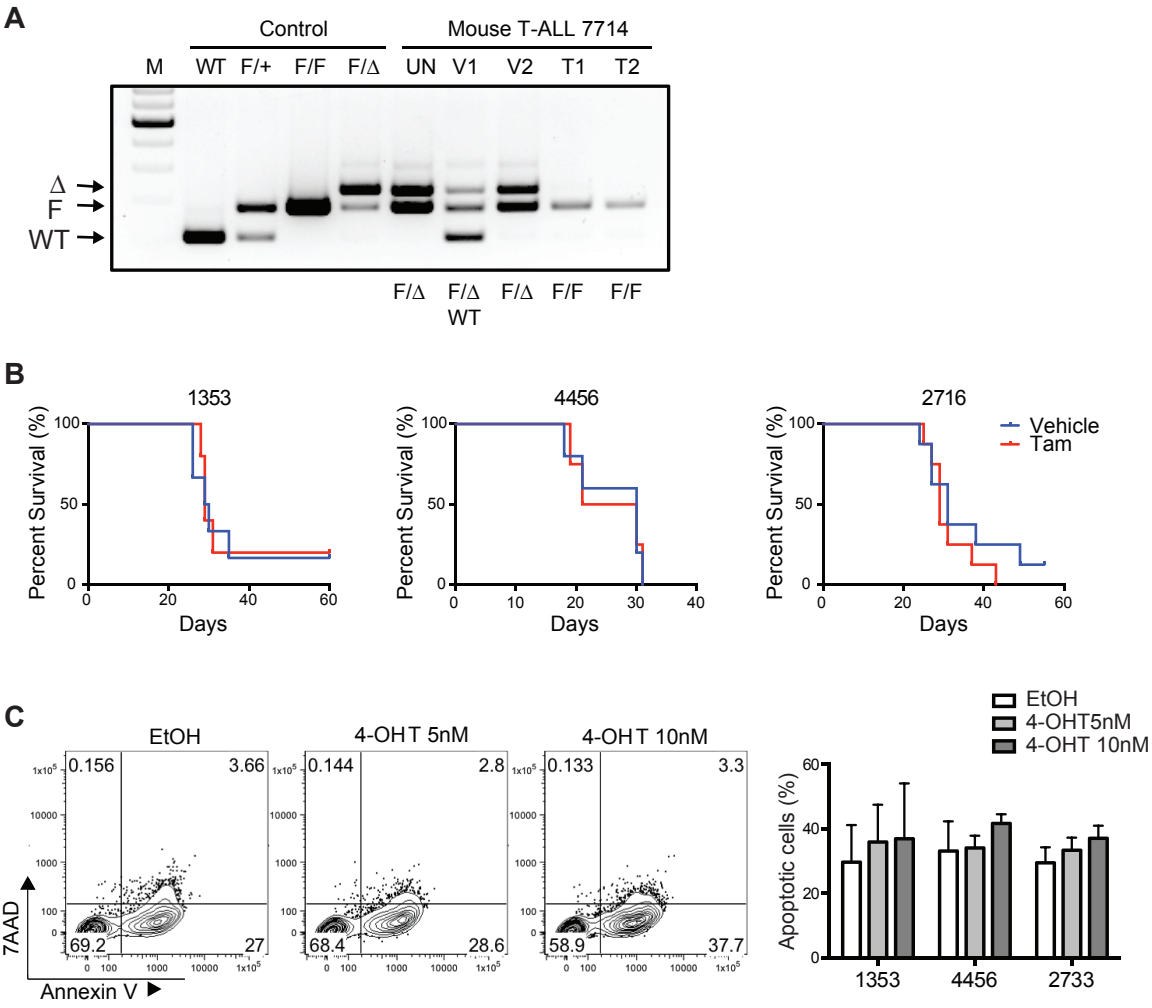
