IKZF1 gene deletions drive resistance to cytarabine in B-cell precursor acute lymphoblastic leukemia

Britt M. T. Vervoort,[1*] Miriam Butler,[1*] Kari J.T. Grünewald,[1] Dorette S. van Ingen Schenau,[1] Trisha M. Tee,[1] Luc Lucas,[2] Alwin D. R. Huitema,[1-3] Judith M. Boer,[1] Beat C. Bornhauser,[4] Jean-Pierre Bourquin,[4] Peter M. Hoogerbrugge,[1] Vincent H.J. van der Velden,[5] Roland P. Kuiper,[1,6] Laurens T. van der Meer[1#] and Frank N. van Leeuwen[1#]

[1]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands; [2]Netherlands Cancer Institute, Amsterdam, the Netherlands; [3]Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands; [4]Department of Pediatric Oncology, Children's Research Center, University Children's Hospital Zürich, Zürich, Switzerland; [5]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands and [6]Department of Genetics, Utrecht University Medical Center, Utrecht University, Utrecht, the Netherlands

BMTV and MB contributed equally as first authors.

#LTvdM and FNvL contributed equally as senior authors.

Correspondence: F.N. van Leeuwen
F.N.vanleeuwen@prinsesmaximacentrum.nl

Received: September 23, 2023.
Accepted: May 22, 2024.
Early view: June 6, 2024.

https://doi.org/10.3324/haematol.2023.284357

Supplemental File

SUPPLEMENTAL METHODS

Cell viability assays

Cell viability was determined using a MTS assay and flow cytometry using amine staining to discriminate between live and dead cells. For the MTS assay 100,000 cells were seeded in a well of a 96 well plate, flow cytometry cells were seeded in a 96-well plate, 24-well plate or in a 6-well plate at 500,000 cells per mL. After the indicated incubation times, relative cell viability was assessed using the CellTiter 2 96® AQueous One Solution Cell Proliferation (MTS) Assay (Promega, Madison, WI). Absorbance was acquired using a plate reader (Infinite F50; TECAN, Männedorf, Switzerland). Alternatively, cells were stained with LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit (Thermo-Fischer, L34960) according to the manufacturer's instructions or by incubating the cells with 7,5 µg/ml Hoechst 33342 (Sigma Aldrich (Zwijndrecht, the Netherlands) for 45 minutes at 37 °C in culture medium. Stained cells were analyzed by Fluorescence Activated Cell Sorting (FACS) using an LSRII flow cytometer (BD Biosciences, Breda, The Netherlands) or CytoFLEX LX (Beckman Coulter). For co-culture experiments, MSCs were gated out via FSC/SSC gating. The data were collected and analyzed by FlowJo V10 software (FlowJo, Ashland, Oregon).

Cell culture

Sem cells (ACC 546) were obtained from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and K562 wildtype and t(3.8) were a gift from Ruud Delwel. Both cell lines are maintained in RPMI (Invitrogen, Thermo Fisher Scientific, Breda, the Netherlands), supplemented with 10% fetal bovine serum (FBS, Greiner Bio-One, Essen, Germany) and 1% penicillin/streptomycin solution (P/S) (Invitrogen) at a cell density between 0.2 and $3x10^6$ cells per milliliter. Cell line identity was confirmed by DNA fingerprinting.

Hek293FT cells were purchased from Invitrogen and maintained in DMEM, supplemented with 10% FBS, 1% Non-essential amino acids (Gibco, Thermo Fischer) and 1% P/S. Cell cultures were tested regularly for the presence of mycoplasma.

Plasmids

The following plasmids were obtained via Addgene: pS-Pax2 (#12260), pMGD2 (#12259), pL-CRISPR.EFS.GFP (#57818), pLKO5.sgRNA.EFS.tRFP (#57823). *IKZF1* KD plasmids were purchased from sigma (pLKO1-Puro). For targeted knockout, gRNA sequences (Supplemental Table 3) were cloned into pL-CRISPR.EFS.GFP and pLKO5.sgRNA.EFS.tRFP using the BsmBI sites and the resulting vector was verified using Sanger sequencing. The hENT1 overexpression vector was obtained from Twist Bioscience (San Francisco, California). The

hENT1 protein sequence (NP_001071643.1) was used as a template to generate and synthesize a DNA sequence that was cloned into the pTwist Lenti SFFV. Sequence was verified using Sanger sequencing. The empty plasmid was used as control.

Reagents

Cytarabine (AraC), Prednisone, Dexamethasone, Vincristine, Methotrexate, Asparaginase, 6TG, Doxorubicin, 6MP, Etoposide, Mitoxantrone, Daunorubicin were ordered from Selleckchem (Munich, Germany) and dissolved as instructed by the manufacturer.

Lentivirus production and transduction

HEK293FT packaging cells (Invitrogen) were co-transfected with a viral backbone encoding the sgRNAs or Cas9 and the helper plasmids for virus production (psPAX2 and pMD2.G), using Polyethylenimine (PEI). Virus containing supernatant was collected 2 days after transfection. Virus was concentrated by centrifugation at 25,000G for 1 hour at 4°C and resuspended in the cell culture medium required by the target cells. Target cells ($0.5\text{-}1x10^6$) were transduced with 1 mL virus (1-10 times concentrated) using spinoculation for 45 min at 700g (30°C) in the presence of 5 ug/mL polybrene (Santa Cruz Biotechnology, Dallas, TX). Selection was started 72-96 hours after transduction (2ug/mL puromycin) or GFP/RFP positive cells were sorted by Flow cytometry using the H800S Cell Sorter (Sony Biotechnology).

Real-time quantitative polymerase chain reaction

Total RNA was extracted using the RNeasy mini-kit (Qiagen). Subsequently, cDNA was synthesized of 500 ng RNA template using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA). mRNA expression levels were determined by use of Power SYBR®Green PCR master mix using gene-specific primers (Supplemental table 4) and the CFX96 TouchTM Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). TBP mRNA expression was used as a reference to obtain the relative fold expression of target genes using the comparative cycle threshold 2(-ΔΔCt) method.

Immunoblotting

Cells were lysed in Laemmli protein buffer and treated with benzonuclease for 30 minutes, prior to boiling. Extracted proteins were separated by SDS-PAGE and transferred to PVDF or nitrocellulose membranes (Amersham Biosciences). After protein transfer, membranes were blocked in TBS-5% low fat milk (Elk, Campina, Amersfoort, the Netherlands) and stained with primary antibodies according to the specific antibody (Supplemental Table 5), washed in TBS-0.02% Tween, followed by IRDye conjugated secondary antibody (Li-cor, Biotechnology). Proteins were visualized with Odyssey®CLx (Li-cor, Biotechnology).

