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Supplemental information

Detection and targeting of splicing deregulation in pediatric acute myeloid leukemia stem cells

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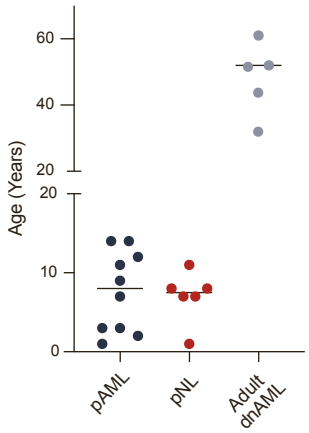
Figure S1

A



B

NGS Hematologic Malignancy Panel							
ABL1	CCND1	DDX41	GNAS	KLHL6	NRAS	RPL10	SUZ12
ASXL1	CD79B	DNM2	GNB1	KMT2A	PAX5	RPL5	TET2
ASXL2	CDKN2A_p16	DNMT1	HRAS	KMT2C	PDGFRA	RUNX1	TLR2
ATM	CDKN2A_p14	DNMT3A	IDH1	KMT2D	PDS5B	SETBP1	TP53
ATRX	CEBPA	DNMT3B	IDH2	KRAS	PHF6	SETD2	TSC2
BCL11B	CHEK2	EED	IKZF1	LUC7L2	PIK3CA	SF1	TYK2
BCOR	CNOT3	EP300	IKZF2	MAP2K1	PIM1	SF3A1	U2AF1
BCORL1	CREBBP	ETNK1	IKZF3	MEF2B	POT1	SF3B1	U2AF2
BIRC3	CRLF2	ETV6	IL2RG	MPL	PPM1D	SH2B3	NSD2
BRAF	CSF1R	EZH2	IL7R	MYD88	PRPF40B	SMARCA4	WT1
BRCC3	CSF3R	FANCL	IRF1	NF1	PRPF8	SMC1A	XPO1
BTK	CSNK1A1	FBXW7	JAK1	NFE2	PTEN	SMC3	ZRSR2
CALR	CTCF	FLT3	JAK2	NOTCH1	PTPN11	SRSF2	
CBL	CTNNB1	GATA1	JAK3	NOTCH2	RAD21	STAG2	
CBLB	CUX1	GATA2	KDM6A	NOTCH3	RB1	STAT3	
CBLC	CXCR4	GATA3	KIT	NPM1	RET	STAT5B	

Figure S1. Patient characteristics and whole exome (next generation) sequencing analyses.

Related to Figure 1.

(A) Graph of the age distribution of pediatric AML (pAML), pediatric non-leukemic (pNL) and adult *de novo* AML (adult dnAML) patient samples included in this study.

(B) NGS hematologic malignancy panel of genes (n=124) developed at UC San Diego.