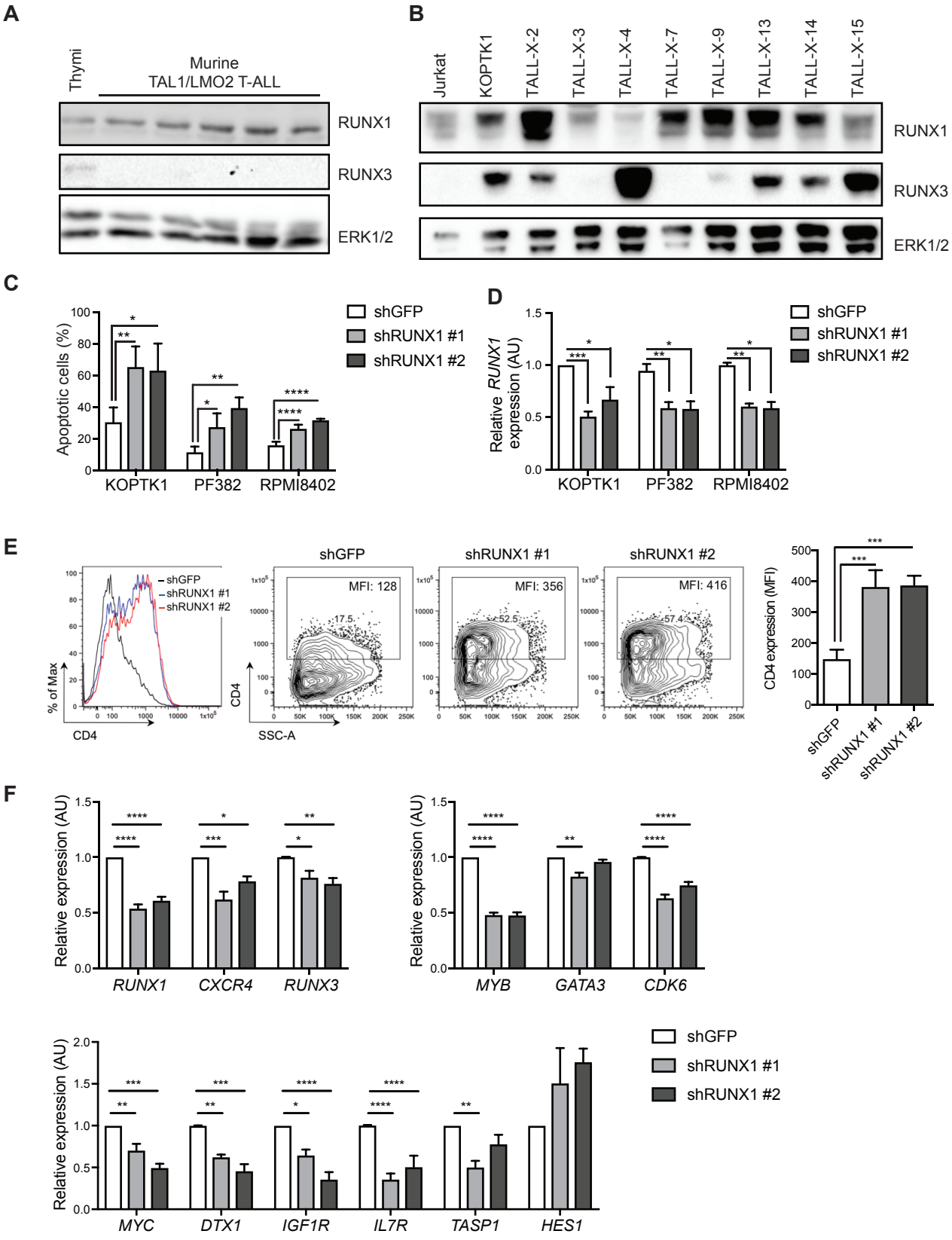