Ex vivo culture of patient derived xenografts

PDXs were generated as described by intrafemoral injection of 0.25 × 10^6 to 1 × 10^6 viable primary BCP-ALL cells in NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice(1). The *ex vivo* co-culture has been described previously(2). hTERT immortalized MSCs (3) were seeded in a 96-wells format (14,000 cells/well) 24 hours prior to the addition of ALL xenografts (140,000 cells/well) or ALL xenografts were seeded as mono-culture in case Zebularine was used (140,000 – 200,00 cells/well). ALL cells were allowed to settle for 24 hours before drugs was added in increasing concentrations. After 3 days of drug incubation, cells were stained with LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit (Thermo-Fischer, L34960) as described above. Cytogenetics of all PDX can be found in supplemental table 6.

In vivo mouse trial

This in vivo experiment was approved by the Animal Experimental Committee of the Radboud University (RU-DEC-2019-0036). Female Mice were housed in groups of 3-5 mice and cages were randomly distributed. For each leukemia, 2 mice NSG mice were randomly assigned and transplanted with 1.0 x 10^6 viable cells via intravenous injection and leukemia load was monitored by flow cytometric detection of human cells by staining blood samples for human CD10, CD45 and CD19 and murine CD45. When a particular sample reached a load of 1% human cells, mice were randomly assigned to treatment or control group and treated with 17,5 mg/kg cytarabine for two weeks (5 days on, 2 off treatment). Mice were weighed daily during treatment and weekly afterwards. Leukemia development was monitored by flow cytometry and the delay in growth was determined by calculating the reduction in time to event, defined as 30% of human cells in the blood. To account for differences in growth speed between leukemias, we first normalized all the times to leukemia and calculated the treatment related delay relative to this normalized growth.

Quantification of incorporated cytarabine by mass spectrometry

The method to determine DNA incorporated AraC was based on a LC-MS/MS method for the analysis of genomic DNA incorporated β-decitabine and 5-methyl-2'-deoxycytidine developed by J. Roosendaal et al(4). Sem wildtype and IKZF1$^{-/-}$ cells were seeded in triplicates and treated with either 1uM AraC for 24 hours or 30nM AraC for 72 hours. Genomic DNA was isolated using the DNA Blood mini kit (Qiagen) following manufactures protocol. Isolated DNA was degraded into single monophosphate nucleotides by adding 4 units of Nuclease P1 (Sigma-Aldrich, St. Louis, MO) and 100 µL digest buffer (0.04 mM deferoxamine mesylate, 3.25 mM ammonium acetate pH 5.0, 0.5 mM zinc chloride) to 50 µL of extracted DNA solution (ranged from 3.7 to 11.4µg DNA/50 µL in the measured samples) and incubated at 65°C for

10 min. The nucleotides were converted into nucleosides by adding 20 µL of 100 mM Trizma®base, pH 8.5 and 4 units of alkaline phosphatase (Roche Life Science, Indianapolis, IN) and incubating at 37°C for 1 h. After incubating, the reaction was stopped by adding 20 µL of 300 mM ammonium acetate pH 5.0 and 6 µL 0.25 mM DFOM/50 mM EDTA to the sample. 10 µL of internal standard working solution (containing 100 ng/mL cytarabine-$^{13}C_3$ and 10,000 ng/mL 2'-deoxycytidine [$^{13}C,^{15}N_2$] in water) was added to each sample before evaporating to dryness (40°C using and a gentle stream of nitrogen). The dry extract was reconstituted in 50 µL 5mM ammonium formate in ACN-water (98:2, v/v) by vortex mixing for 1 min.

Sample analysis was performed by injecting 10 µL of the processed sample onto the LC-MS system which consisted of an UPLC Acquity I Class pump, autosampler and column oven (Waters, Milford, MA,USA) coupled to a QTRAP5500 tandem mass spectrometer (Sciex, Framingham, MA, USA). Cytarabine and 2'-deoxycytidine were chromatographic separated on a Nova-Pak silica column (150 x 3.9 mm ID, 4 µm particle size from Waters, Milford, MA,USA) using a gradient elution with 5 mM ammonium formate in water (mobile phase A) and 5mM ammonium formate in ACN-water (98:2, v/v) (mobile phase B). The flow rate was 1.4 mL/min and a spitter (1:4) was used to introduce 25% of the flow in the QTRAP5500. The QTRAP5500 was equipped with a turbo ionspray interface and operated in positive ionization mode. Cytarabine (244.1→112.1), 2'-deoxycytidine (228.0→112.0), cytarabine-$^{13}C_3$ (347.1→115.1) and 2'-deoxycytidine [$^{13}C,^{15}N_2$] (231.0→115.0) were measured by multiple reaction monitoring. Since cytarabine and cytidine (endogenous nucleoside) are diastereomers, cytidine (244.101→112.1) was monitored to confirm that they were separated chromatographically. A correction for the amount of isolated and digested DNA was performed by expressing the DNA content of cytarabine as a cytarabine/2'-deoxycytidine ratio. Data acquisition was performed using Analyst 1.6.3 software (Sciex).

RNA sequencing

mRNA was isolated on triplicate samples using an RNeasy minikit (74106; Qiagen, Venlo, The Netherlands) after treatment with 1uM AraC for 16 hours. Sample sequencing and data analysis, including differential gene expression analysis, were performed by NovoGene (Cambridge, United Kingdom). Differentially expressed genes (FDR <0.05) of the following comparisons were combined for overrepresentation analysis: *IKZF1*^{wt} treated/*IKZF1*^{wt} untreated, *IKZF1*$^{-/-}$ untreated/*IKZF1*$^{-/-}$ treated, *IKZF1*^{wt} untreated/*IKZF1*$^{-/-}$ untreated and *IKZF1*^{wt} treated/*IKZF1*$^{-/-}$ treated. Overrepresentation analysis was performed in Rstudio using the clusterProfiler package, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. FDR values were calculated using the Benjamini&Hochberg multiple testing method. Heatmaps were created from FPKM values transformed into Z-scores and the ComplexHeatmap package.

Patient RNA expression data extracted from GSE87070

Log transformed microarray gene expression data from newly diagnosed pediatric ALL patients was derived from NCBI's Gene Expression Omnibus (GEO) data set GSE87070(5, 6) using additional subtype annotations and *IKZF1* deletion status (as available in R2, Supplemental Table 1). *IKZF1* copy number was annotated as either wildtype or deleted with no distinction between type of deletion. We included those BCP-ALL subtypes that have ≥ 4 patients with an *IKZF1* deletion making a cohort of in total n=252 patients wildtype for *IKZF1* and n=100 patients with an *IKZF1* deletion. Subtypes included are the high hyperdiploids (≥ 51 chromosomes) (Wildtype n=130, $IKZF1^{del}$ n=17), *BCR-ABL1* (Wildtype n=24, $IKZF1^{del}$ n=15), and B-other, defines as BCP-ALL negative for *ETV6-RUNX1*, high hyperdiploidy, *BCR-ABL1*, *KMT2A*-rearranged, *TCF3-PBX1* and *BCR-ABL1*-like signature (Wildtype n=198, $IKZF1^{del}$ n=68). Expression levels of SLC29A1 (hENT1) and MECOM (Evi1) were visualized using ggplot. The cut off for high *MECOM* expression was defined by the 95th percentile of *MECOM* expression in the *IKZF1* wildtype population. A Chi-square test was used to determine dependency of *IKZF1* status on MECOM expression. Expression levels of stemness markers were visualized using the ComplexHeatmap package and patients ordered based on MECOM expression.

