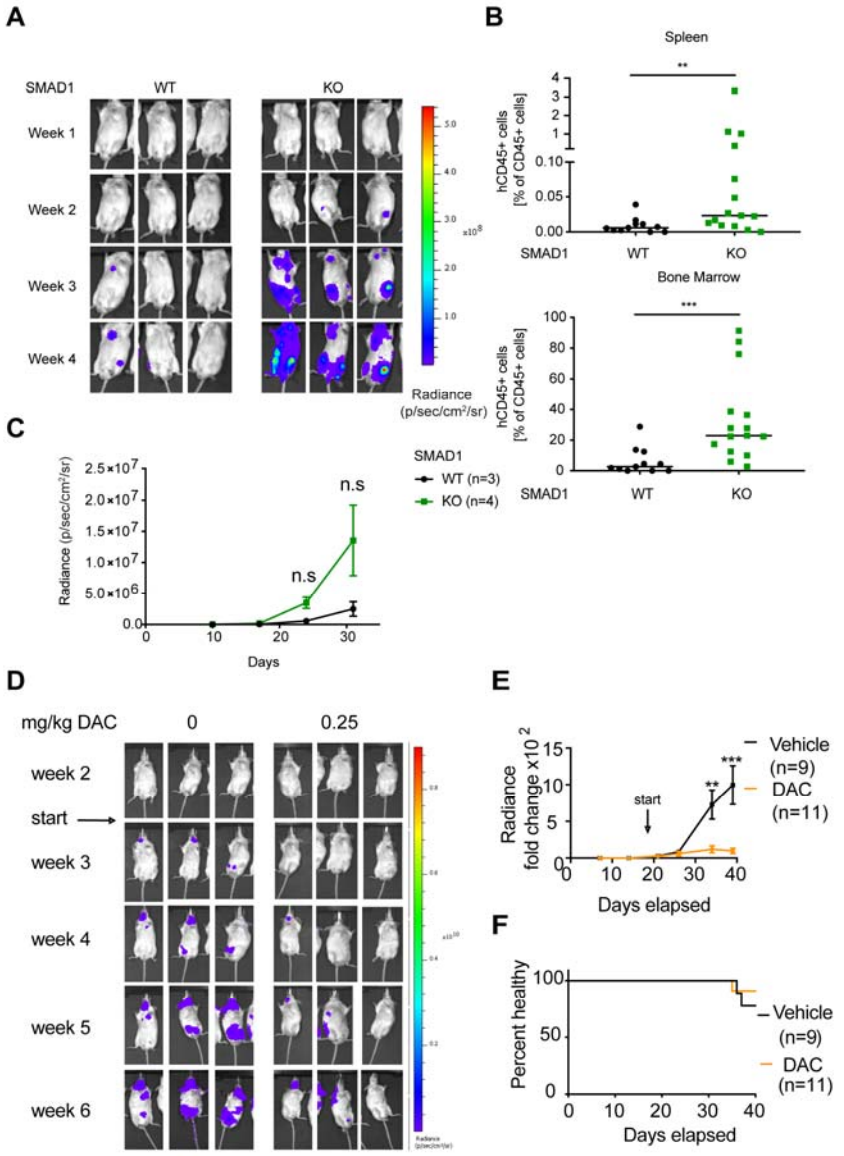

Figure S4. Genetic SMAD1 ablation renders cells more aggressive in an orthotopic DLBCL model using MISTRG mice. (A-C) SU-DHL-6 cells were subjected to CRISPR/Cas9-mediated knockout of *SMAD1* and lentiviral gene transfer of luciferase. Two SMAD1^{KO} clones and three SMAD1^{WT} clones were injected i.v. into MISTRG mice. Representative IVIS images (A) and radiance (C) of SMAD1^{KO} versus SMAD1^{WT} cells as assessed during a four-week time course post injection. Endpoint values for hCD45^{+} cells as a percentage of all CD45^{+} cells in spleen and bone marrow are shown in B. Results in B are pooled from three independent experiments and one dot represents one mouse with horizontal lines representing median values. p-values were calculated using the Mann-Whitney test. The data in C are from one representative experiment. (D-F) SU-DHL-4 cells were subjected to lentiviral gene transfer of luciferase and i.v. injected into MISTRG mice. Mice were treated with 0 or 0.25 mg/kg decitabine starting from two weeks after injection. IVIS images of mice (D) and radiance (E) over 4 weeks. (F) Survival curve showing the percentage of injected mice without symptoms of disease (paralysis, weight loss). Data in E and F are pooled from two independent studies. **p<0.01, ***p<0.001