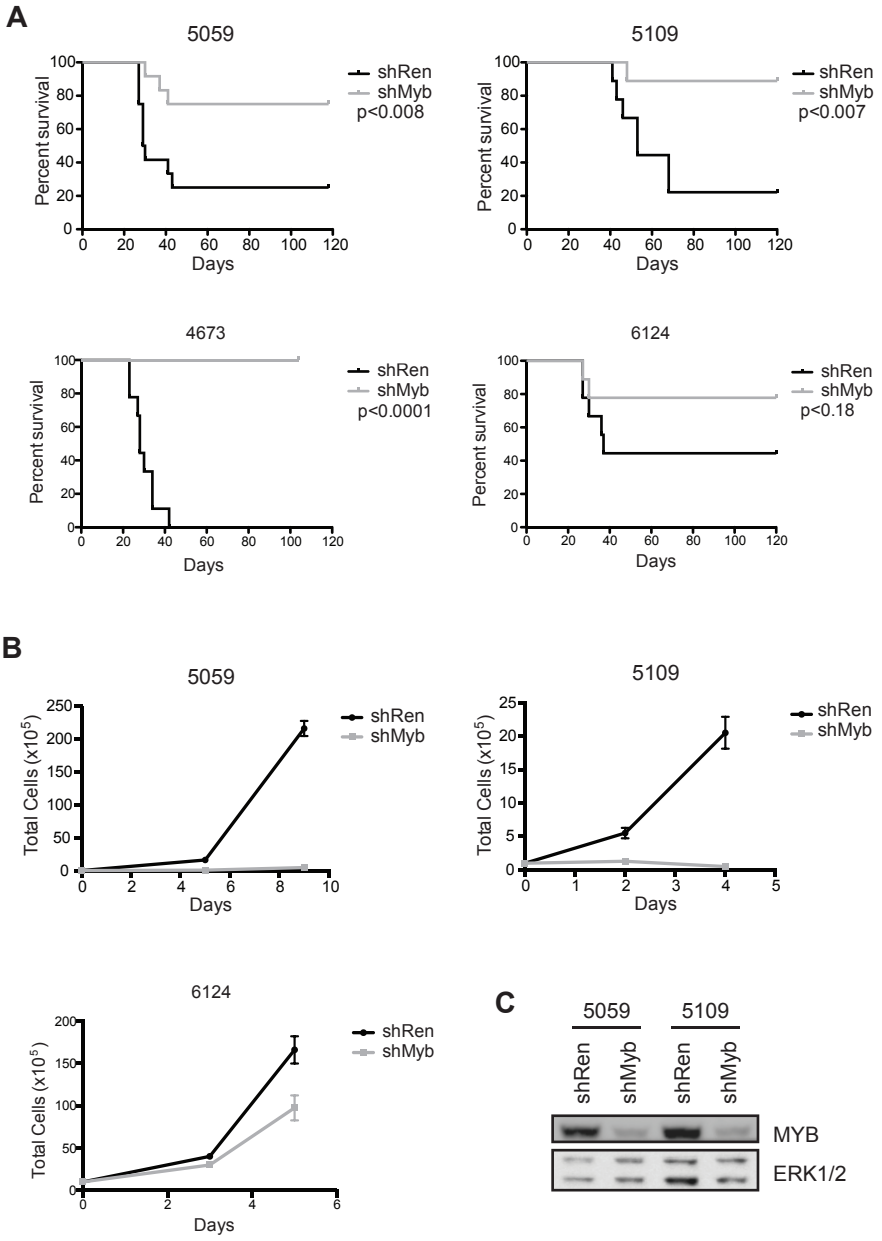
Supplementary Figure 1