Crispr/Cas9 screen

The kinase sgRNA library is used as earlier described(7). For this Crispr/Cas9 screen, Sem $IKZF1^{-/-}$ cells, previously transduced with Cas9, were now transduced with the kinase library at an MOI<1 and cells were selected using blasticidin. Cas9 expression was induced for 2 weeks by adding 1 ug/mL of doxycycline to the cells. Subsequently, cells were split in 3 pools of which 1 was left untreated and the others were exposed to 30nM and 50nM for a duration of 22 days, all in triplicates. To maintain library complexity, transduced cells were always cultured with a minimum of 1000 cells per guide, totaling to a minimum of 5 million cells per condition. After selection, genomic DNA was isolated from these pools and by PCR using Illumina barcode primers the sgRNA region was amplified (Supplemental Table 4). Then, sgRNA abundance was determined by deep sequencing on the Illumina Nextseq500 by the Utrecht sequencing facility, The Netherlands (USEQ). We confirmed that the library complexity was sufficiently maintained in samples at the start of the screen, before and after doxycycline treatment. Then, enriched and depleted genes were identified using the MAGeCK Test alogorithm (Galaxy Version 0.5.8.1)(8) (Supplemental Table 2).

Statistical analyses

All statistical analyses were performed using PRISM6 (GraphPad Software, La Jolla, CA). For the Amine staining, AUC was calculated of the dose response curves and either a two-tailed student's t test or ANOVA with ad hoc Turkey's multiple comparison test was performed. For

qRT-PCR, either a two-tailed student's t test was performed or ANOVA with ad hoc Turkey's multiple comparison test. On *in vivo* PDX data a two-sided student's T test is performed. For MRD patient data significance was tested by two sided Mann Whitney. For patient expression data extracted from R2, a Shapiro-Wilk test was performed to determine normality, followed by two sided Mann Whitney.

References

1. Schmitz M, Breithaupt P, Scheidegger N, Cario G, Bonapace L, Meissner B, Mirkowska P, Tchinda J, Niggli FK, Stanulla M. Xenografts of highly resistant leukemia recapitulate the clonal composition of the leukemogenic compartment. Blood, The Journal of the American Society of Hematology. 2011;118(7):1854-1864.

2. Frismantas V, Dobay MP, Rinaldi A, Tchinda J, Dunn SH, Kunz J, Richter-Pechanska P, Marovca B, Pail O, Jenni S. Ex vivo drug response profiling detects recurrent sensitivity patterns in drug-resistant acute lymphoblastic leukemia. Blood, The Journal of the American Society of Hematology. 2017;129(11):e26-e37.

3. Mihara K, Imai C, Coustan-Smith E, Dome JS, Dominici M, Vanin E, Campana D. Development and functional characterization of human bone marrow mesenchymal cells immortalized by enforced expression of telomerase. British journal of haematology. 2003;120(5):846-849.

4. Roosendaal J, Rosing H, Lucas L, Oganesian A, Schellens JH, Beijnen JH. Development, validation, and clinical application of a high-performance liquid chromatography-tandem mass spectrometry assay for the quantification of total intracellular β-decitabine nucleotides and genomic DNA incorporated β-decitabine and 5-methyl-2'-deoxycytidine. Journal of Pharmaceutical and Biomedical Analysis. 2019;164:16-26.

5. Polak R, Bierings MB, van der Leije CS, Sanders MA, Roovers O, Marchante JR, Boer JM, Cornelissen JJ, Pieters R, Den Boer ML. Autophagy inhibition as a potential future targeted therapy for ETV6-RUNX1-driven B-cell precursor acute lymphoblastic leukemia. Haematologica. 2019;104(4):738.

6. Jerchel IS, Boer JM, Hoogkamer AQ, Beverloo HB, Pieters R, den Boer ML. High PDGFRA expression does not serve as effective therapeutic target in ERG-deleted B-cell precursor acute lymphoblastic leukemia. Blood. 2017;130:1274.

7. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. Science. 2014;343(6166):80-84.

8. Li W, Xu H, Xiao T, Cong L, Love MI, Zhang F, Irizarry RA, Liu JS, Brown M, Liu XS. MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. Genome Biology. 2014 2014/12/05;15(12):554. doi:10.1186/s13059-014-0554-4.

SUPPLEMENTAL TABLES

Supplemental Table 1 Microarray gene expression data from NCBI's Gene Expression Omnibus (GEO) data set GSE87070 using additional subtype annotations and *IKZF1* deletion status

Supplemental Table 2 CRISPR-Cas9 screen results from MaGeck Test analysis: Ranking gene lists of depleted and enriched genes

Supplemental Table 3 *IKZF1* targeting gRNA sequences

Application	Name	Sequence
sgRNA	gRNA_IKZF1_exon3.1_FW	TCATCTGGAGTATCGCTTAC
	gRNA_IKZF1_exon3.2_FW	GACCTCTCCACCACCTCGGG
	gRNA_IKZF1_exon3.3_FW	CTCCAAGAGTGACAGAGTCG
	gRNA_control_FW	GTAGCGAACGTGTCCGGCGT