Suppl Figure 2 van der Werf, et al

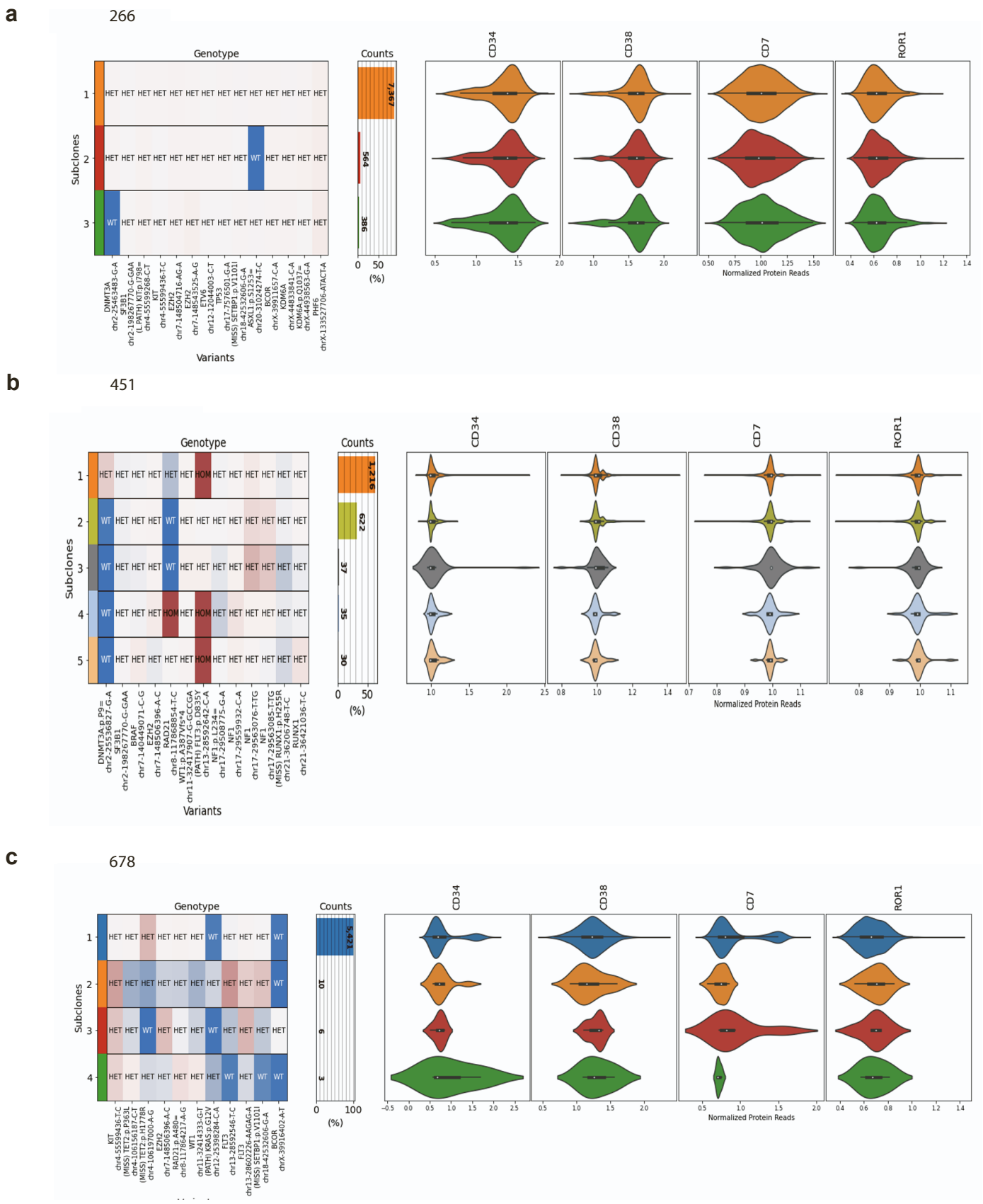


Figure S2. Single cell proteogenomic detection of clonal heterogeneity in pediatric AML. Related to Figure 2.
Single cell proteogenomic analyses of three pediatric AML (pAML) patients show DNA mutation genotype, individual cell counts in each clone, and violin plots.
(A) Patient 266. Detection of SNP variants with cell counts (left) and violin plots of immunophenotyping expression of CD34, CD38, CD7 and ROR1 are shown (right).
(B) Patient 451. Detection of SNP variants with cell counts (left) and violin plots of immunophenotyping expression of CD34, CD38, CD7 and ROR1 are shown (right).
(C) Patient 678. Detection of SNP variants with cell counts (left) and violin plots of immunophenotyping expression of CD34, CD38, CD7, and ROR1 are shown (right).

