Susceptibility of pediatric acute lymphoblastic leukemia to STAT3 inhibition depends on p53 induction

Luca Gasparoli,[1] Clemence Virely,[1] Alexia Tsakaneli,[1] Noelia Che,[1] Darren Edwards,[2] Jack Bartram,[2] Michael Hubank,[3] Deepali Pal,[4] Olaf Heidenreich,[5] Joost H.A. Martens,[6] Jasper de Boer[1] and Owen Williams[1]

[1]Cancer Section, Developmental Biology and Cancer Department, UCL Great Ormond Street Institute of Child Health, London, UK; [2]Department of Pediatric Hematology, Great Ormond Street Hospital for Children, London, UK; [3]Center for Molecular Pathology, The Royal Marsden, Sutton, UK; [4]Department of Applied Sciences, Northumbria University, Newcastle upon Tyne, UK; [5]Princess Maxima Center for Pediatric Oncology, Utrecht, the Netherlands and [6]Department of Molecular Biology, Faculty of Science, Radboud Institute for Molecular Life Sciences, Radboud University, Nijmegen, the Netherlands

Correspondence: O. Williams
owen.williams@ucl.ac.uk

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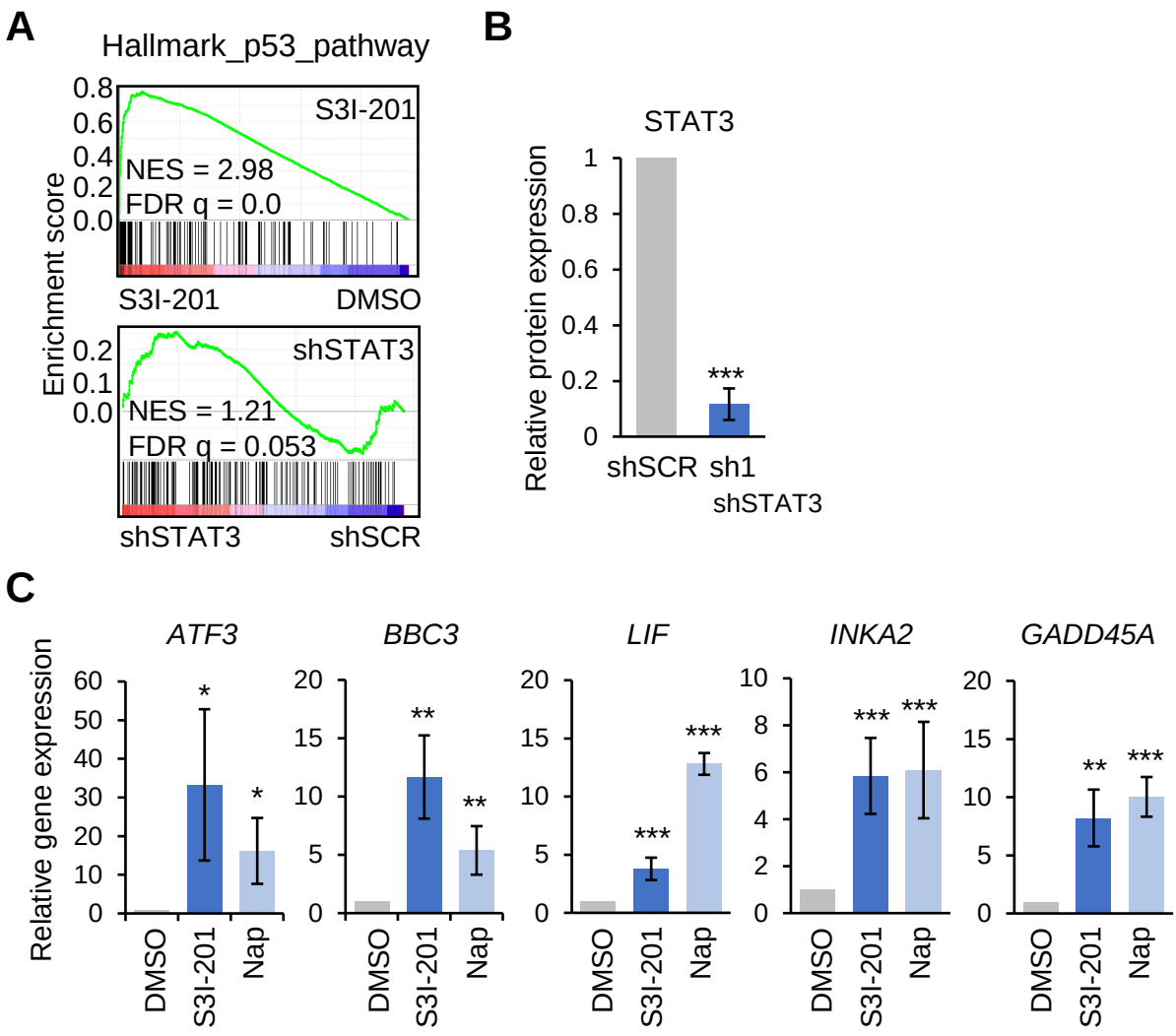
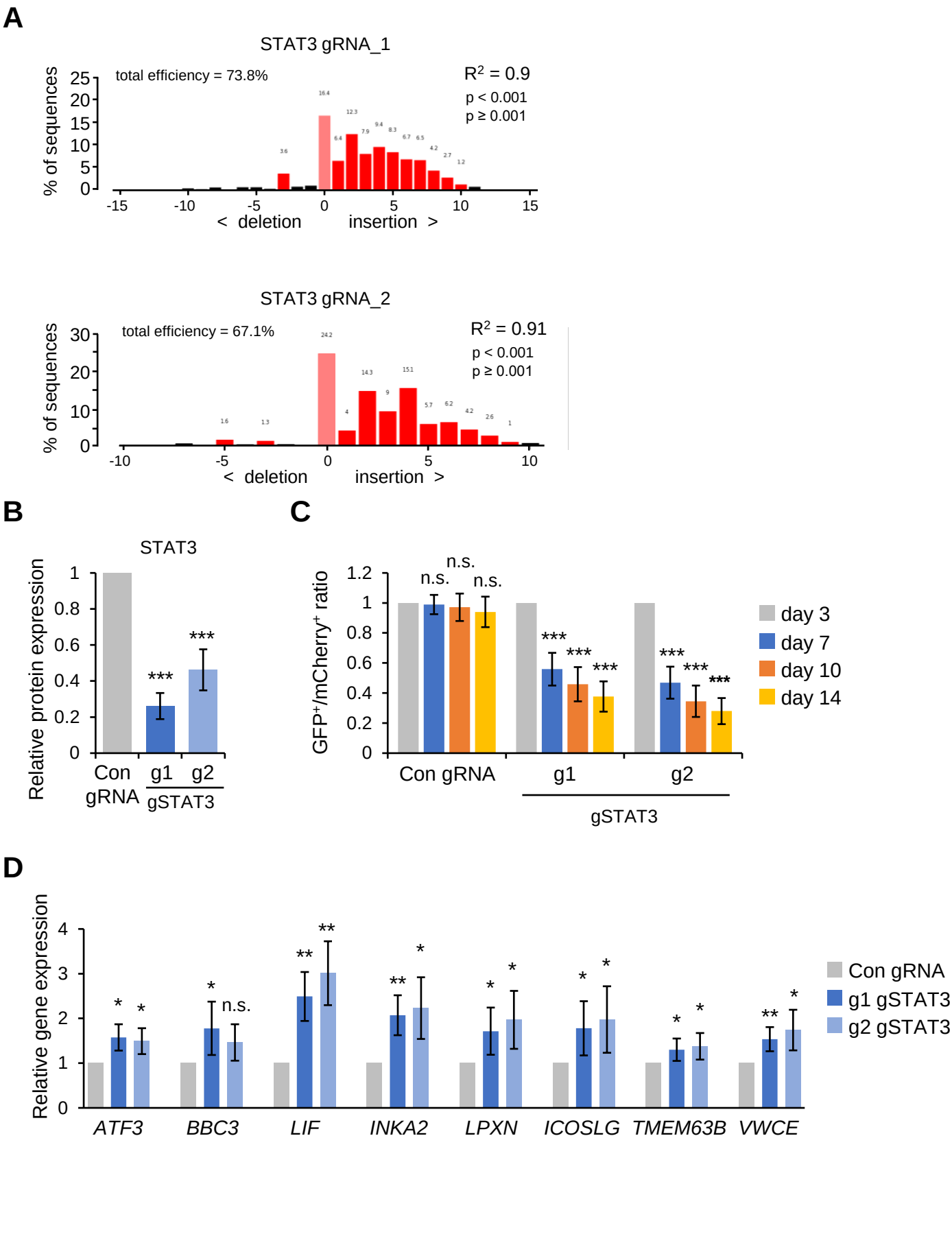