Supplemental Table 4 RT-qPCR and PCR primer sequences

Target	Use	Name	Sequence
ENT1	RT-qPCR	Forward primer	ACCTCAACTCCTTGCATCAGA
	RT-qPCR	Reverse primer	ACCAGGATGGCAGTGATCAGAAAC
Evi1	RT-qPCR	Forward primer	AGTGCCCTGGAGATGAGTTG
	RT-qPCR	Reverse primer	TTTGAGGCTATCTGTGAAGTGC
Kinase CRISPR/Cas9 screen	PCR	FW primer	AATGATACGGCGACCACCGAGATC TACACCGACTCGGTGCCACTTTT
	PCR	RV Barcode primer 1	CAAGCAGAAGACGGCATACGAGATC **ATCACG**TTTCTTGGGTAGTTTGCAGTTTT
	PCR	RV Barcode primer 2	CAAGCAGAAGACGGCATACGAGATC **CGATGT**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 3	CAAGCAGAAGACGGCATACGAGATC **TTAGGC**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 4	CAAGCAGAAGACGGCATACGAGATC **TGACCA**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 5	CAAGCAGAAGACGGCATACGAGATC **ACAGTG**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 6	CAAGCAGAAGACGGCATACGAGATC **GCCAAT**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 7	CAAGCAGAAGACGGCATACGAGATC **CAGATC**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 8	CAAGCAGAAGACGGCATACGAGATC **ACTTGA**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 9	CAAGCAGAAGACGGCATACGAGATC **GATCAG**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 10	CAAGCAGAAGACGGCATACGAGATC **TAGCTT**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 11	CAAGCAGAAGACGGCATACGAGATC **GGCTAC**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 12	CAAGCAGAAGACGGCATACGAGATC **CTTGTA**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 13	CAAGCAGAAGACGGCATACGAGATC **AGTCAA**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 14	CAAGCAGAAGACGGCATACGAGATC **AGTTCC**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 15	CAAGCAGAAGACGGCATACGAGATC **ATGTCA**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 16	CAAGCAGAAGACGGCATACGAGATC **CCGTCC**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 18	CAAGCAGAAGACGGCATACGAGATC **GTCCGC**TTTCTTGGGTAGTTTGCAGTTTT
		RV Barcode primer 19	CAAGCAGAAGACGGCATACGAGATC **GTGAAA**TTTCTTGGGTAGTTTGCAGTTTT
	Illumina sequencing	Read1 primer	CGGTGCCACTTTTTCAAGTTGATAACGGACTAG CCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
	Illumina sequencing	Index primer	TTTCAAGTTACGGTAAGCATATGATAGTCCATTTTA AAACATAATTTTAAAACTGCAAACTACCCAAGAAA

Supplemental Table 5 List of antibodies

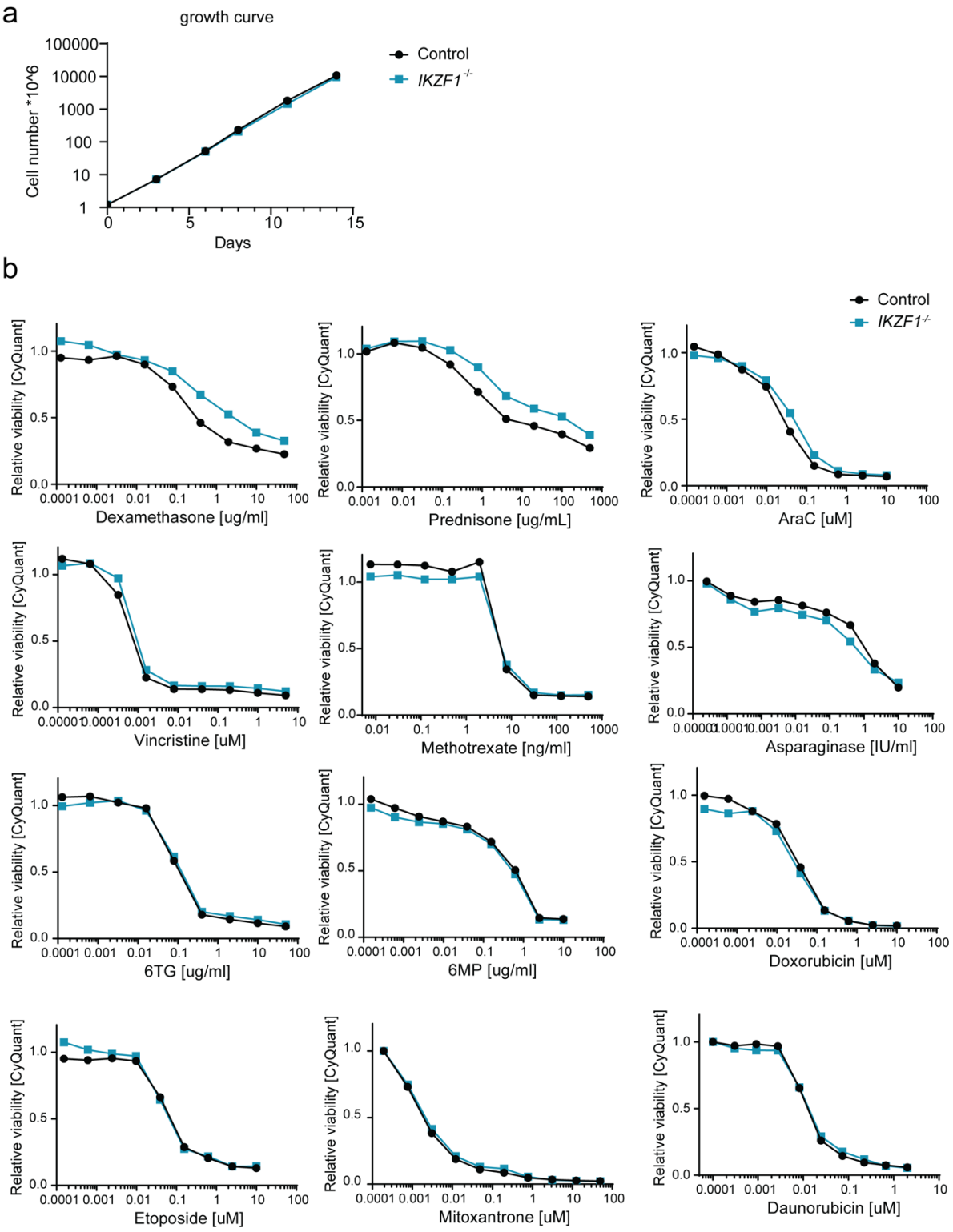
Blocking and staining conditions for antibodies used in western blotting

Antibody	Supplier	Cat. number	Solvent	Dilution
actin	Sigma Aldrich	A4700	1% milk in TBS	1:10000
ENT1	Protein tech	11337-1-AP	1% milk in TBS	1:1000
Evi1	Cell Signaling	2265	1% milk in TBS	1:1000
Ikaros	Santa Cruz	sc-398265	1% milk in TBS	1:1000
PARP	Cell Signaling	9542	1% milk in TBS	1:4000
tubulin	Genetex	GTX628802	1% milk in TBS	1:10000
vinculin	Sigma Aldrich	V9131	1% milk in TBS	1:4000