Figure S3

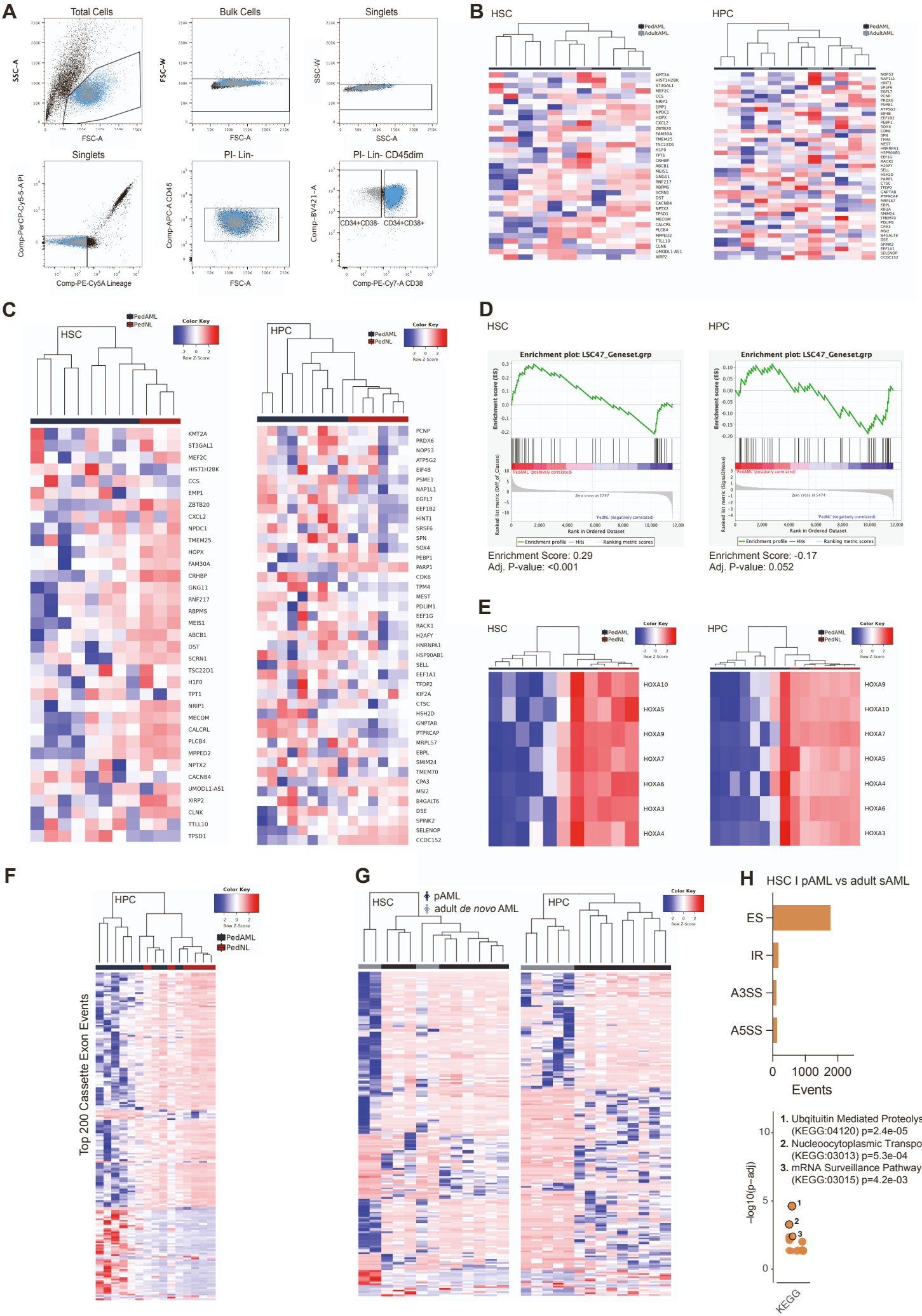

A
Total Cells
Bulk Cells
Singlets
SSC-A
FSC-A
FSC-W
SSC-W
Singlets
PI- Lin-
PI- Lin- CD45dim
Comp-PerCP-Cy5-5-A PI
Comp-PE-Cy5A Lineage
Comp-APC-A CD45
Comp-BV421-A
CD34+CD38-
CD34+CD38+
Comp-PE-Cy7-A CD38
B
HSC
HPC
PedAML
AdultAML
C
HSC
HPC
PedAML
PedNL
Color Key
Row Z-Score
KMT2A
ST3GAL1
MEF2C
HIST1H2BK
CCS
EMP1
ZBTB20
CXCL2
NPDC1
TMEM25
HOPX
FAM30A
CRHBP
GNG11
RNF217
RBPMS
MEIS1
ABCB1
DST
SCRN1
TSC22D1
H1F0
TPT1
NRIP1
MECOM
CALCRL
PLCB4
MPPED2
NPTX2
CACNB4
UMODL1-AS1
XIRP2
CLNK
TTLL10
TPSD1
PCNP
PRDX6
NOP53
ATP5G2
EIF4B
PSME1
NAP1L1
EGFL7
EEF1B2
HINT1
SRSF6
SPN
SOX4
PEBP1
PARP1
CDK6
TPM4
MEST
PDLIM1
EEF1G
RACK1
H2AFY
HNRNPA1
HSP90AB1
SELL
EEF1A1
TFDP2
KIF2A
CTSC
HSH2D
GNPTAB
PTPRCAP
MRPL57
EBPL
SMIM24
TMEM70
CPA3
MSI2
B4GALT6
DSE
SPINK2
SELENOP
CCDC152
D
HSC
HPC
Enrichment plot: LSC47_Geneset.grp
Enrichment score (ES)
Ranked list metric (Diff_of_Classes)
Ranked list metric (Signal2Noise)
'PedAML' (positively correlated)
'PedNL' (negatively correlated)
Zero cross at 5797
Zero cross at 5474
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
Enrichment Score: 0.29
Adj. P-value: <0.001
Enrichment Score: -0.17
Adj. P-value: 0.052
E
HSC
HPC
PedAML
PedNL
Color Key
Row Z-Score
HOXA10
HOXA5
HOXA9
HOXA7
HOXA6
HOXA3
HOXA4
HOXA9
HOXA10
HOXA7
HOXA5
HOXA4
HOXA6
HOXA3
F
HPC
PedAML
PedNL
Color Key
Row Z-Score
Top 200 Cassette Exon Events
G
HSC
pAML
adult de novo AML
HPC
Color Key
Row Z-Score
H
HSC I pAML vs adult sAML
ES
IR
A3SS
A5SS
0
1000
2000
Events
1. Ubqituitin Mediated Proteolysis (KEGG:04120) p=2.4e-05
2. Nucleoocytoplasmic Transport (KEGG:03013) p=5.3e-04
3. mRNA Surveillance Pathway (KEGG:03015) p=4.2e-03
−log10(p−adj)
10
5
0
KEGG

Figure S3. RNA-sequencing based hematopoietic stem cell and hematopoietic progenitor cell gene expression patterns in pediatric AML. Related to Figure 3.