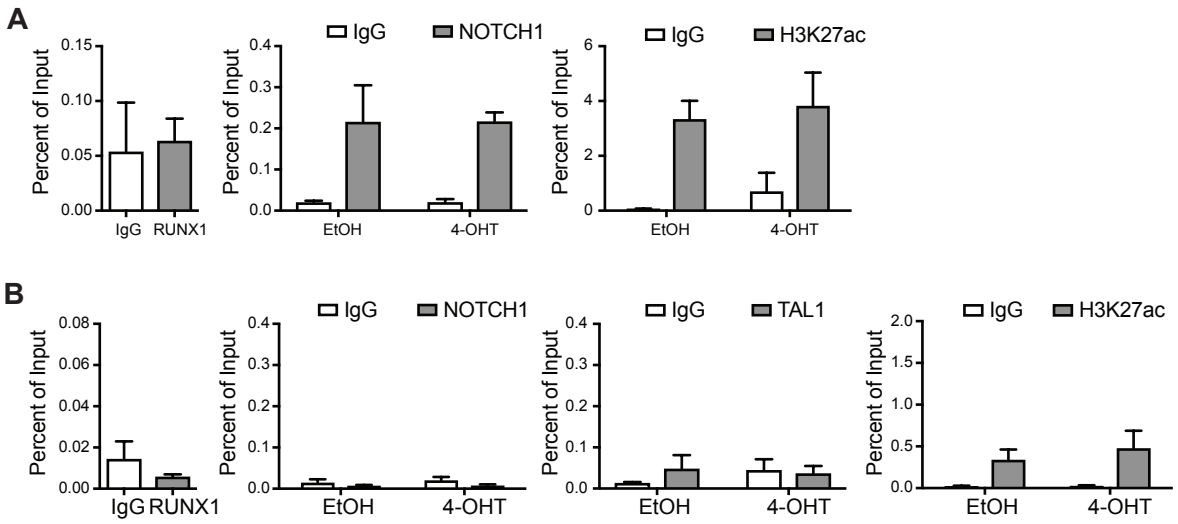
Supplementary Figure 2



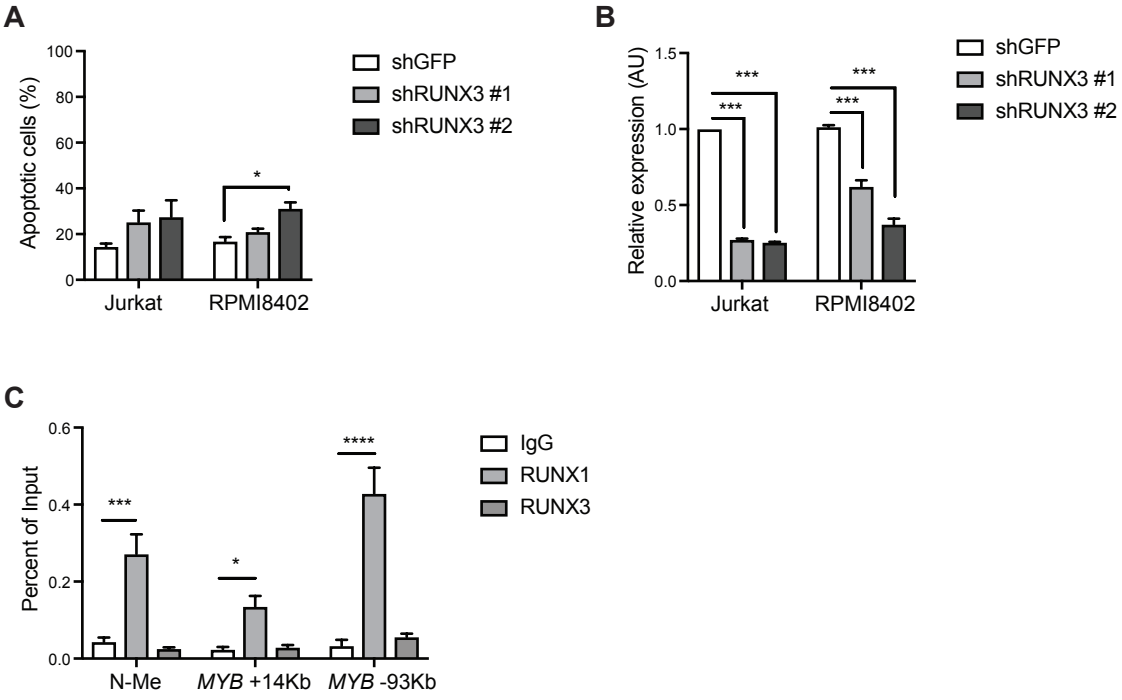
Supplementary Figure 3



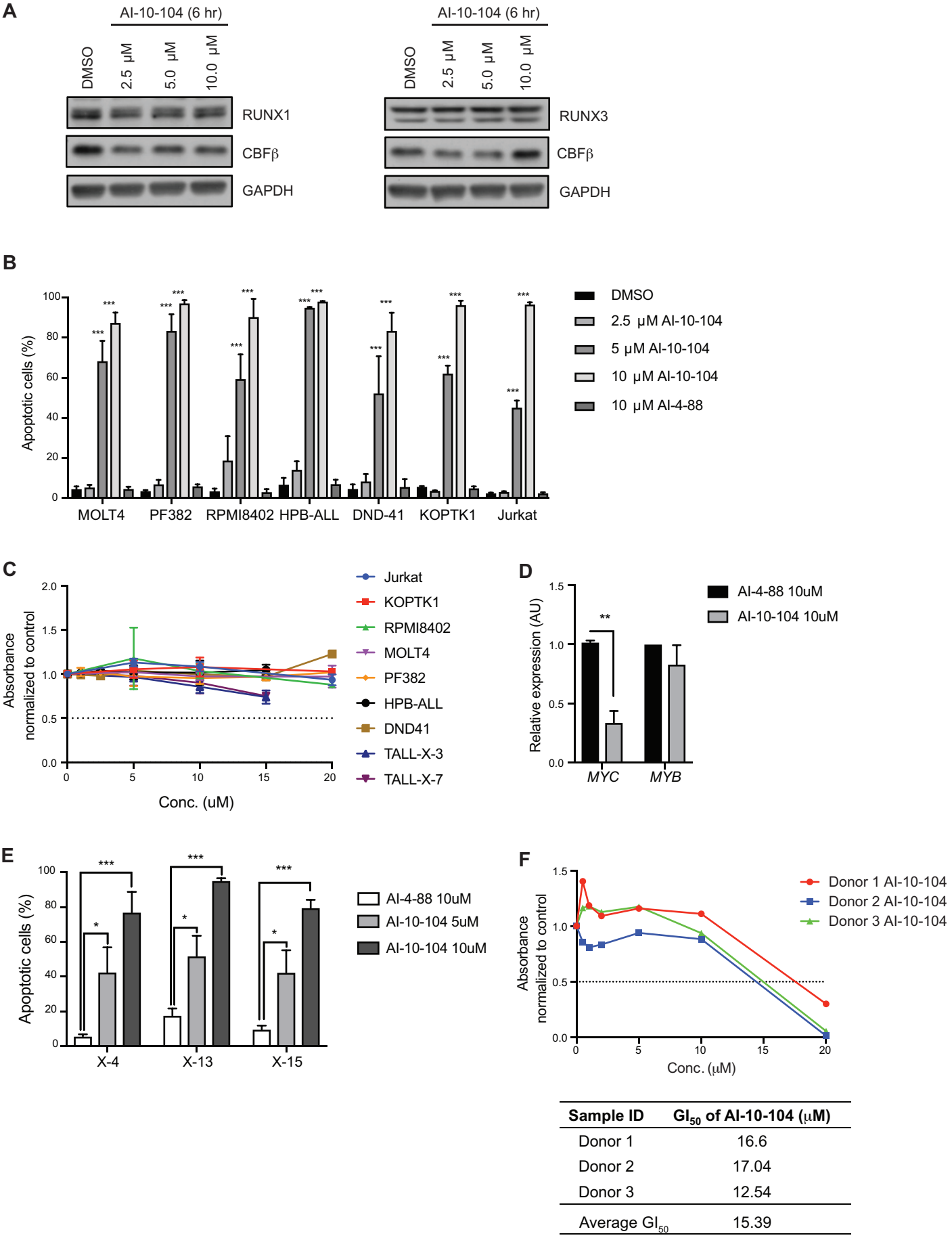
Supplementary figure 4



Supplementary Figure 5



Supplementary Figure 6



Sample ID	GI_{50} of AI-10-104 (μM)
Donor 1	16.6
Donor 2	17.04
Donor 3	12.54
Average GI_{50}	15.39

Supplementary Table 1. Expression and mutation status in patient samples and human T-ALL cell lines.