Supplementary Figure S1



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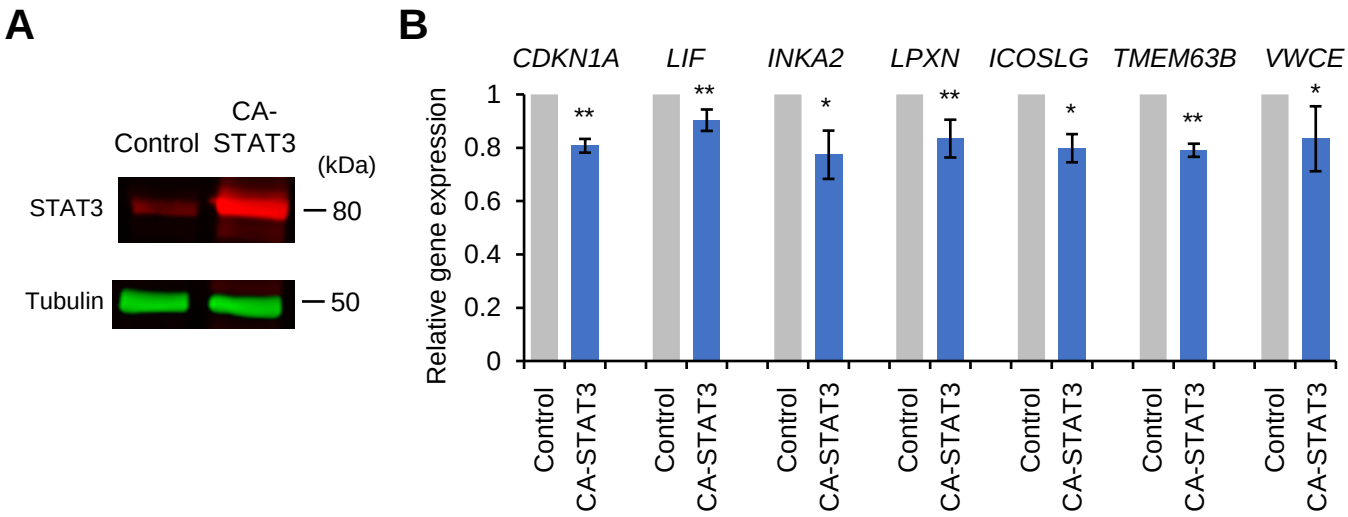

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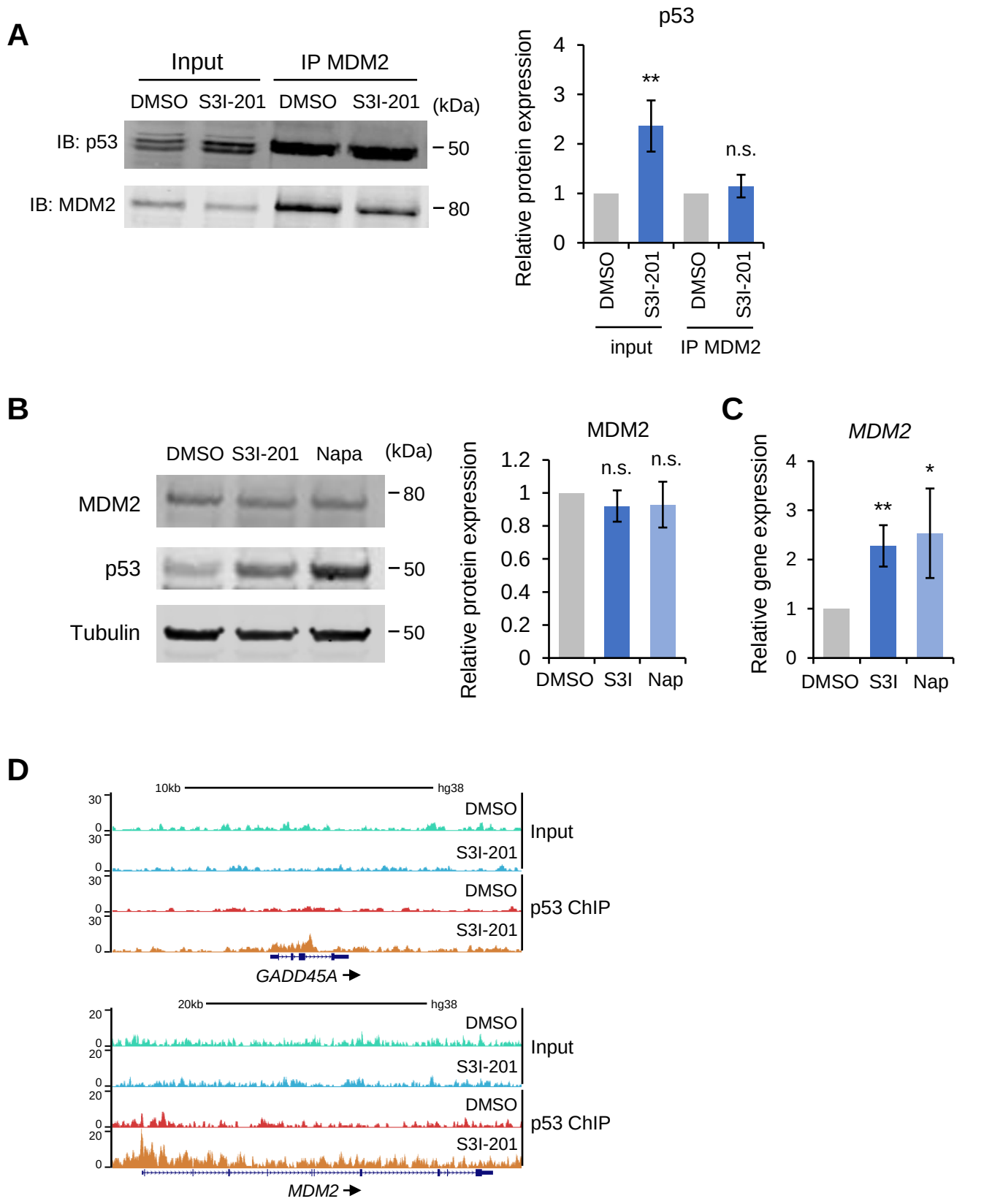