(A) FACS gating strategy of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs).

(B) RNA sequencing (RNA-seq) derived heatmap of normalized gene expression levels after unsupervised hierarchical clustering of pediatric AML (pAML) versus adult *de novo* AML (dnAML) HSCs (left) and HPCs (right). Genes were selected for the heatmap based on a single-cell RNA-seq study of adult dnAML.[29]

(C) Heatmap of normalized expression levels after unsupervised hierarchical clustering of pAML versus pediatric non-leukemic (pNL) HSCs (left) and HPCs (right).[29]

(D) GSEA enrichment plot of the LSC47 gene signature shows significant enrichment in pAML versus age-matched non-leukemic derived HSCs (left) and HPCs (right).

(E) Expression of HOXA genes in pAML versus age-matched non-leukemic HSCs (left) and HPCs (right). Note, LSC47 signature is based on a study in adults.

(F) Heatmap of top 200 distinct splice isoforms in HPCs derived from pAML versus age-matched non-leukemic cells.

(G) Heatmap of top 200 distinct splice isoforms in HSCs (left) and HPCs (right) derived from pAML adult dnAML.

(H) Amount of differential splicing events in HSCs derived from pAML versus adult secondary AML (sAML; top). Functional enrichment analysis of HSCs derived from pediatric AML versus HSCs derived from adult sAML (bottom). Hematopoietic stem cells (HSCs); Hematopoietic progenitor cells (HPCs).