Supplemental Table 6 Characteristics of BCP-ALL PDXs

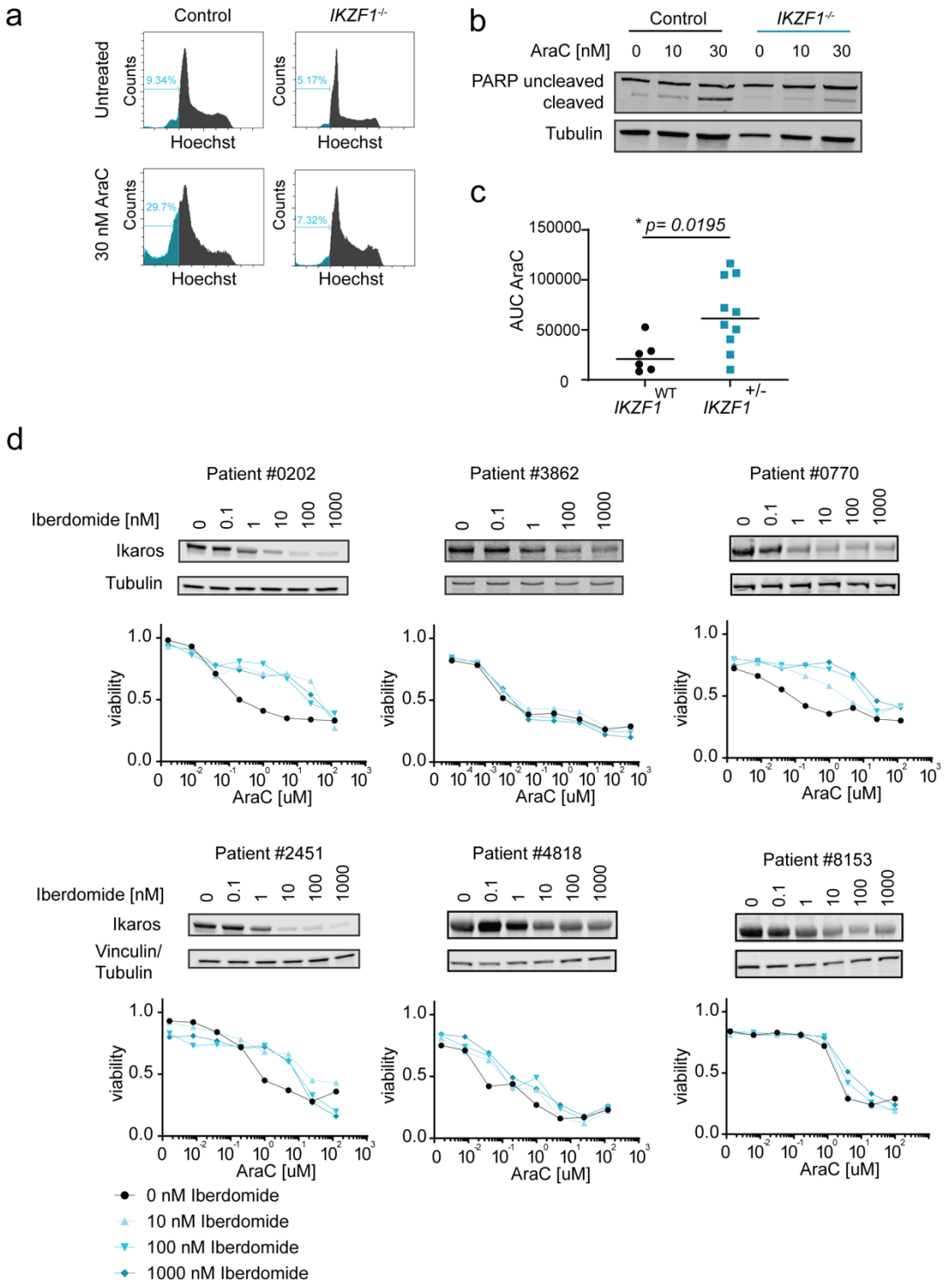
Patient number	Cytogenetics	IKZF1 status	Sample type
#0202	TCF3-PBX1_ t(1::19)	Wildtype	Diagnosis
#2451	B-other	Wildtype	Diagnosis
#4818	Not available	Wildtype	Diagnosis
#0770	TP53 deletion	Wildtype	Diagnosis
#8153	TCF3-PBX1_ t(1::19)	Wildtype	Diagnosis
#6523	TCF3-PBX1_ t(1::19)	Wildtype	Diagnosis
#3862	TP53 deletion (25%), PAX5/CDKN2A	Wildtype	Diagnosis
#7148	TCF3-HLF_t(17::19)	Wildtype	Diagnosis
#2796	TCF3-HLF, t(17::19)	Wildtype	Diagnosis
#8076	TYK2	Wildtype	Diagnosis
#1502	ETV6-RUNX1	Wildtype	Diagnosis
#2103	TCF3-PBX1, t(1::19), PAX5 deletion 1-10	Wildtype	Diagnosis
#7811	BTG1 + IKZF1 deletion	Deletion	Diagnosis
#6609	IKZF1 deletion	Deletion	Diagnosis
#6439	BTG1 + IKZF1 deletion	Deletion	Diagnosis
#2072	IKZF1 deletion	Deletion	Diagnosis
#2400	IKZF1 deletion, PAX5 translocation	Deletion	Diagnosis
#3134	low hypodiploid, TP53 WT, IKZF1 deletion	Deletion	Diagnosis
#7558	IKZF1 deletion	Deletion	Diagnosis
#4707	EBF1deletion1-16, PAX5 deletion2-8, ETV6 deletion 1-2, IL3 deletion, CSF2RA, IKZF1deletion 4-7	Deletion	Diagnosis
#2377	ABL1+, CDKN2A/B deletion, PAX5 deletion, IKZF1 deletion 1-8, TP53 deletion	Deletion	Diagnosis
#6273	IKZF1 deletion 4-7, CDKN2A deletion	Deletion	Diagnosis
#2179	CRLF2, ETV6, IKZF1 deletion 2-3	Deletion	Diagnosis

SUPPLEMENTAL FIGURES


a
growth curve
Cell number *10^6
100000
10000
1000
100
10
1
0
5
10
15
Days
Control
IKZF1-/-
b
Control
IKZF1-/-
Relative viability [CyQuant]
Dexamethasone [ug/ml]
Prednisone [ug/mL]
AraC [uM]
Vincristine [uM]
Methotrexate [ng/ml]
Asparaginase [IU/ml]
6TG [ug/ml]
6MP [ug/ml]
Doxorubicin [uM]
Etoposide [uM]
Mitoxantrone [uM]
Daunorubicin [uM]

Supplemental Figure 1: CRISPR/Cas9-mediated targeting of *IKZF1* drives resistance to AraC