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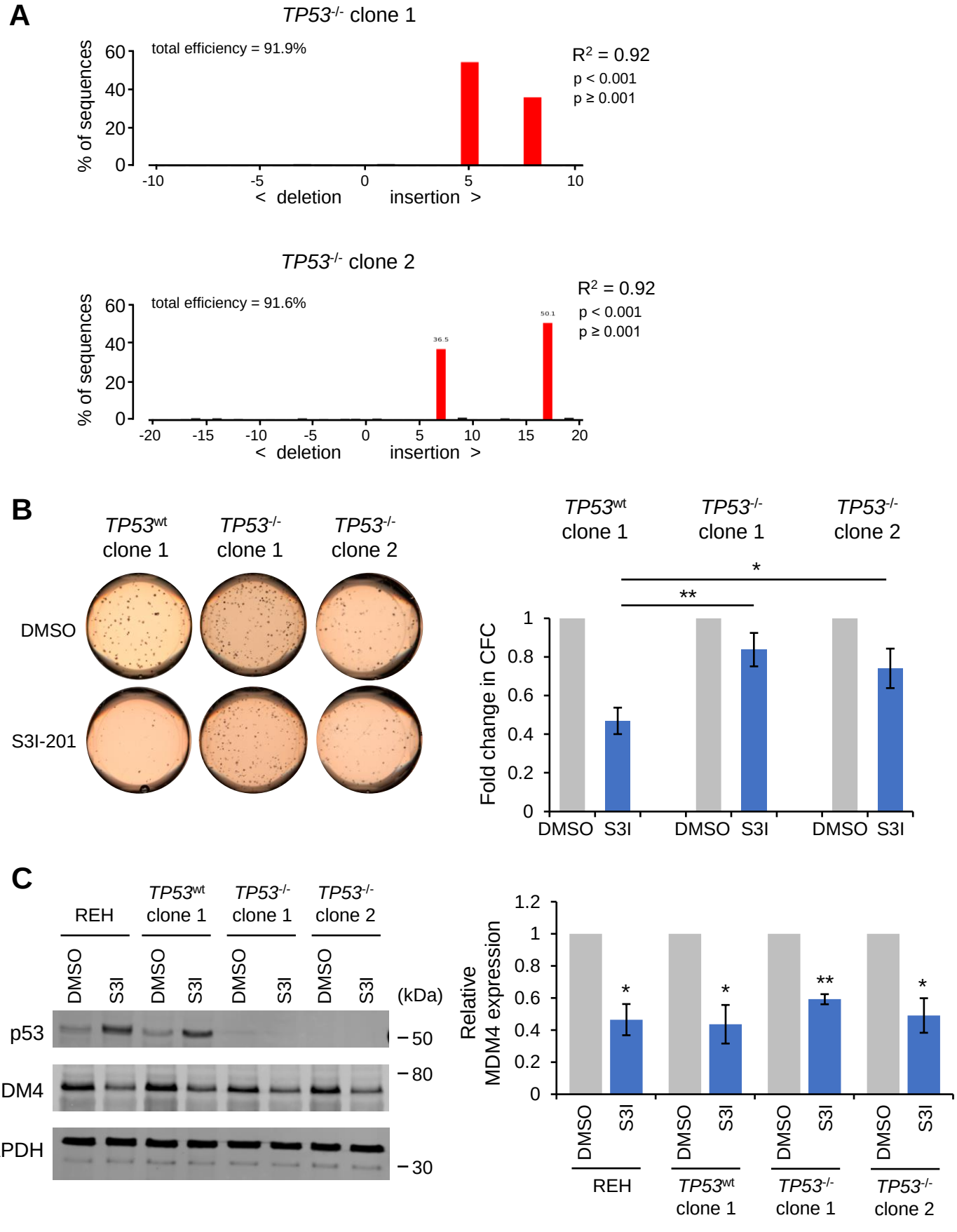