Figure S4

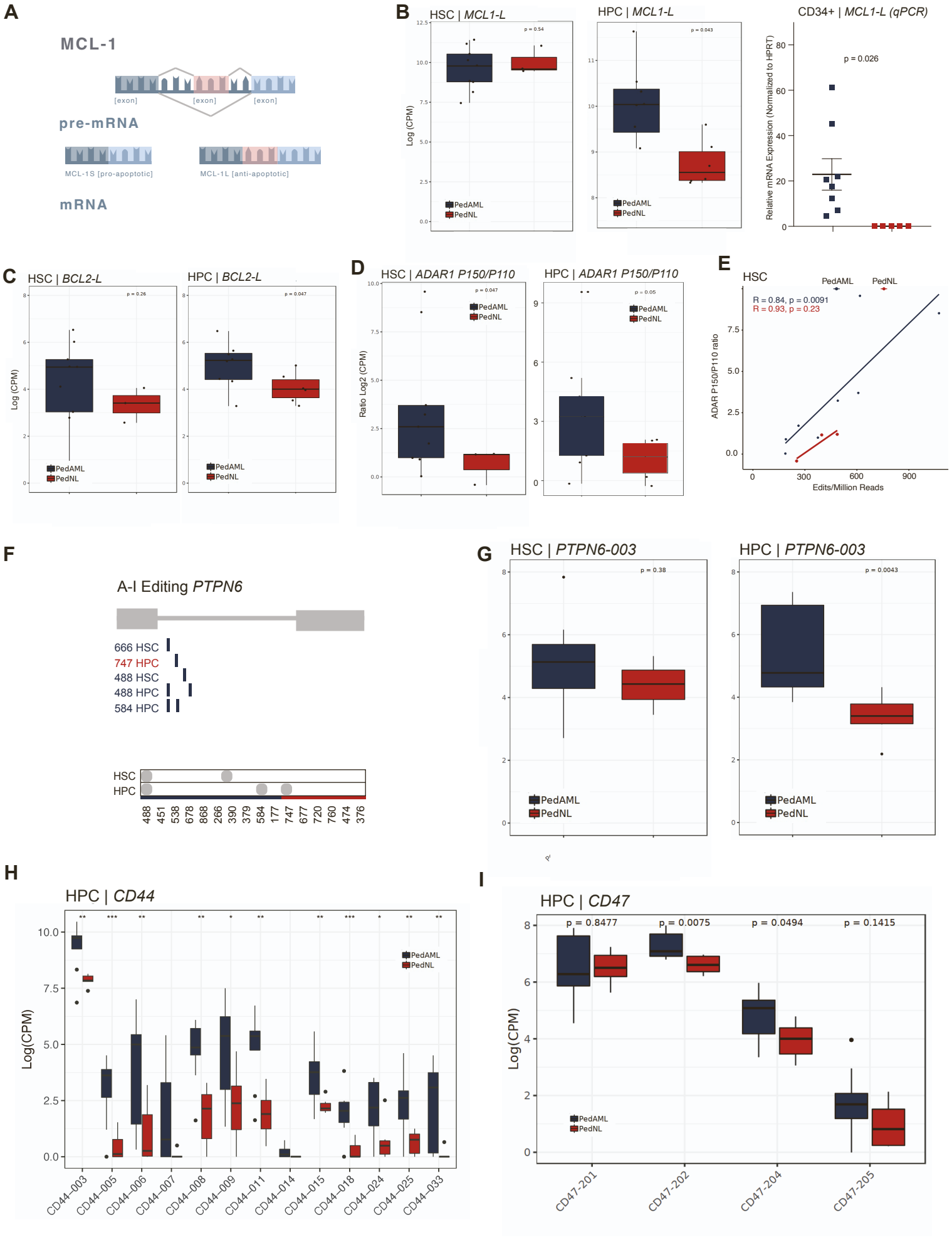

A
MCL-1
[exon]
[exon]
[exon]
pre-mRNA
MCL-1S [pro-apoptotic]
MCL-1L [anti-apoptotic]
mRNA
B
HSC | MCL1-L
HPC | MCL1-L
CD34+ | MCL1-L (qPCR)
Log (CPM)
Relative mRNA Expression (Normalized to HPRT)
p = 0.026
PedAML
PedNL
C
HSC | BCL2-L
HPC | BCL2-L
D
HSC | ADAR1 P150/P110
HPC | ADAR1 P150/P110
Ratio Log2 (CPM)
E
HSC
R = 0.84, p = 0.0091
R = 0.93, p = 0.23
ADAR P150/P110 ratio
Edits/Million Reads
F
A-I Editing PTPN6
666 HSC
747 HPC
488 HSC
488 HPC
584 HPC
HSC
HPC
488 451 538 678 868 266 390 379 584 177 747 677 720 760 474 376
G
HSC | PTPN6-003
HPC | PTPN6-003
H
HPC | CD44
Log(CPM)
CD44-003 CD44-005 CD44-006 CD44-007 CD44-008 CD44-009 CD44-011 CD44-014 CD44-015 CD44-018 CD44-024 CD44-025 CD44-033
I
HPC | CD47
p = 0.8477
p = 0.0075
p = 0.0494
p = 0.1415
CD47-201
CD47-202
CD47-204
CD47-205

Figure S4. Differential splicing in pediatric AML hematopoietic stem cells and hematopoietic progenitor cells. Related to Figure 4.

(A) Schematic of MCL-1 splicing.

(B) Transcript levels of *MCL1-L* based on RNA-seq of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) in pediatric AML (pAML, n=8 biological replicates) and age-matched controls (pNL, n=5 biological replicates), and qRT-PCR of $CD34^+$ selected cells derived from pAML and cord blood.

(C) RNA-seq-based expression levels of *BCL2-L* in HSCs and HPCs derived from pAML and pNL.

(D) RNA-seq-based expression levels the ratio *ADAR1 p150* to *ADAR1 p110* in HSCs and HPCs derived from pAML and pNL.

(E) Correlation of the ADAR ratio versus the number of edits per million in pAML and pNL HSCs.

(F) Adenosine to inosine (A-I) RNA editing events detected in *PTPN6*.

(G) RNA-seq-based expression levels of *PTPN6-003* in pAML and pNL HSCs and HPCs.

(H) RNA-seq-based expression of splice isoforms of *CD44* in HPCs (* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.005$).

(I) RNA-seq-based quantification of splice isoforms of *CD47* in HPCs.