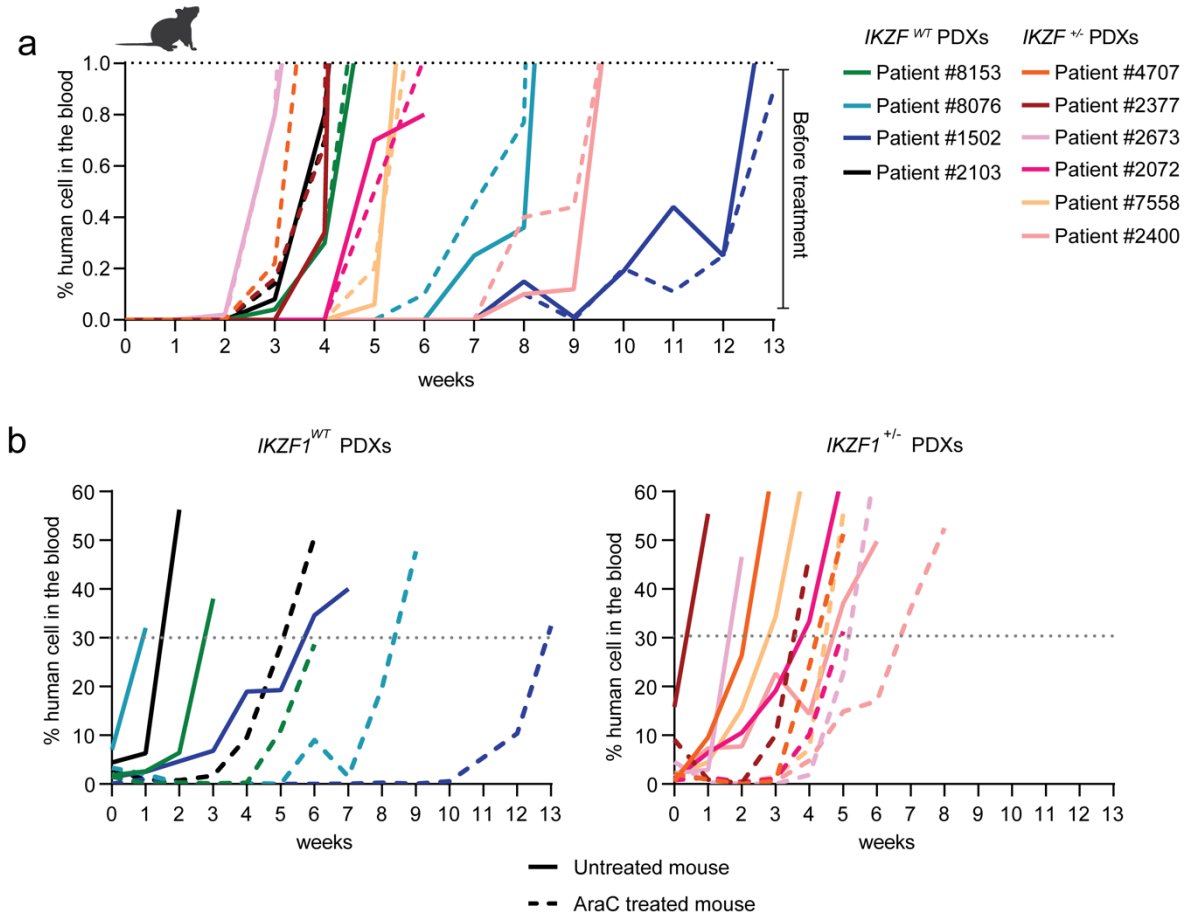
(a) Growth Curve of the Sem control versus *IKZF1*$^{-/-}$ cells (Mean ± SEM, n=3). **(b)** Relative viability as determined by a live cell staining using CyQuant in Sem control and Sem *IKZF1*$^{-/-}$ cells after a 3-day treatment with increasing concentrations (as indicated in figure) of different drugs used in the BCP-ALL treatment protocol.



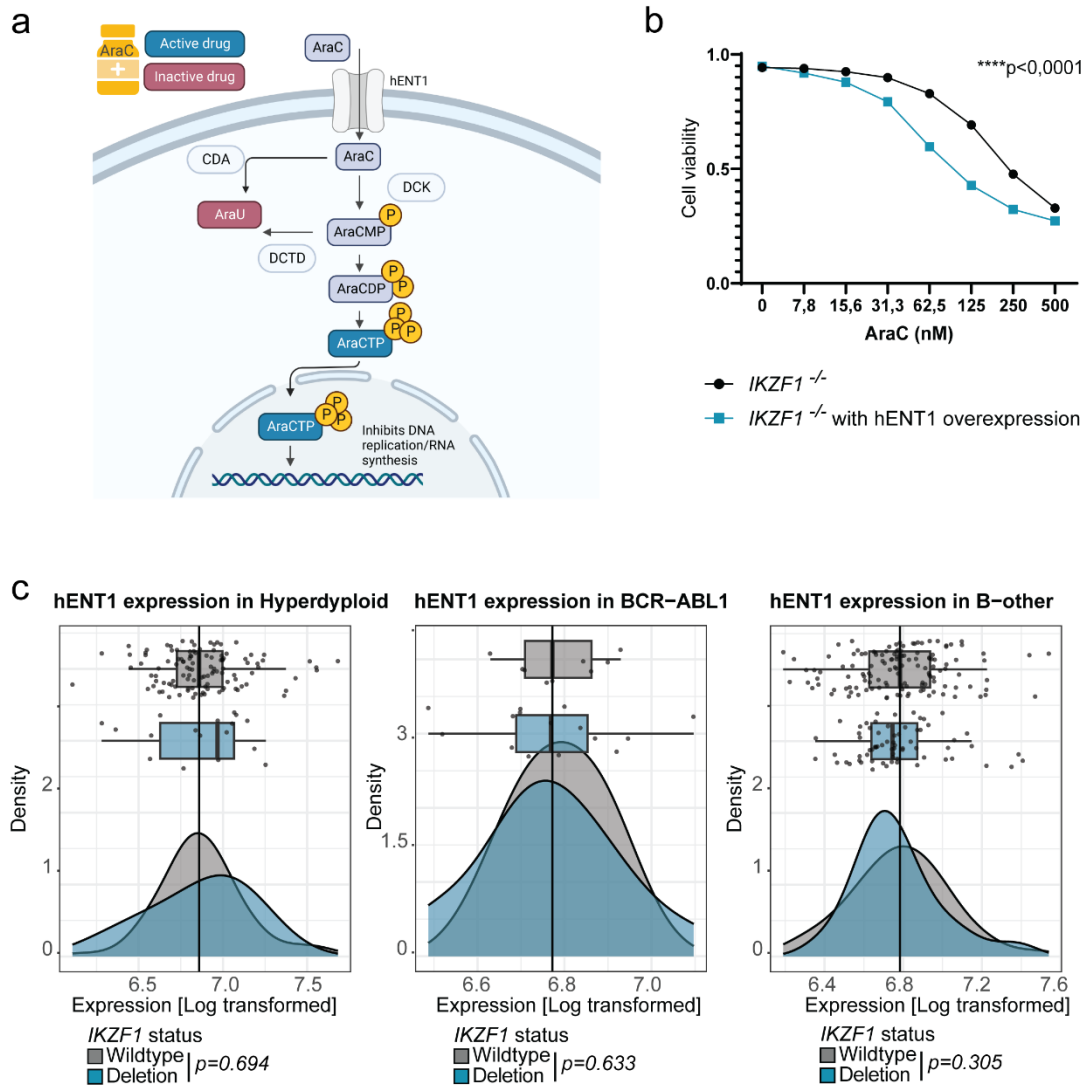
Supplemental Figure 2: Deletion of *IKZF1* drives resistance to AraC in Vitro