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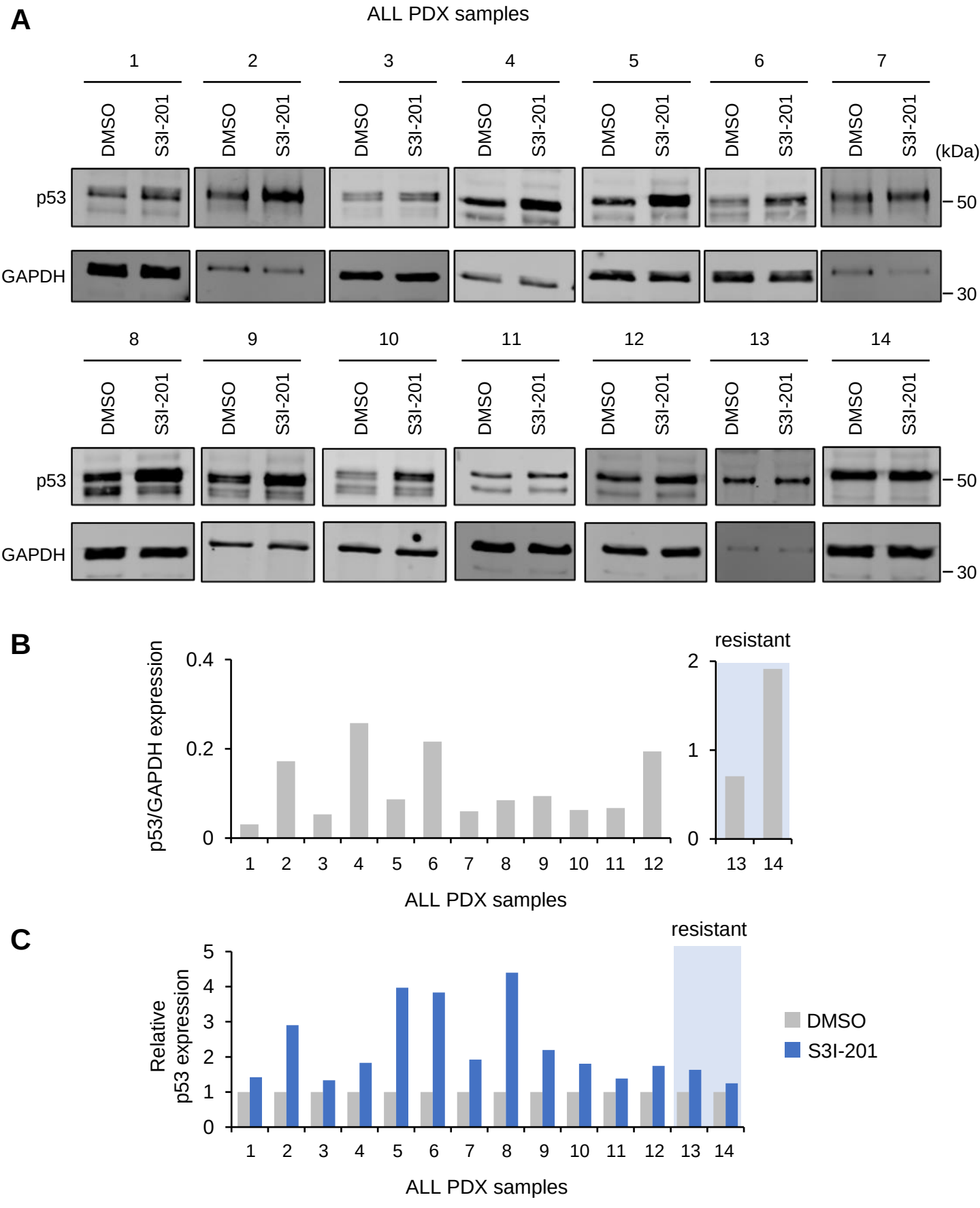
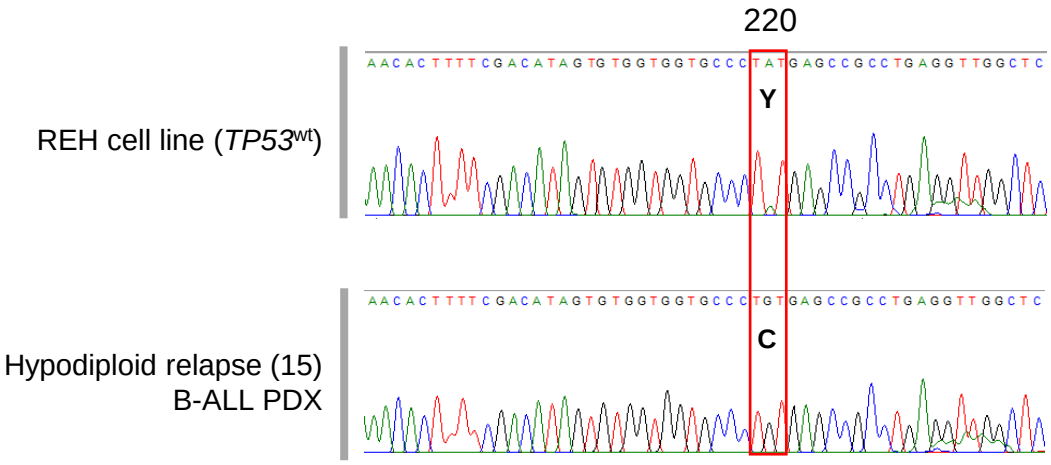