Figure S5

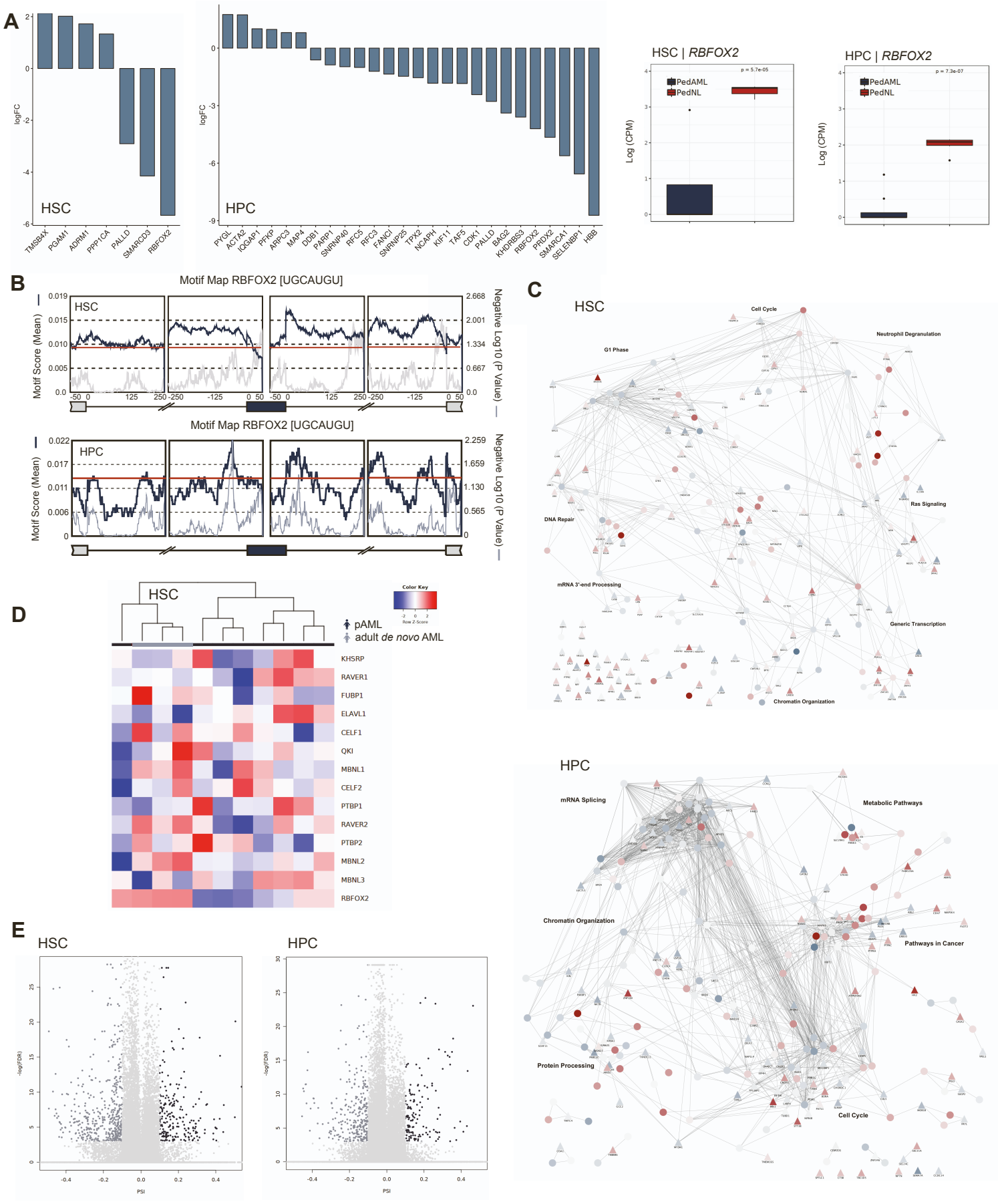

A
HSC
HPC
logFC
TMSB4X
PGAM1
ADRM1
PPP1CA
PALLD
SMARCD3
RBFOX2
PYGL
ACTA2
IQGAP1
PFKP
ARPC3
MAP4
DDB1
PARP1
SNRNP40
RFC5
RFC3
FANCI
SNRNP25
TPX2
NCAPH
KIF11
TAF5
CDK1
PALLD
BAG2
KHDRBS3
RBFOX2
PRDX2
SMARCA1
SELENBP1
HBB
HSC | RBFOX2
HPC | RBFOX2
PedAML
PedNL
Log (CPM)
B
Motif Map RBFOX2 [UGCAUGU]
Motif Score (Mean)
Negative Log10 (P Value)
C
HSC
Cell Cycle
Neutrophil Degranulation
G1 Phase
Ras Signaling
DNA Repair
mRNA 3'-end Processing
Generic Transcription
Chromatin Organization
HPC
mRNA Splicing
Metabolic Pathways
Chromatin Organization
Pathways in Cancer
Protein Processing
Cell Cycle
D
HSC
Color Key
Row Z-Score
pAML
adult de novo AML
KHSRP
RAVER1
FUBP1
ELAVL1
CELF1
QKI
MBNL1
CELF2
PTBP1
RAVER2
PTBP2
MBNL2
MBNL3
RBFOX2
E
HSC
HPC
-log(FDR)
PSI

Figure S5. Decreased expression of RBFOX2 in pediatric AML and LSC survival gene expression. Related to Figure 5.

(A) RNA-seq based quantification of significant differentially expressed human-specific spliceosome components in hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) between pediatric AML (pAML) and pediatric non-leukemic (pNL). Genes with FDR <0.05 are shown (left). RNA-seq quantification of *RBFOX2* transcript levels in HSCs and HPCs. P-values are based on student's t-test (right).