Supplemental Material and Methods

Cell culture

Cell lines were maintained at 37°C, 5% CO_2 in a humidified atmosphere in IMDM (OCI-Ly3 and RIVA) or RPMI (U2932, SU-DHL-2, SU-DHL-4, SU-DHL-10, SU-DHL-6, SU-DHL-5, SU-DHL-8,SU-DHL-16 and RC-K8) supplemented with 10% (OCI-Ly3,SU-DHL-2, SU-DHL-5 and RIVA) or 20% (U2932, SU-DHL-4, SU-DHL-6 ,SU-DHL-8 SU-DHL-10, SU-DHL-16 and RC-K8) heat-inactivated FBS and antibiotics. Cell numbers were counted using commercial counting chambers and only live cells were counted by Trypan blue (Sigma Aldrich, Cat No T8154) staining.

In vitro decitabine and TGF-β treatment

$0.5x10^6$ cells cultured in appropriate cell culture medium were subjected for 96 hours to decitabine treatment (5-aza-2'-deoxycytidine, Sigma Aldrich) diluted in DMSO. TGFβ-1 (PreproTech, Cat No 100-21-100UG) reconstituted according to manufacturer`s instructions was added for an additional 24 hours.

Annexin V staining

Cells were stained with PE-Cy7-labelled Annexin V (BioLegend, Cat No 559934) in 1x AnnexinV Binding buffer (BD Bioscience, Cat No 556454) and data was acquired on a CyAn ADP (Beckman Coulter) flow cytometer and analyzed with the FlowJo software (TreeStar).

RNA extraction and qRT-PCR

Cells were harvested or tumors homogenized using a FastPrep24 homogenizer (MP) and RNA was extracted according to the manufacturer`s protocol using the Nucleospin RNA kit (Macherey-Nagel, Cat No. 740955.250). RNA concentration was measured using NanoDrop (ThermoScientific) and a total of 1 µg RNA was transcribed using SuperScriptIII reverse transcriptase (Invitrogen, Cat No 18080044). For qRT-PCR, Lightcycler 480 Cyber Green Master I (Roche, Cat. No 04887352001) was used followed by analysis on a Lightcycler 480 instrument (Roche). Samples were measured in technical duplicates. For all primer pairs, the efficiency was calculated by performing dilution series experiments. Target mRNA abundance was subsequently calculated relative to human RPLP32 or RPLP0. The following primer sequences were used: RPLP32 fw: GAAGTTCCTGGTCCACAACG, rev:

GCGATCTCGGCACAGTAAG; RPLP0 fw: CCAGCTCTGGAGAAACTGCTG, rev: CAGCAGCTGGCACCTTATTGG; S1PR2 fw: CCACTCGGCAATGTACCTGT, rev: ACGCCTGCCAGTAGATCG; SMAD1 fw: ACCTGCTTACCTGCCTCCTG, rev: CATAAGCAACCGCCTGAACA.

Western blotting

Protein extracts were made in 50 µl RIPA buffer (Sigma-Aldrich, Cat No R0278-50ML) supplemented with 1x complete protease inhibitor cocktail (Roche). Protein concentrations were determined using Bradford assay (Bio-Rad, Cat No 5000002) and equal amounts were separated by SDS/PAGE (10% gel) followed by transfer onto nitrocellulose membranes (GE Healthcare Life Sciences, Cat No 10600023). Membranes were probed with antibodies against α-tubulin (DM1A, Sigma Aldrich, Cat No T9026-100UL) and SMAD1 (Cell signaling, Cat No 9743).

CRISPR/Cas 9 genomic editing

SU-DHL-6 cells were genome-edited by transfection with a plasmid encoding Cas9 and SMAD1-specific guide RNA (SMAD1 gRNA: TTAGCTCAGTTCCGTAACTT). The PX458 plasmid used for this purpose was obtained from Addgene (#48138: pSpCas9 (BB)-2A-GFP). Nucleoporation was performed using the Amaxa Nucleofector II device. Specifically, 1-3x10^6 SU-DHL-6 cells were nucleoporated with 0.5-3µg DNA. the Cells were single cell-sorted into 96 well plates based on their GFP expression 48h post transfection. Clones were subjected to Western blot analysis using a specific SMAD1 antibody (Cell signaling, Cat No 9743) to verify the SMAD1 knockout on protein level.