Supplementary Figure S6

A



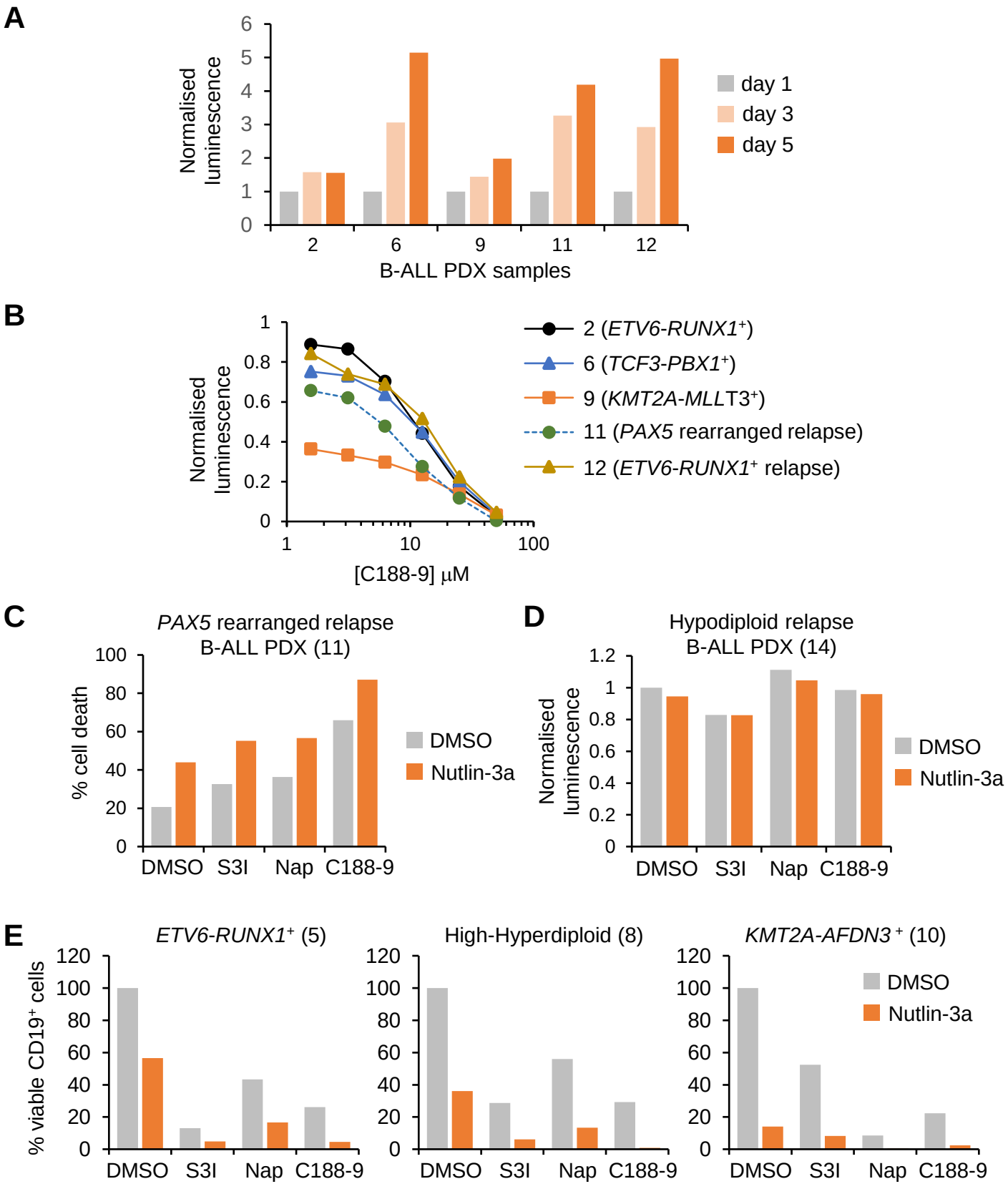
B

ETV6-RUNX1^{+} relapse (14) B-ALL PDX

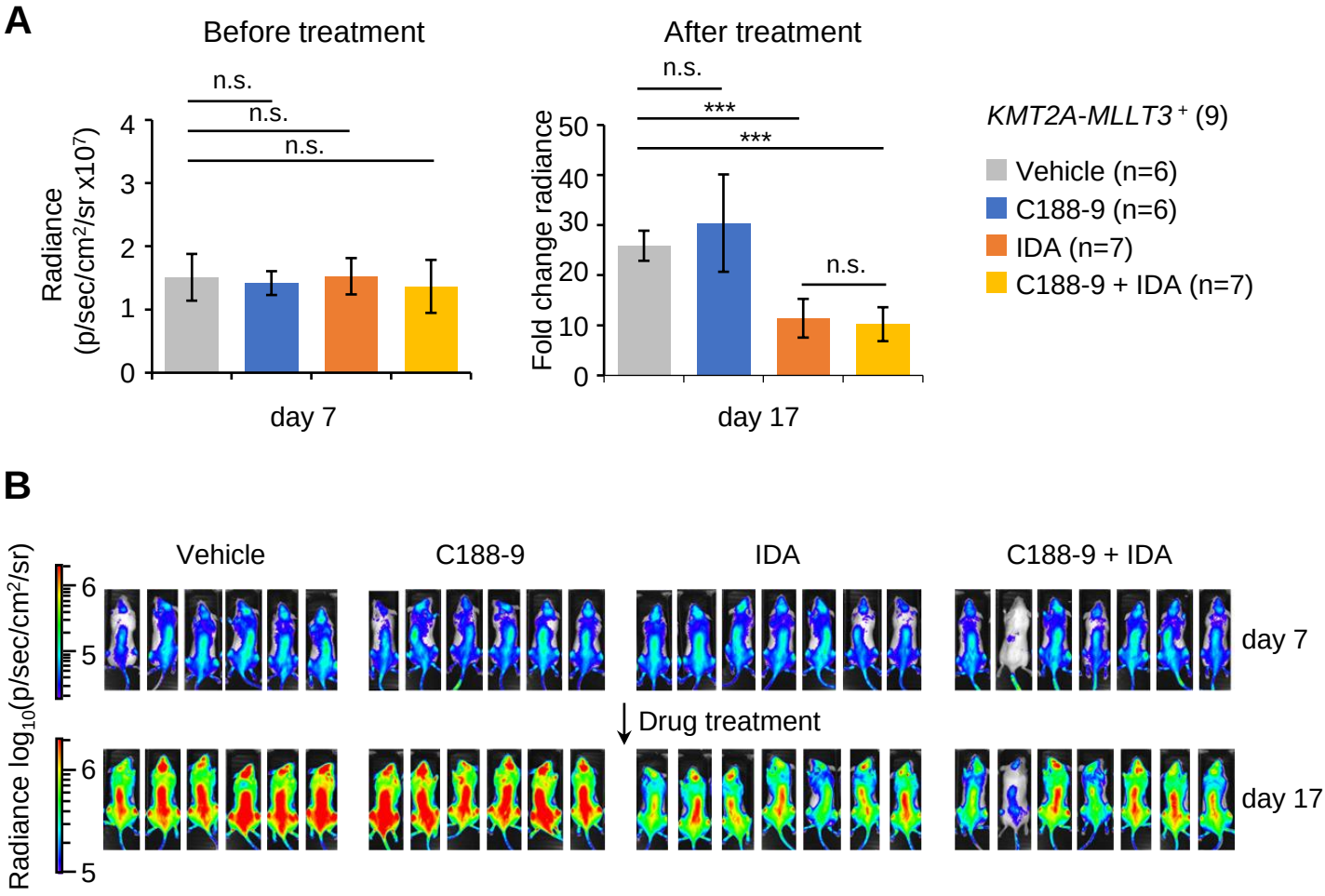
Gene	Consequence	Location	HGVSc	HGVSp	VAF	TIER
TP53	**Missense variant**	chr17:7572938	c.1171G>C	p.Asp391His	0.416	TIER 3
TP53	**Splice acceptor variant**	chr17:7573009	c.1101-1G>A	NA	0.394	TIER 3
TP53	**Missense variant**	chr17:7577538	c.743G>A	p.Arg248Gln	0.470	TIER 2

Tier 2: potential clinical significance
Tier 3: variant of unknown clinical significance

Supplementary Figure S7



Supplementary Figure S8



Supplementary Figure S9

Supplementary Figure Legends

Supplementary Figure S1. A GSEA demonstrating enrichment of the MSigDB hallmark p53 pathway gene set in S3I-201 (top panel) and shSTAT3 (lower panel) induced gene expression changes. **B** Western blot quantification of STAT3 protein expression in REH cells 5 days post transduction with control or shSTAT3 shRNA. Gene expression data are normalised to control shSCR transduced REH cells. Bars and error bars are means and SD of four independent experiments. ***$P < 0.001$, one sample *t* test. **C** qRT-PCR analysis of p53 target genes in REH cells following 6h treatment with either S3I-201 (50 μM) or Napabucasin (10 μM). Gene expression data are normalised to DMSO-treated cells. Bars and error bars are means and SD of n=5 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, one sample *t* test.

Supplementary Figure S2. A TIDE (Tracking of Indels by Decomposition) analysis of CRISPR-Cas9 generated indels in REH cells transduced with Cas9 and gSTAT3 gRNA in a representative experiment**. B** Western blot quantification of STAT3 protein expression in REH cells 7 days post transduction with Cas9 and control or gSTAT3 gRNA. Bars and error bars are means and SD of n=5 independent experiments. Data are normalized to GAPDH loading control and to control gRNA transduced REH cells. ***$P < 0.001$, one sample *t*-test. **C** Data representing flow cytometric quantification of the relative proportions of GFP^+ or $mCherry^+$ REH cells at day 3, 7, 10 and 14 following transduction of REH cells with Cas9 and control or gSTAT3 gRNA. Cells were transduced with GFP-expressing GFP-CRISPR-Cas9-gRNA constructs or with an mCherry-expressing control empty vector (LeGO-iC2), mixed in a 1:1 ratio three days after transduction and resulting cultures subsequently monitored by flow cytometry. Bars and error bars are means and SD of n=4 independent experiments. ***$P < 0.001$;

n.s. not significant, one sample *t*-test. **D** qRT-PCR analysis of p53 target genes in REH cells 7 days post transduction with Cas9 and control or gSTAT3 gRNA. Gene expression data are normalised to control gRNA transduced REH cells. Bars and error bars are means and SD of n=4 independent experiments. *$P < 0.05$; **$P < 0.01$, one sample *t*-test.