(B) rMAPS motif enrichment analysis output for RBFOX2 consensus binding site for all identified splice isoforms in HSCs (top) and HPCs (bottom; false discovery rate; FDR <0.05, percent spliced in; PSI >0.1). The blue line indicates motif score while the red line indicates Log10 (p-value), and the grey line represents the background. The RBFOX2 motif score is significantly enriched if Log10 (p-value) is 1.3, reminiscent of a p-value lower than 0.05.

(C) Network analysis of differentially spliced RBFOX2 target genes in pAML compared to pNL HSCs (left) and HPCs (right). Top 200 exon skipping events in this comparison were used as seeds for network propagation on the STRING high confidence interactome. Differential expression log fold change is mapped to node color. Triangles depict seed list genes and circles present associated genes.

(D) Heatmap of expression levels after unsupervised clustering of RBFOX2 associated splicing factors in pediatric AML versus adult de novo AML derived HSCs.

(E) Volcano plot highlighting RBFOX2 target genes that present differential exon usage in pediatric AML (FDR<0.05, PSI>0,1). A negative PSI reflects exon inclusion in pediatric AML while a positive PSI reflects exon skipping in pediatric AML versus adult AML-derived HSCs (left) and HPCs (right).

Figure S6

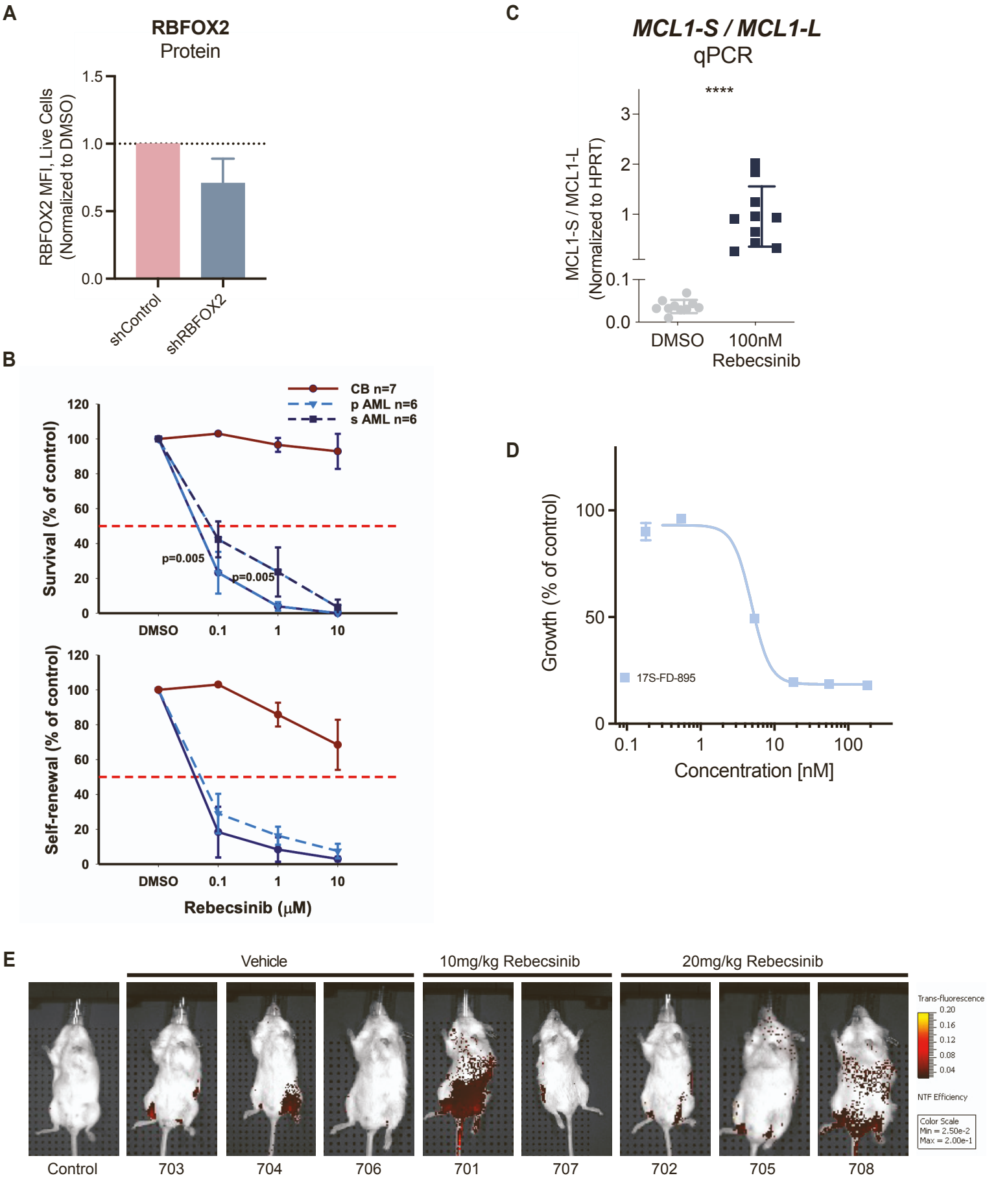

A
RBFOX2
Protein
RBFOX2 MFI, Live Cells
(Normalized to DMSO)
1.5
1.0
0.5
0.0
shControl
shRBFOX2
C
MCL1-S / MCL1-L
qPCR
****
MCL1-S / MCL1-L
(Normalized to HPRT)
3
2
1
0.1
0.0
DMSO
100nM
Rebecsinib
B
CB n=7
p AML n=6
s AML n=6
Survival (% of control)
120
100
80
60
40
20
0
p=0.005
p=0.005
DMSO
0.1
1
10
Self-renewal (% of control)
Rebecsinib (μM)
D
Growth (% of control)
100
50
0
17S-FD-895
0.1
1
10
100
Concentration [nM]
E
Vehicle
10mg/kg Rebecsinib
20mg/kg Rebecsinib
Trans-fluorescence
0.20
0.16
0.12
0.08
0.04
NTF Efficiency
Color Scale
Min = 2.50e-2
Max = 2.00e-1
Control
703
704
706
701
707
702
705
708

Figure S6. Rebecsinib-mediated inhibition of pediatric AML *in vitro* and *in vivo*. Related to Figure 6.

