APPENDIX METHODS

Gene set enrichment analysis (GSEA)

To identify gene sets that are enriched among *SOX2*-co-regulated genes in primary EwS tumours (mRNA-cohort) for which microarray data were available, all genes were ranked by their Pearson correlation coefficient with *SOX2* expression, and a pre-ranked GSEA with 1,000 permutations was carried out (MSigDB, c2.all.v6.1) as previously described [1].

Cell lines and reagents

The human EwS cell line RDES as well as HEK293T cells were obtained from the German Collection of Microorganisms and Cell lines (DSMZ). The human EwS cell lines EW1 and POE were kindly provided by O. Delattre (Institute Curie Research Center, Paris, France). EwS cells were grown at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium with stable glutamine (Biochrom, Berlin, Germany) containing 10% tetracycline-free fetal calf serum (Sigma-Aldrich) and 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom). Cell line identity was verified by STR-profiling, and the absence of Mycoplasma was routinely checked by nested PCR.

RNA extraction, reverse transcription, and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel). 1 µg of total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR reactions were performed using SYBR green Mastermix (Applied Biosystems) mixed with diluted cDNA (1:10) and 0·5 µM forward and reverse primer on a Bio-Rad CFX Connect instrument and analysed using Bio Rad CFX Manager 3.1 software. Gene expression values were calculated using the ΔΔCt method relative to the housekeeping gene

RPLP0 as internal control. Oligonucleotides were purchased from MWG Eurofins Genomics (Ebersberg, Germany):

RPLP0 forward 5'-GAAACTCTGCATTCTCGCTTC-3';

RPLP0 reverse 5'-GGTGTAATCCGTCTCCACAG-3';

SOX2 forward 5′-CACACTGCCCCTCTCAC-3′;

SOX2 reverse 5′-TCCATGCTGTTTCTTACTCTCC-3′.

Generation of cells with doxycycline (dox)-inducible knockdown of SOX2

POE cells with dox-inducible knockdown of SOX2 were generated by employing the pLKO-TET-ON all-in-one vector system (Plasmid #21915, Addgene) as previously described [2]. The sequence of the shRNA targeting *SOX2* was 5'-CTGCCGAGAATCCATGTATAT-3' (TRCN0000003253). The sequence of the non-targeting control shRNA (shCTR) was 5'-CAACAAGATGAAGAGCACCAA-3' (Sigma MISSION SHC002). Vectors were amplified in Stellar Competent Cells (Clontech) and integrated shRNAs were verified by Sanger sequencing using following forward sequencing primer:

5'-GGCAGGGATATTCACCATTATCGTTTCAGA-3'.

Lentiviral particles were generated in HEK293T cells. Virus-containing supernatant was collected to infect POE cells (thereafter termed POE/TR/shSOX2 or POE/TR/shCTR, respectively). Successfully infected cells were selected with 1·5 µg/ml puromycin (InVivoGen). The shRNA expression for *SOX2* knockdown in POE/TR/shSOX2 cells was achieved by adding 1 µg/ml dox (Sigma-Aldrich) to the medium.

Proliferation analysis

POE/TR/shSOX2 and POE/TR/shCTR were seeded in triplicate wells in a 96-well plate at density of 2×10^3 cells/well. After 96 h of incubation at 37°C and 5% CO_2 with or without dox,

proliferation was assessed with a Resazurin assay (Sigma-Aldrich). The fluorescent signal was measured with a Varioscan reader (Thermo Fisher).

Clonogenic growth and anchorage-independent growth analysis

POE/TR/shSOX2 and POE/TR/shCTR were seeded in triplicate wells at density of 200 cells per well in a 12-well plate in presence and absence of dox. Every 48 h, dox was refreshed. After 10–14 d of incubation, colonies were stained with crystal violet and counted. For analysis of anchorage-independent growth, cells were plated in triplicate wells at a density of 500 cells per well in a 96-well ultra-low attachment plate (Corning). After 10–15 d, visible spheres were with an Axiocam 105 Color camera (Carl Zeiss), and sphere numbers and diameters measured by ImageJ software. The sphere index was calculated by multiplying the average number of spheres by their average diameter.

Xenograft mouse model

2×10^6 POE/TR/shSOX2 or POE/TR/shCTR cells were injected subcutaneously in the flanks of NOD/Scid/gamma (NSG) mice. Dox (Beladox, Bela-pharm) dissolved in drinking water containing 5% sucrose (Sigma-Aldrich) was given *ad libitum* to mice once first tumours were palpable. Dox-treatment was maintained until mice were sacrificed by cervical dislocation 77 days after injection of the tumour cells or before if tumours exceeded a mean diameter of 18 mm. Xenografts were fixed in 4%-formalin and embedded in paraffin for IHC analysis of SOX2. In parallel, a fraction of each xenograft was used to extract RNA for validation of *SOX2* knockdown by qRT-PCR. Animal experiments were approved by local authorities and conducted in accordance with the recommendations of the European Community (86/609/EEC) and UKCCCR (guidelines for the welfare and use of animals in cancer research).