Supplementary Figure S3. **A** Representative western blot analysis of STAT3 protein expression in REH 7 days following overexpression of constitutively activated STAT3 cDNA (*CASTAT3*). Tubulin was used as a loading control. **B** qRT-PCR analysis of p53 target genes in REH cells, 7 days following transduction with control empty vector or *CASTAT3*. Gene expression data are normalised to empty vector transduced REH cells. Bars and error bars are means and SD of n=3 independent experiments. *$P < 0.05$; **$P < 0.01$, one sample *t*-test.

Supplementary Figure S4. **A** Western blot analysis (left panel) and p53 protein expression quantification (right panel) of input and anti-MDM2 immunoprecipitates from REH cells, following 6 hours treatment with S3I-201 (50 μM). Blots were stained with anti-p53 (DO-1) or anti-MDM2 (OP-143). Data are normalized to DMSO-treated REH cells. Bars and error bars are means and SD of n=5 independent experiments. **$P < 0.01$; n.s. not significant, one sample *t*-test. **B** Western blot analysis (left panel) and quantification (right panel) of MDM2 and p53 protein expression in REH cells following 6h treatment with either S3I-201 (50 μM) or Napabucasin (10 μM). Tubulin was used as a loading control. Bars and error bars are means and SD of n=4 independent experiments. Data are normalized to Tubulin loading control and to DMSO-treated REH cells. n.s. not significant, one sample *t*-test. **C** qRT-PCR analysis

of *MDM2* gene expression level in REH cells following 6h treatment with either S3I-201 (50 μM) or Napabucasin (10 μM). Gene expression data are normalised to DMSO-treated cells. Bars and error bars are means and SD of n=5 independent experiments. *$P < 0.05$; **$P < 0.01$, one sample t-test. **D** Exemplar ChIP-Seq tracks for p53 peaks at target genes in DMSO and S3I-201 treated REH cells.

Supplementary Figure S5. **A** TIDE analysis of CRISPR-Cas9 generated indels in *TP53*$^{-/-}$ REH clones 1 and 2**. B** Representative example (left panel) and quantification (right panel) of *TP53*^{wt} or *TP53*$^{-/-}$ REH clone colony formation in methylcellulose cultures in the presence of DMSO or S3I-201 (50 μM). Fold change in colony forming capacity (CFC) relative to DMSO-treated cells is shown. Bars and error bars are means and SD of n=5 independent experiments. *$P < 0.05$, **$P < 0.01$, unpaired Student's *t* test between S3I-201-treated *TP53*^{wt} and *TP53*$^{-/-}$ clones. **C** Western blot analysis (left panel) and quantification (right panel) of MDM4 protein expression in REH cells, *TP53*^{wt} and *TP53*$^{-/-}$ clones following 6h treatment with S3I-201 (50 μM) or DMSO control. GAPDH was used as a loading control. Bars and error bars are means and SD of n=3 independent experiments. Data are normalized to GAPDH loading control and to DMSO-treated REH cells. *$P < 0.05$; **$P < 0.01$, one sample t-test.

Supplementary Figure S6. A Western blot analysis of p53 protein expression in B-ALL PDX samples 6h after exposure to S3I-201 (50 μM). **B** Quantification of p53 protein expression, relative to GAPDH loading control and **C** of p53 induction in S3I-201 treated cells relative to DMSO treated controls, in the PDX samples.

Supplementary Figure S7. A Sanger sequencing of a PCR amplified region of the *TP53* gene from the B-ALL REH cell line (upper panel) and Hypodiploid relapse (15) B-ALL PDX sample (lower panel). The TAT>TGT (Y220C) mutation was confirmed in this B-ALL PDX sample. **B** *TP53* somatic variants, identified via targeted capture panel sequencing, in the *RUNX1-ETV6*$^{+}$ relapse (14) B-ALL PDX sample. HGVSc (Human Genome Variation Society, coding); HGVSp (Human Genome Variation Society, protein); VAF (Variant Allele Frequency).

Supplementary Figure S8. A Luminescence of luciferase-expressing B-ALL PDX samples on days 1, 3 and 5 after plating onto human MSC. Data are normalised to luminescence values measured on day 1 of the co-cultures. B-ALL PDX samples: *ETV6-RUNX1*$^{+}$ (2), *TCF3-PBX1*$^{+}$ (6), *KMT2A-MLLT3*$^{+}$ (9), *PAX5* rearranged relapse (11), *ETV6-RUNX1*$^{+}$ relapse (12). **B** Luminescence of luciferase-expressing B-ALL PDX samples, grown in co-culture with human MSC, 5 days after exposure to the indicated concentrations of the STST3 inhibitor C188-9. Data are normalised to DMSO-treated cells. **C** Induction of apoptosis (% Annexin V^{+} cells) in the *PAX5* rearranged relapse (11) B-ALL PDX sample, grown in co-culture with human MSC, 72 hours after exposure to Nutlin-3a (5 μM) alone or in combination with STAT3 inhibitors S3I-201 (50 μM), Napabucasin (1.5 μM) and C188-9 (10 μM). Data are normalised to DMSO-treated cells. **D** Luminescence of luciferase-expressing Hypodiploid relapse (14) B-ALL PDX sample, grown in co-culture with human MSC, 5 days after exposure to Nutlin-3a (5μM) alone or in combination with STAT3 inhibitors S3I-201 (50 μM), Napabucasin (1.5 μM) and C188-9 (10 μM). Data are normalised to DMSO-treated cells. **E** Viability of untransduced B-ALL PDX samples, grown in co-culture with human MSC, 5 days after exposure to MDM2 inhibitor Nutlin-3a (5 μM) alone or in

combination with STAT3 inhibitors S3I-201 (50 μM), Napabucasin (1.5 μM) and C188-9 (10 μM), as determined by flow cytometry. Corresponding B-ALL PDX sample number from Figure 5 is indicated in brackets. Each bar represents the number of viable human $CD19^+$ ALL cells as a percentage of DMSO treated control cultures. The mean percentage of viable $CD19^+$ cells in DMSO treated triplicate control cultures at day 5 was 75.3% (PDX 5), 88.2% (PDX 8) and 85.9% (PDX 10).

Supplementary Figure S9. A Bioluminescence signal (radiance = photons/s/cm2/steradian) in NSG recipient mice 7 days after injection of the luciferase-expressing *KMT2A-MLLT3*$^+$ B-ALL PDX sample and before drug treatment (left panel), and fold change in bioluminescence signal 17 days after injection, following 7 daily treatments with vehicle or the indicated drug combinations (right panel). Bars and error bars are means and SD of values form each treatment group, the number of mice in each group indicated in brackets. ***$P < 0.001$; n.s. not significant, unpaired Student's *t* test between indicated groups. **B** Bioluminescence imaging of NSG recipient mice before (day 7) and after (day 17) drug treatment. Bars for luminescence signal represent photons/s/cm2/steradian.