(a) AraC induced cell death as determined by quantification of cells in subG1 using flow cytometry of Hoechst-stained cells after a treatment of 30 nM AraC. Representative cell cycle distribution of three independent experiments. **(b)** Apoptosis induction upon AraC treatment measured by immunoblot analysis of PARP cleavage in Sem wt and Sem *IKZF1*$^{-/-}$ cells after a 3-day treatment with 0, 10 or 30 nM AraC. Representative blot of three independent

experiments. **(c)** Data from Figure 1f depicted as Area Under the Curve (AUC), (Mean ± SEM, **p*= *0.0195* unpaired T-test, two-tailed). **(d)** Collection of BCP-ALL PDX samples pharmacologically targeted by Iberdomide for degradation of the Ikaros protein. Immunoblot analysis of IKZF1 protein expression showing degradation of *IKZF1* protein in response to Iberdomide treatment after 24 hours. in different PDX samples. AraC induced cell death was determined by quantification of cells positive for amine-reactive dyes using flow cytometry after a 3-day treatment with increasing concentrations of AraC in the presence or absence of indicated doses of Iberdomide. Other tested PDX Samples (n=6) can be found in Supplemental figure 2d.

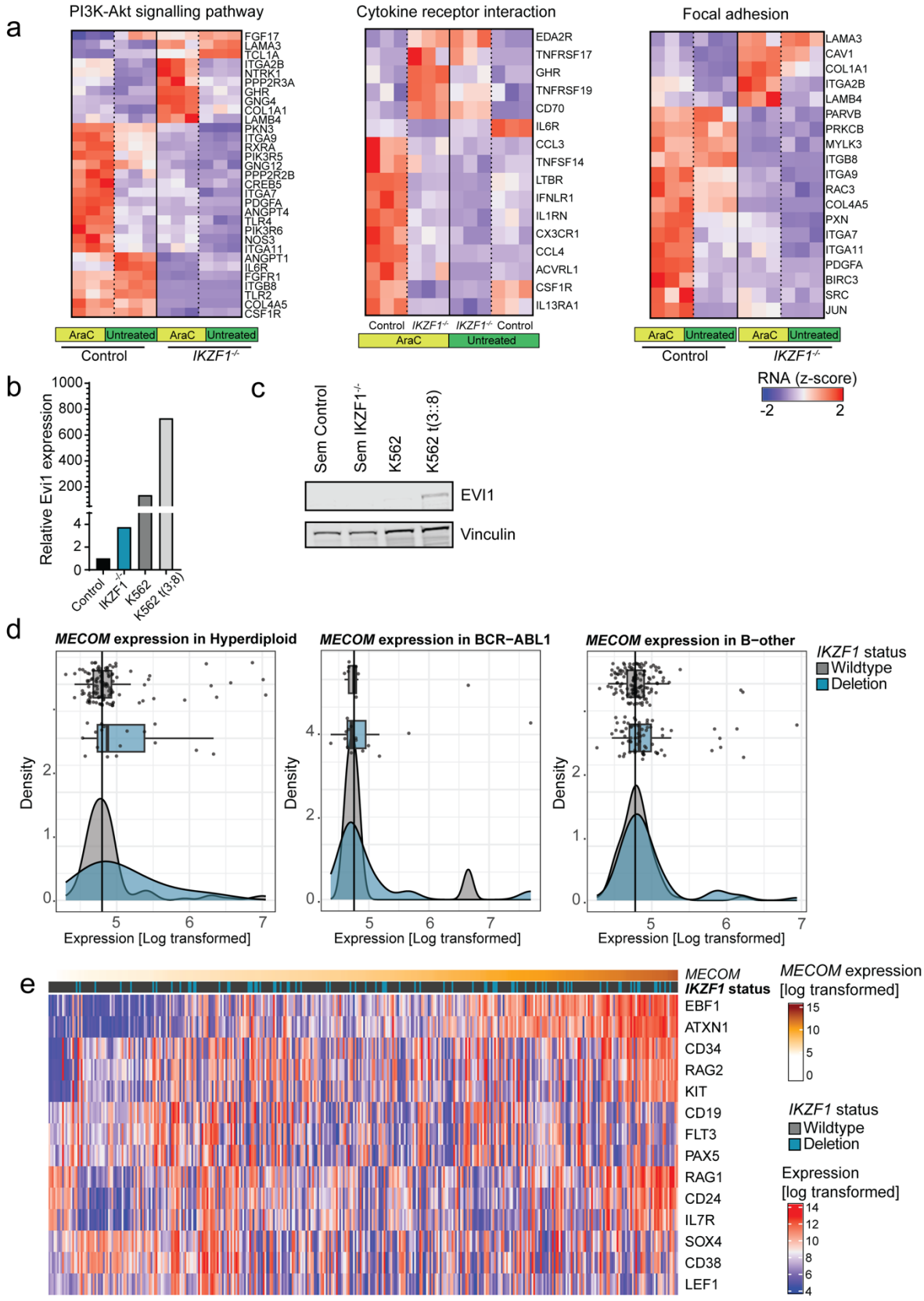


Supplemental Figure 3: In vivo Xenograft growth curves upon treatment with AraC **(a)** In vivo PDX growth curves, based on weekly measurement of % human cells in the blood from moment of Intravenous injection until start of treatment. Including here 4 PDX samples WT for *IKZF1* and 6 *IKZF1*$^{+/-}$ samples. **(b)** In vivo PDX growth curves, based on weekly measurement of % human cells in the blood, after treatment with either AraC or vehicle for 2 weeks until human endpoint was reached by either >50% of human cells in the blood, or based on condition of the mouse.