(A) Flow cytometric analysis of RBFOX2 protein levels following lentiviral shControl or lentiviral shRBFOX2 transduction of cord blood derived $CD34^+$ selected cells for 48h (n=7 biological replicates).

(B) Survival and self-renewal of $CD34^+$ cells derived from pediatric AML (pAML, n=6 biological replicates), cord blood or adult secondary AML (sAML, n=9 biological replicates) in long-term stromal co-cultures. These data represent the mean +/- SD for each treatment condition. Statistical analysis was performed by one-way ANOVA**.**

(C) qRT-PCR mRNA expression of the ratio *MCL1-S* to *MCL1-L* in pAML samples treated with 100 nM Rebecsinib; p values are based on the Mann-Whitney U test (n=10 biological replicates).

(D) MTT assay of MOLM13 cells incubated with a range of Rebecsinib (n=3 technical replicates)**.**

(E) Live animal epifluor imaging 24h after Rebecsinib treatment.

Table S1. Patient Demographics. Related to Figure 1.

Sample ID	Gender	Age	Cell Source	Blast %	Diagnosis	Cytogenetics
488	M	7	PB	100	AML	Missing
451	M	9	PB	91	AML	Missing
538	F	14	PB	54	AML	inv(16)
678	M	1	PB	21	AML	inv(16)
868	M	2	PB	90	AML	t(15;17)
266	F	11	BM	65	AML	CEBPA
390	M	12	BM	54	AML	t(8;21)
379	F	3	PB	91	AML	Missing
584	M	14	PB	52	AML	Missing
177	M	3	BM	30	AML	t(8;21)
1474	M	52	PB	83	de novo AML	inv(16), KIT, NRAS
251	F	32	BM	32	de novo AML	inv(16), NRAS, KRAS
023	M	52	BM	56	de novo AML	CEBPA, NRAS, WT1
220	M	44	BM	37	de novo AML	MLL-PTD, STAG2
682	M	61	BM	34	de novo AML	t(8;21)
747	F	7	BM	NA	Complete Remission	NA
677	F	7	BM	NA	Healthy Donor	NA
720	F	8	BM	NA	Healthy Donor	NA
760	M	1	BM	NA	Lymphoma without BM involvement	NA
474	M	11	BM	NA	Lymphoma without BM involvement	NA
376	M	8	BM	NA	Lymphoma without BM involvement	NA

Male (M); Female (F); Age in years; Bone marrow (BM); Peripheral blood (PB); Acute myeloid leukemia (AML)

Table S4. Primer Sequences. Related to Figure 6.

Transcript	Species	Primer FW sequence (5'-3')	Primer REV sequence (5'-3')
HPRT	Human	TCAGGGATTTGAATCATGTTTGTG	CGATGTCAATAGGACTCCAGATG
MCL1-L	Human	AGACCTTACGACGGGTTGG	AATCCTGCCCCAGTTTGTTA
MCL1-S	Human	GAGGAGGACGAGTTGTACCG	ACTCCACAAACCCATCCTTG