Supplementary Table 1. Patient sample characteristics.

PDX sample	Classification	Sex	Age (yrs)	Cytogenetics
4	*ETV6-RUNX1*$^{+}$	F	1.92	ETV6-RUNX1 rearrangement detected in 60% of interphase nuclei.
5	*ETV6-RUNX1*$^{+}$	M	3.37	ETV6-RUNX1 fusion detected, showing clonal evolution involving gain of RUNX1 signal, by interphase FISH analysis.
6	*TCF3-PBX1*$^{+}$	F	10.95	Abnormal karyotype with a der(19)t(1;19) unbalanced translocation by G-banded analysis. Unbalanced TCF3-PBX1 rearrangement in 52% of nuclei and mono-allelic loss of CDKN2A in 5.5% nuclei by interphase FISH analysis.
7	Hyperdyploid	M	2.36	Gain of DXZ1, D9Z3, ABL1, RUNX1 and relative loss of one CDKN2A signal by FISH. Gain of chromosomes X, 9 and 21 by G-banded analysis.
8	High-Hyperdiploid	F	2.48	Abnormal high-hyperdiploid karyotype with gain of chromosomes X, 3, 5, 8, 10, 11, 12, 14, 17, 18, 21 and 22. Favourable prognosis.
9	*KMT2A-MLLT3*$^{+}$	F	2.52	KMT2A rearrangement with concomitant deletion of the 3'KMT2A region. t(9;11)(p22;q23).
10	*KMT2A-AFDN*$^{+}$	M	0.28	A t(6;11)(q27;q23) translocation identified in all metaphase spreads. FISH analysis confirmed a KMT2A rearrangement in 56% of nuclei.
11	*PAX5* rearranged (relapse)	M	5.10	PAX5 rearrangement in 92% cells by interphase FISH analysis. Consistent with CNS relapse of ALL.
12	*ETV6-RUNX1*$^{+}$ (relapse)	M	7.91	ETV6-RUNX1 rearrangement and bi-allelic deletion of CDKN2A detected in 69% and 72.5% nuclei, respectively. Additional copy of chromosome 21
13	*ETV6-RUNX1*$^{+}$ (relapse)	M	4.62	An ETV6-RUNX1 rearrangement detected in 76% nuclei examined by interphase FISH analysis.
14	Hypodiploid (relapse)	M	11.8	Hypodiploid with a doubled-up clone in a sub-population showing additional structural abnormalities, indicative of clonal evolution. FISH analysis showed loss of ETV6(73%), relative loss of centromere 17 (54%), and gain of signals for 11 and 17 centromeres (27%).

*PDX samples *ETV6-RUNX1* (1-3): patient samples characteristics previously reported in Mangolini et al (2013)

Supplementary Methods

Cell culture

Human B-ALL cell line REH was purchased from the ATCC. Cell lines were authenticated by short tandem repeat profiling using the PowerPlex 16 system (Promega, Southampton, UK) and mycoplasma negative status confirmed using the MycoAlert Mycoplasma Detection Kit (Lonza, Verviers, Belgium). Primary human MSC cells were obtained as described previously[1]. REH cells were cultured in Roswell Park Memorial Institute (RPMI), supplemented with 10% Foetal Bovine Serum (FBS), 100 U/ml Penicillin/100 µg/ml Streptomycin (Merck Life Science, Dorset, UK) and 2 mM L-glutamine (Merck Life Science). The HEK293FT packaging cell line was purchased from Thermofisher Scientific (Hemel Hempstead, UK) and cultured in Dublecco's Modified Medium (DMEM), supplemented with 10% FBS, 100 U/ml Penicillin/100 µg/ml Streptomycin and 2 mM L- glutamine. Primary human MSC cells were cultured in DMEM low glucose, supplemented with 20% FBS, 100 U/ml Penicillin/100 µg/ml Streptomycin, 2 mM L- glutamine and 8ng/ml Human FGF-basic (FGF-2/bFGF) (aa 10-155) recombinant protein (Thermofisher Scientific).

RNA sequencing (RNA-seq) and Gene set enrichment analysis (GSEA)

Total cellular RNA was purified from control and S3I-201-treated cells or shSTAT3 shRNA-transduced cells from three independent experiments each and submitted to UCL Genomics for RNA-sequencing. GSEA (https://software.broadinstitute.org/gsea/) was used to examine enrichment of the p53 target gene set[3] in RNA-seq data. Total cellular RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Manchester, UK) according to manufacturer's instructions. 100 ng of RNA per sample were analysed using Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA) to verify RNA

integrity prior to amplification. Samples were processed using the Illumina TruSeq RNA sample prep kit Version2 (p/n RS-122-2001) according to manufacturer's instructions (Illumina, Cambridge, UK). Briefly, mRNA was selected using paramagnetic dT beads and fragmented by metal hydrolysis to approximately 150 bp lengths. Random primed cDNA was then generated and adapters compatible with Illumina sequencing were ligated before being enriched by 12 cycles of PCR. Libraries were quantified, normalised, and pooled before sequencing on an Illumina NextSeq 500, generating approximately 20 million 43 bp read pairs per sample. Fastq was then demultiplexed and generated using Illumina bcl2fastq v2.19 before pre-processing (trimmomatic) to remove adapter read-through and poor-quality sequences. Pre-processed data were then aligned to the genome (UCSC hg38) with Bowtie 2 and deduplicated using Picard. Reads-per-transcript were counted by FeatureCounts, before differential expression analysis by SARTools, a DESeq2 wrapper. All tools were invoked through the Galaxy Project for NGS analysis.

Colony-forming assays

Colony-forming ability was assessed by plating REH cells in methylcellulose (HSC002, Bio-Techne). Colonies were stained with 1 mg/ml p-iodonitrotetrazolium after 10 days of culture.

Synergy Experiments

$5x10^4$ REH cells were treated for 72h with S3I-201 or Napabucasin in combination with Nutlin-3a at indicated concentrations. Cell viability was evaluated with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). ZIP synergy score was calculated using SynergyFinder (version 2.0) online software.[2]

Lentiviral transduction of human cell lines

293FT packaging cells (Thermo Fisher Scientific) were transiently co-transfected with the lentiviral expression vectors, the pCMV-PAX2 construct and the pVSV-G envelope construct (kind gifts of Prof D. Trono, Lausanne, Switzerland). Human leukemia cells were transduced with lentiviral supernatant by spinoculation at 700g, 25°C for 45 minutes in the presence of 5 μg/ml polybrene. After 2 days, transduced cells were selected in 1 μg/ml puromycin for 3 days. Lentiviral MISSION pLKO.1 shRNA constructs targeting *STAT3* (sh1, Clone ID:NM_003150.2-458s1c1; sh2, Clone ID:NM_003150.2-361s1c1) and the scramble (SCR) non-silencing control (SHC002) were purchased from Merck Life Science. CRISPR-Cas9 mediated knockout of *TP53* or *STAT3* in REH cells was performed with gRNA targeting *TP53* (CCATTGTTC AATATCGTCCGGGG) and *STAT3* (g1_STAT3 gRNA TGGGTGGAGAAGGACATC AGCGG; g2_STAT3 gRNA ACCCTGAGGGAGCAGAGATGTGG). *TP53*$^{-/-}$ REH clones were obtained by isolating individual colonies from methylcellulose cultures of bulk transduced REH cells.