Transient RNA interference (RNAi)

For RNAi experiments, 3×10^5 EW1, RDES, and POE cells were transiently transfected in wells of 6-well plates with Lipofectamine™ RNAiMAX according to the manufacturer's protocol (Invitrogen). Cells were reversely transfected with either a non-targeting sipool or two different sipools specifically directed against *SOX2* (all from siTOOLs, Biotech) at a final concentration of 5 nM. All sipools consisted of 30 different short interfering RNAs (siRNAs), which eliminates off-target effects [3]. Knockdown efficacy was validated by qRT-PCR.

RNA sequencing (RNA-Seq)

To identify differentially expressed genes upon *SOX2* knockdown, RNA-Seq was performed 72 h after transient transfection of EW1, POE, and RDES with siPOOLs. RNA was extracted using the RNA isolation kit from Macherey-Nagel, which includes an on-column 15 min DNase treatment. RNA quality was controlled by a Bioanalyzer (BioRad). RNA-Seq libraries were prepared from 100-200 ng total RNA using the Illumina TruSeq RNA Sample Kit (Illumina, San Diego, CA, USA). RNA-Seq was carried out on an Illumina HiSeq2500 instrument and it was performed by using single-end approach (SE50) with 50 bp read-length and coverage of 50 million reads per sample. RNA-Seq data were deposited at Gene Expression Omnibus (GEO; accession code: GSE124221).

APPENDIX REFERENCES

[1] Orth MF, Gerke JS, Knösel T, Altendorf-Hofmann A, Musa J, Alba-Rubio R, et al. Functional genomics identifies AMPD2 as a new prognostic marker for undifferentiated pleomorphic sarcoma. Int J Cancer 2018;144:859–67. doi:10.1002/ijc.31903.
[2] Wiederschain D, Wee S, Chen L, Loo A, Yang G, Huang A, et al. Single-vector inducible lentiviral RNAi system for oncology target validation. Cell Cycle Georget Tex 2009;8:498–504. doi:10.4161/cc.8.3.7701.
[3] Hannus M, Beitzinger M, Engelmann JC, Weickert M-T, Spang R, Hannus S, et al. siPools: highly complex but accurately defined siRNA pools eliminate off-target effects. Nucleic Acids Res 2014;42:8049–61. doi:10.1093/nar/gku480.

APPENDIX FIGURES

Appendix Figure 1 Sannino *et al.*

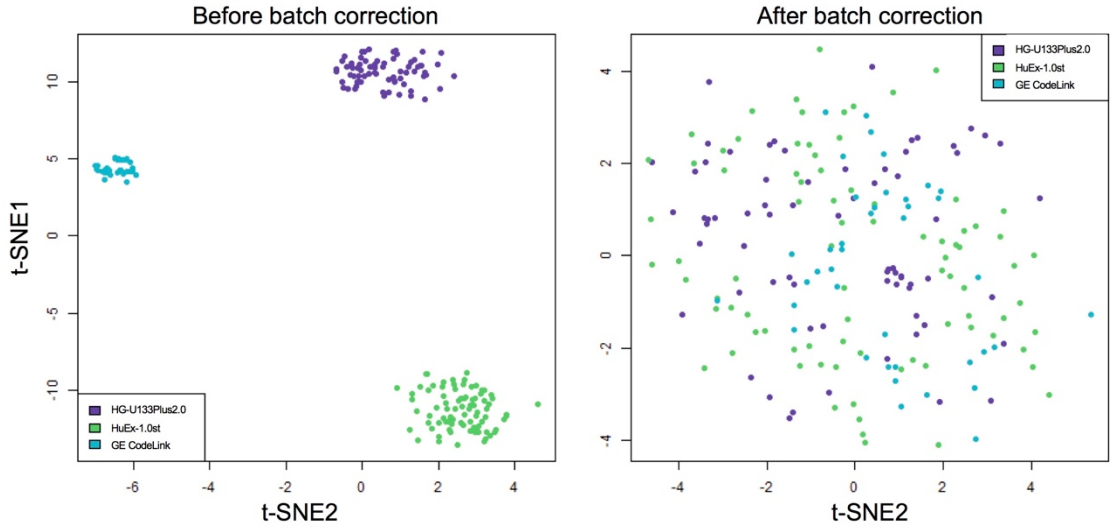

Figure S1: t-SNE analysis of gene expression data before and after batch correction.
t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis on the normalised transcriptome data of the three different microarray platforms that have been used for assembly of the mRNA-cohort (see methods section). t-SNE plots before and after batch correction are depicted.