SU-DHL-4 cells were edited via lentiviral transfer. Lentiviral packaging was performed in HEK 293T cells (cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics) by polyethylenimine transfection (PEI, Polysciences, MW 25000). 4 µg of viral plasmid containing SMAD1 or Scr guide sequences (SMAD1: TTAGCTCAGTTCCGTAACTT, Scr control: GCTGATCTATCGCGGTCGTC),2 µg of helper plasmid psPAX2 (Addgene plasmid #12260) and 1 µg of envelope plasmid pCMV-VSV-G (Addgene plasmid #8454) were used for transfection. Cells were infected with lentiviral particles by spinoculation at 32°C, 800 g for 60 minutes at presence of 5 µg mL^{-1} polybrene (Hexadimethrine bromide, Sigma-Aldrich). Transduced cells were bulk-selected using Neomycin and subjected to decitabine treatment, followed by Western blotting for confirmation of the knock out.

Bisulfite sequencing

Genomic DNA was isolated using the NucleoSpin® Tissue kit (Macherey-Nagel). DNA was bisulfite converted with the EpiTect® 48 Bisulfite Kit (Qiagen) or EZ DNA Methylation-Lightning™ Kit (Zymo). Five different regions within the SMAD1 promoter region were chosen based on a previous publication[1] and amplified using the HotStarTaq® Plus DNA Polymerase kit (Qiagen). Amplicon regions and primer sequences are shown in table 1. Assembly GRCh38.p12 chr.4 (GCF_000001405.38) (NCBI) and transcript Transcript ENST00000515385.1 (Ensemble ID) were used. Amplified regions were extracted from an agarose gel using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel). Purified amplicons were transformed into competent cells using the pGEM®-T easy vector systems kit (Promega). Clones were picked and isolated after overnight incubation using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). Clones were sequenced using an in-house sequencing service. Obtained sequences were analyzed using the QUMA software.[2]

Table1. Gene locations and primer sequences of bisulfite sequenced regions.

No	Start location	End location	Forward primer	Reverse primer	size
1	145481114	145481348	GGAGTTGGTATTATTTAAAA GATTGTAG	CCAATAATTTACAAACCCCA AAAAT	234
2	145481539	145481824	TTAAAAAGGTAGGGTTAGGA AAGTTTG	TACCCAACCTCCACTCCTCA ACTAC	285
3	145482636	145482970	GGTTTTAGTAAGTTTTTTTGG GGT	AAACCTTCATACAATTTCAA CAACTAAA	334
4	145481119	145481564	TGGTATTATTTAAAAGATTGT AGAAATTGG	AAACTTTCCTAACCCTACCT TTTTAATC	445
5	145482853	145483167	TTTGTTTTTTGTTTTTGTTAGT GTT	ACTTTCTCAAAAAATAATTC TAAAACC	314

Lentiviral ZsGreen gene transfer

Lentiviral packaging was performed in HEK 293T cells (cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics) by polyethylenimine transfection (PEI, Polysciences, MW 25000). 4 µg of viral plasmid pHIV-Luc-ZsGreen (Addgene plasmid #39196), 2 µg of helper plasmid psPAX2 (Addgene plasmid #12260) and 1 µg of envelope plasmid pCMV-VSV-G (Addgene plasmid #8454) were used for transfection. Cell lines were infected with lentiviral particles by spinoculation at 32°C, 800 g for 60 minutes at presence

of 5 µg mL^{-1} polybrene (Hexadimethrine bromide, Sigma-Aldrich). Transduced cells (high ZsGreen-expressing) were sorted using FACSAria III 6 days after transduction.

Flow cytometric analysis of $hCD45^+$ populations in humanized mice

Cells isolated from mouse spleens or bone marrow were treated with ACK red blood cell lysis buffer pH 7.2-7.4 (150mM NH4Cl, 10mM KHCO3, 0.1mM Na2EDTA) and pushed through a 40µM cell strainer to produce single-cell suspensions, which were stained using the following fluorescent-labeled antibodies: Fixable Viability Dye eFluor® 780, Pacific Blue conjugated hCD45 (HI30, ebioscience) and PE conjugated mCD45 (30-F11, Biolegend). Data was acquired on a CyAn ADP (Beckman Coulter) flow cytometer and analyzed with the FlowJo software package (TreeStar).

References

1. Clozel T, Yang S, Elstrom RL, et al. Mechanism-based epigenetic chemosensitization therapy of diffuse large B-cell lymphoma. *Cancer Discov*. 2013;3(9):1002-1019.
2. Kumaki Y, Oda M, Okano M. QUMA: quantification tool for methylation analysis. *Nucleic Acids Res*. 2008;36(Web Server issue):W170-175.