Flow cytometry

Apoptosis was measured using the Annexin V Apoptosis Detection Kit (Thermo Fisher Scientific). Cells were acquired on a CytoFLEX (Beckman Coulter, High Wycombe, UK) and the data was analysed with CytExpert Software (Beckman Coulter).

Western blot and immunoprecipitation (IP) analysis

The following primary antibodies were used: anti-STAT3 (12640, Cell Signaling Technology, Leiden, The Netherlands), anti-TP53 (sc-126, Santa Cruz Biotechnology,

Texas, USA), anti-MDM2 (OP-143, Merck Life Science), anti-MDM4 (BL-3-2F2, Cambridge Bioscience, Cambridge, UK), anti-Tubulin (sc-53029, Santa Cruz Biotechnology), anti-GAPDH (sc-32233, Santa Cruz Biotechnology). IP analysis in REH cells was performed using the Pierce™ Classic Magnetic IP/Co-IP Kit (ThermoFisher Scientific) according to manufacturer's instructions. 2 μg of anti-$p53^{K372me1}$ (ab16033, Abcam, Cambridge, UK) or 2 μg of anti-MDM2 (sc-965, Santa Cruz Biotechnology) were used in each immunoprecipitation reaction. Protein samples were resolved on 10 % polyacrylamide gels (0.36 M bis-Tris, 8-10 % acrylamide/bis) in MOPS-SDS running buffer (50 mM Tris, 50 mM MOPS, 1 mM EDTA, 0.1 % SDS). Gels were transferred onto nitrocellulose (LI-COR Biosciences, Cambridge, UK) membranes. Proteins were detected using appropriate IRDye 800CW and IRDye 680RD labelled secondary antibodies (LI-COR Biosciences). Quantification was performed on fluorescent images using the Odyssey® CLx and Image Studio software (LI-COR Biosciences).

Chromatin immunoprecipitation (ChIP), ChIP-quantitative PCR (ChIP-qPCR) and sequencing (ChIP-seq)

$25x10^6$ REH cells were treated for 6h with either S3I-201 (50 μM) or DMSO and then were cross-linked with 2 mM DSG for 30 minutes at room temperature. After washing the samples 3 times with PBS, cells were fixed with 1% formaldehyde for 10 minutes at room temperature. The reaction was quenched by adding glycine to final concentration 0.125 M for 5 minutes at room temperature. Fixed cells were washed twice with cold PBS, and then sequentially in lysis buffer A (0.25% TritonX100, 10 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]) and lysis buffer B (150 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6], incubating for 10 minutes in

rotation at 4°C each time. Cells were then resuspended in incubation buffer (0.15% SDS, 1% TritonX100, 150 mM NaCl, 10 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]) plus protease inhibitors (Roche, Merck) and incubated for 30 minutes on ice. Lysates were then sonicated for 20 cycles (30 sec on / 30 sec off) in the Bioruptor Pico water bath sonicator (Diagenode, Liege, BE). Cleared supernatant was stored at -80°C. 250 µl of sonicated chromatin were diluted in incubation buffer supplemented with 0.1% BSA and protease inhibitors and were incubated rotating at 4°C overnight, with protein A magnetic beads (Merck Life Science) and 2 µg of anti-TP53 (C15410083 ,Diagenode) antibody. Beads were washed for 10 minutes twice in low salt buffer (0.1% SDS, 0.1% DOC, 1% Triton X-100, 0.1 mM EDTA pH 8.0, 0.5 mM EGTA, 10 mM Tris-HCl [pH 8.0], 150 mM NaCl), and once in high salt buffer (0.1% SDS, 0.1% DOC, 1% Triton X-100, 0.1 mM EDTA pH 8.0, 0.5 mM EGTA, 10 mM Tris-HCl [pH 8.0], 500 mM NaCl), LiCl Buffer (250 mM LiCl, 0.5% DOC, 0.5% NP-40, 0.1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl [pH 8.0]) and in TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.5 mM EGTA). DNA was eluted from the ChIP samples by 20 minutes incubation in rotation at RT with elution buffer (1% SDS, 100 mM $NaHCO_3$). Chromatin was reverse-crosslinked and subjected to RNase and proteinase K digestion and extracted by using the MinElute PCR purification kit (Qiagen). ChIP-qPCR experiments were performed on the purified DNA using SensiFAST™ SYBR® Hi-ROX Kit (Meridian Bioscience, TN, United States). Primers were designed with Primer3web (version 4.1.0) and were as follows: *CDKN1A* forward: CTCTGGCATAGAAGAGGCTGG; *CDKN1A* reverse: GGCTAAGGTTTACCTGGGGTC; *BBC3* forward: TACTGTGCGTTGAGGTCGT; *BBC3* reverse: CGGGGAGGAGGAACAGTG; *ATF3* forward: CACACACCTGGGACTCTCAC; *AFT3* reverse: TATCTGCACAAGTGGCTCCA;

Gene Desert 21 forward: GGGGGATCAGATGACAGTAAA; Gene Desert 21 reverse: AATGCCAGCATGGGAAATA. For ChIP-seq, libraries were prepared from 1ng of ChIP DNA using the NEBNext DNA Ultra II assay (New England Biolabs, Hitchin, UK) with bead-based size selection for 200 bp fragments and 12 cycles of amplification. Samples were then sequenced on an Illumina NextSeq 500, using a 43bp, paired end configuration. Fastq was then demultiplexed and generated using Illumina bcl2fastq v2.19 before pre-processing (trimmomatic) to remove adapter read-through and poor-quality sequences. Pre-processed data were aligned to the genome (UCSC hg38) with BWA14 and deduplicated. Peak calling was conducted using MACS1.3.3[4] at a *P*-value cut-off of 10^{-6}. Bigwig files were generated using bam2bw. Tags within a given region were counted and adjusted to represent the number of tags within a 1 kb region. Subsequently the percentage of these tags as a measure of the total number of sequenced tags of the sample was calculated and displayed as a heat map as before.[5,6]

In vivo transplantation

6-10 week old female NOD-SCID-$\gamma^{-/-}$ (NSG) mice were used as recipients for transplantation. Group sizes were chosen based on previous estimates of disease progression in transplanted mice and experiments in the literature performing similar studies. No samples or animals were excluded from analysis.

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