Appendix Figure 2 Sannino *et al.*

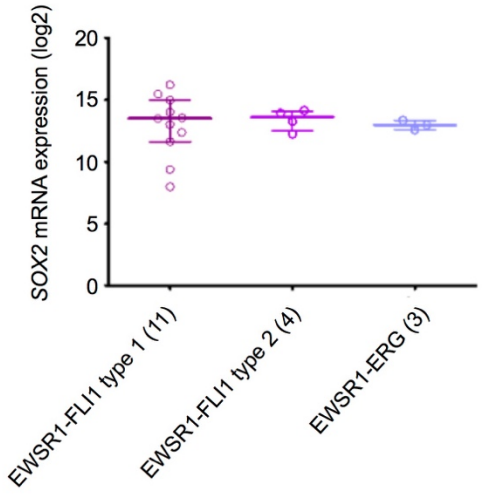

Figure S2: *SOX2* mRNA expression in EwS cell lines with different *EWSR1-ETS* fusions
SOX2 expression was determined in 18 EwS cell lines in triplicates by using Affymetrix Clariom D microarrays. Microarray data were simultaneously normalised using Transcriptome Analysis Console 4.0 software (Thermofisher). The mean *SOX2* expression was calculated for each cell line and displayed in a scatter plot. Horizontal bars represent the median and whiskers the interquartile range. The number of analysed samples per group is given in parentheses.

Appendix Figure 3 Sannino *et al.*

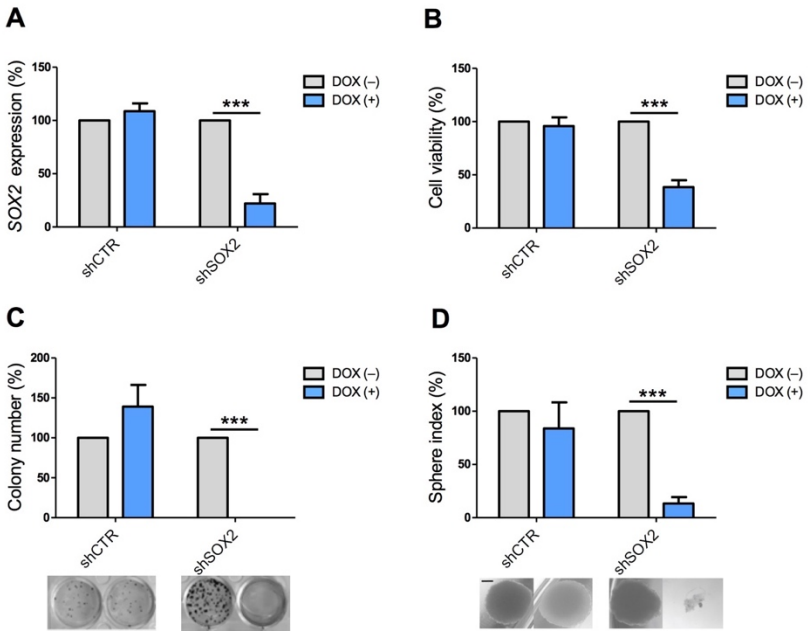

Figure S3: SOX2 knockdown inhibits proliferation, clonogenic growth and anchorage-independent growth of EwS cells

(A) qRT-PCR analysis of SOX2 expression POE/TR/shCTR and POE/TR/shSOX2 cells. *SOX2* expression is normalised to that of *RPLP0*. Data are mean and S.E.M., n=3. Unpaired two-tailed student's T-test.

(B) Analysis of proliferation of POE/TR/shCTR and POE/TR/shSOX2 cells using Resazurin assay. Data are mean and S.E.M., n=3. Unpaired two-tailed student's T-test.

(C) Analysis of 2D clonogenic growth of POE/TR/shCTR and POE/TR/shSOX2. Data are mean and S.E.M., n=6. Unpaired two-tailed student's T-test.

(D) Analysis of 3D anchorage-independent growth of POE/TR/shCTR and POE/TR/shSOX2. Data are mean and S.E.M., n=4. Scale bar = 500 μm. Unpaired two-tailed student's T-test. *** $p<0{\cdot}001$.