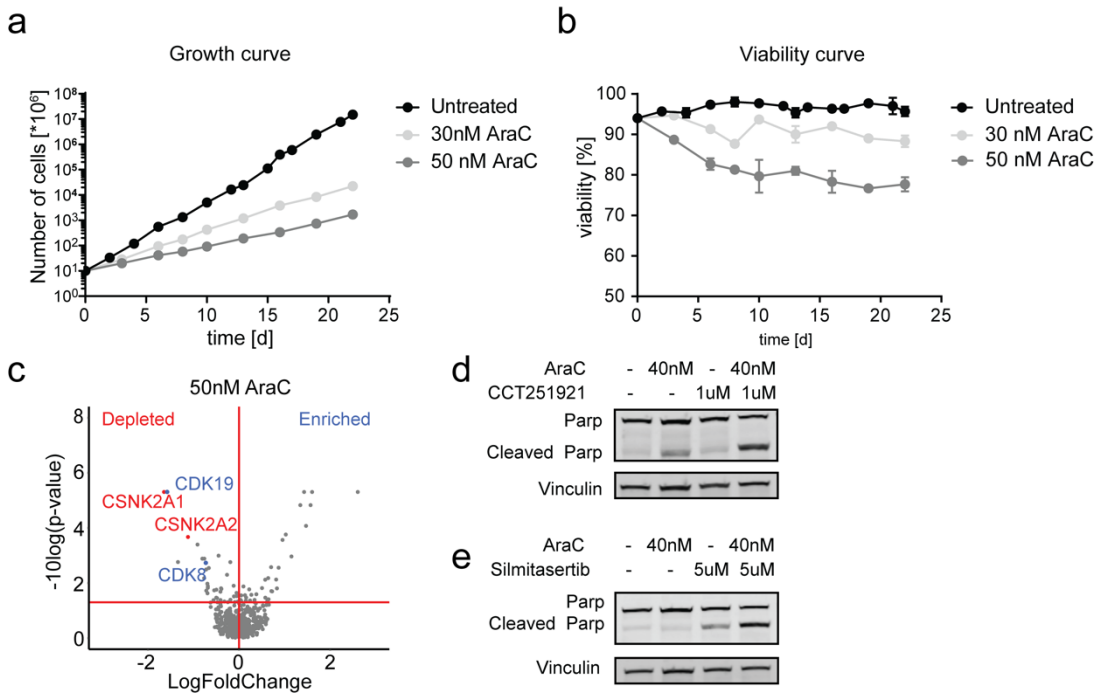
Supplemental Figure 4: the role of hENT1 in AraC metabolism and its expression in BCP-ALL subgroups

(a) Schematic representation of AraC metabolism by the cell, from cellular uptake to incorporated into DNA and RNA. Created with Biorender. **(b)** Dose response curve to 3 day AraC treatment in *IKZF1*$^{-/-}$ cells and IKZF-/- cells with an overexpression of EVI1. Each data point represents a mean (+/- SEM) of 2 independent experiments after 2 rounds of transduction. Student's T-test is performed on the AUC values (****p<0.0001) IC50 values shift from 141.6 nM to 59.4 nM AraC in cells with a hENT1 overexpression **(c)** hENT1 expression extracted from the GEO database, containing microarray gene expression data on patients harboring an *IKZF1* deletion versus patients wildtype for *IKZF1*. These plots illustrate the 3 subgroups hyperdiploid (n=130), BCR-ABL (n=24), B-other (n=198) that include >3 patients with an *IKZF1*^{del} deletion and are combined into one plot for Figure 3g.


a
PI3K-Akt signalling pathway
FGF17
LAMA3
TCL1A
ITGA2B
NTRK1
PPP2R3A
GHR
GNG4
COL1A1
LAMB4
PKN3
ITGA9
RXRA
PIK3R5
GNG12
PPP2R2B
CREB5
ITGA7
PDGFA
ANGPT4
TLR4
PIK3R6
NOS3
ITGA11
ANGPT1
IL6R
FGFR1
ITGB8
TLR2
COL4A5
CSF1R
AraC
Untreated
AraC
Untreated
Control
IKZF1-/-
Cytokine receptor interaction
EDA2R
TNFRSF17
GHR
TNFRSF19
CD70
IL6R
CCL3
TNFSF14
LTBR
IFNLR1
IL1RN
CX3CR1
CCL4
ACVRL1
CSF1R
IL13RA1
Control
IKZF1-/-
IKZF1-/-
Control
AraC
Untreated
Focal adhesion
LAMA3
CAV1
COL1A1
ITGA2B
LAMB4
PARVB
PRKCB
MYLK3
ITGB8
ITGA9
RAC3
COL4A5
PXN
ITGA7
ITGA11
PDGFA
BIRC3
SRC
JUN
AraC
Untreated
AraC
Untreated
Control
IKZF1-/-
RNA (z-score)
-2
2
b
Relative Evi1 expression
1000
800
600
400
200
4
2
0
Control
IKZF1-/-
K562
K562 t(3;8)
c
Sem Control
Sem IKZF1-/-
K562
K562 t(3;8)
EVI1
Vinculin
d
MECOM expression in Hyperdiploid
MECOM expression in BCR−ABL1
MECOM expression in B−other
IKZF1 status
Wildtype
Deletion
Density
Expression [Log transformed]
e
MECOM
IKZF1 status
EBF1
ATXN1
CD34
RAG2
KIT
CD19
FLT3
PAX5
RAG1
CD24
IL7R
SOX4
CD38
LEF1
MECOM expression
[log transformed]
15
10
5
0
IKZF1 status
Wildtype
Deletion
Expression
[log transformed]
14
12
10
8
6
4

Supplemental Figure 5: Evi1 expression in different BCP-ALL subgroups and comparison to expression in AML

(a) Heatmaps created by unsupervised clustering of the PI3K-Akt, Cytokine receptor signaling and Focal adhesion pathway, only showing genes differentially expressed between control and *IKZF1*$^{-/-}$ cells upon AraC treatment. Heatmap is created using the FPKM values transformed into Z-scores. **(b)** Relative expression of Evi1 by RT-qPCR to determine relative expression of Evi1 between BCP-ALL cell line Sem and AML cell line K562. (Mean, n=2 technical replicate). **(c)** Immunoblot analysis of Evi1 protein expression in the BCP-ALL cell line Sem and the AML cell line K562. This is a representative blot of two independent experiments. **(d)** *MECOM* expression extracted from RNA sequencing on patients harboring an *IKZF1* deletion versus patients wildtype for *IKZF1*. These plots illustrate the 3 subgroups (hyper diploid (n=130), BCR-ABL (n=24), B-other (n=198) that include >3 patients with an *IKZF1*^{del} deletion and are combined into one plot for Figure 4G. **(e)** Log transformed RNA expression levels of stemness markers in patients either Wildtype for *IKZF1* or harboring a deletion. Patients of all BCP-ALL subtypes (hyper diploid, BCR-ABL, B-other) were combined and ordered based on *MECOM* expression (n=352).



Supplemental Figure 6: Drug selection with AraC for kinase CRISPR/Cas9 screen

(a) Growth Curve of the Sem *IKZF1*$^{-/-}$ cells under selection of AraC for the CRISPR/Cas9 screen (Mean ± SEM, n=3). **(b)** Cell viability of the of the Sem *IKZF1*$^{-/-}$ cells under selection of AraC for the CRISPR/Cas9 screen (Mean ± SEM, n=3). **(c)** Vulcano-plot of gRNA targets that significantly modulate AraC response under selection of 50nM AraC. Genes are plotted based on p-value and log fold change as analyzed using the MaGeCK Test algorithm. **(d)** Apoptosis induction as measured by westernblot analysis of PARP cleavage in Sem *IKZF1*$^{-/-}$ cells after a 5-day treatment with AraC in combination with CCT251921. Representative blot of three independent experiments. **(e)** Apoptosis induction as measured by westernblot analysis of PARP cleavage in SEM *IKZF1*$^{-/-}$ cells after a 5-day treatment with AraC in combination with Silmitasertib. Representative blot of three independent experiments.