Appendix Figure 4 Sannino *et al.*

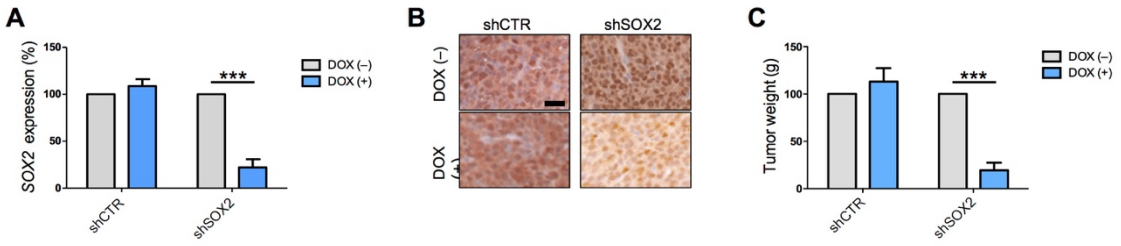

Figure S4: SOX2 knockdown reduces tumour growth *in vivo*
(A) qRT-PCR analysis of SOX2 expression POE/TR/shCTR or POE/TR/shSOX2 xenografts of NSG mice treated with either dox (DOX +) or vehicle (DOX –). Data are mean and S.E.M., n=4 per group. Unpaired two-tailed student's T-test.
(B) Representative images for SOX2 stain in POE/TR/shCTR and POE/TR/shSOX2 xenografts treated with either dox (DOX +) or vehicle (DOX –). Scale bar = 100 μm.
(C) Tumour weight of POE/TR/shCTR and POE/TR/shSOX2 xenografts of NSG mice treated with either dox (DOX +) or vehicle (DOX –). Data are mean and S.E.M., n=4 per group. Unpaired two-tailed student's T-test. *** $p<0{\cdot}001$.

Appendix Figure 5 Sannino *et al.*

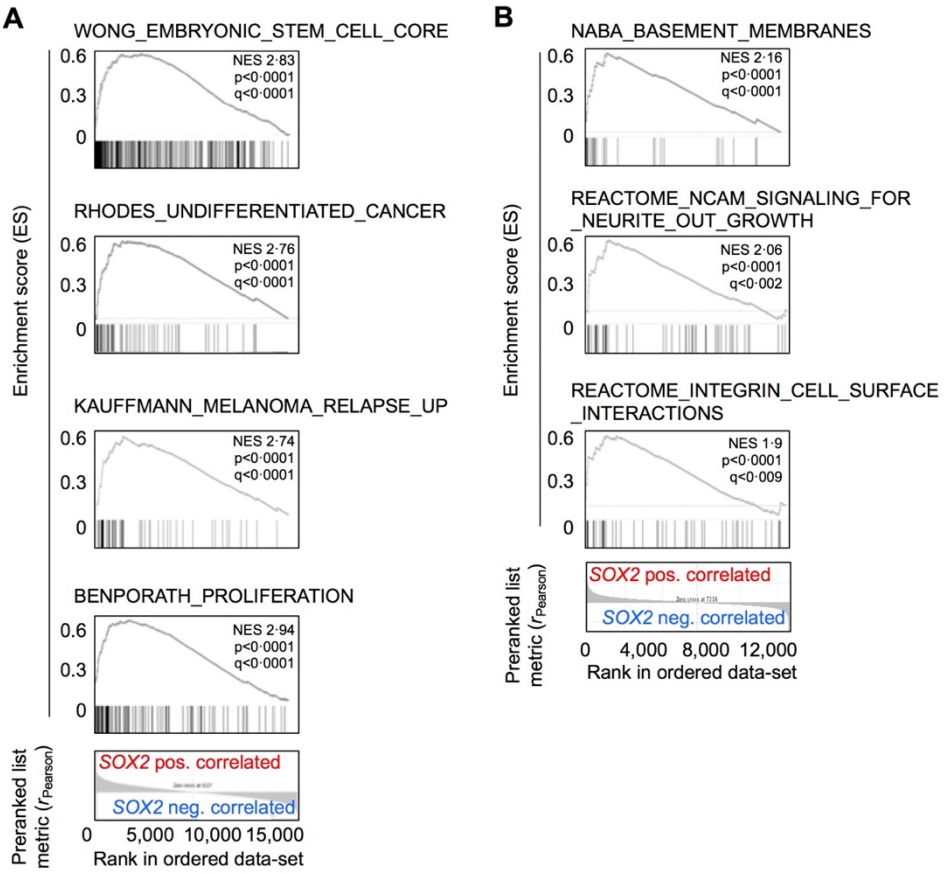

Figure S5: GSEA analysis in primary EwS tumours and EwS cell lines
(A) GSEA results obtained from analysis of *SOX2* co-expressed genes in primary EwS.
(B) GSEA results obtained from analysis of up-regulated genes upon *SOX2* knockdown in three EwS cell lines (EW1, POE, and RDES). NES = normalised enrichment score.