Patient samples

	RUNX1	TAL1 detected[+]	NICD detected[+]	NOTCH1	FBW7
TALL-X-1	WT	Yes	No	WT	WT
TALL-X-2	WT	Yes	Yes	p.P2514fs, p.F1592S	WT
TALL-X-3	WT	Yes	Yes	p.L1593fs	WT
TALL-X-4	WT	Yes	Yes	p.F1592S	WT
TALL-X-5	WT	Yes	Yes	p.L1585P	WT
TALL-X-7	WT	Yes	Yes	p.L1600P	p.R479Q
TALL-X-8	WT	Yes	Yes	p.R1598P	p.T450fs, p.R689W
TALL-X-9	WT	No	Yes	p.R1598P	WT
TALL-X-13	WT	Yes	Yes	WT	WT
TALL-X-14	WT	No	No	WT	p.R465H
TALL-X-15	WT	Yes	Yes	WT	WT

Human cell lines

	RUNX1	RUNX3	NOTCH1	FBW7
Jurkat	p.A122T	WT	WT	p.R505C
KOPTK1	N/A	N/A	p.L1600P, p.P2514fs*4	WT
RPMI8402	WT	WT	p.E1583_Q1584insPVELMPPE, p.Q1584>HRGADAAGA	p.R465H
MOLT4	WT	WT	p.L1600P, p.P2515fs	WT
PF382	WT	WT	p.L1574P, p.P2493fs*>67	WT
HPB-ALL	WT	WT	p.L1574P, p.D2443fs*39	p.R465H, p.D527G
DND41	WT	WT	p.L1593P, p.D1609V, p.V2444fs*35	WT
TALL-1	WT	WT	WT	WT
LOUCY	WT	WT	WT	WT

RUNX3 mutation status in patient samples is not available.

[+]As determined by western blot analysis.

N/A, Not available

Supplementary Table 2. Human T-ALL cell lines and primary patient samples are sensitive to AI-10-104 treatment.

	GI_{50} ± SEM(μM)
Cell Line	
Jurkat	8.2 ± 0.4
KOTPK1	5.9 ± 0.5
PRMI8402	4.3 ± 0.4
MOLT4	5.7 ± 0.3
PF382	5.8 ± 0.3
HPB-ALL	6.6 ± 0.5
DND-41	5.2 ± 0.5
TALL-1	7.0 ± 0.5
LOUCY*	11.0 ± 1.1
Average	6.1
Patient sample	
Diagnostic	
TALL-X-1	0.9 ± 0.1
TALL-X-2	2.8 ± 0.6
TALL-X-3	1.1 ± 0.1
TALL-X-4	4.3 ± 6.2
TALL-X-5	4.4 ± 1.0
TALL-X-7	0.7 ± 0.1
TALL-X-8	2.7 ± 0.8
TALL-X-9	0.9 ± 0.1
Relapsed	
TALL-X-13	1.3 ± 0.1
TALL-X-14	3.0 ± 0.5
TALL-X-15	4.3 ± 0.8
Average	2.4

GI_{50} values of the inhibitor are shown for each cell line and patient sample analyzed 2 or 3 times.

*GI_{50} of LOUCY cell line is excluded from the average of GI_{50}.

Supplementary Table 3. qPCR primers for mouse genes analyzed in Figure 3

Gene symbol	Direction	Primer sequence (5' to 3')
Actin	For	CGAGGCCCAGAGCAAGAGAG
	Rev	CGGTTGGCCTTAGGGTTCAG
Runx1	For	GCCATGAAGAACCAGGTAGC
	Rev	GCCGTCCACTGTGATTTTG
Cxcr4	For	GAAGTGGGTTCTGGAGACTAT
	Rev	TTGCCGACTATGCCAGTCAAG
Bcl2	For	ATGCCTTTGTGGAACTATATGGC
	Rev	GGTATGCACCCAGAGTGATGC
Cd4	For	TCCTAGCTGTCACTCAAGGGA
	Rev	TCAGAGAACTTCCAGGTGAAGA
Cdkn1a	For	CCTGGTGATGTCCGACCTG
	Rev	CCATGAGCGCATCGCAATC
Cdk6	For	GGCGTACCCACAGAAACCATA
	Rev	AGGTAAGGGCCATCTGAAAACT
Myb	For	GAGCACCCAACTGTTCTC
	Rev	CACCAGGGGCCTGTTCTTAG
Gata3	For	GAAGGCATCCAGACCCGAAAC
	Rev	ACCCATGGCGGTGACCATGC
Notch1	For	CCCTTGCTCTGCCTAACGC
	Rev	GGAGTCCTGGCATCGTTGG
Myc	For	TCTGTGGAGAAGAGGCAAACCC
	Rev	TAGTTGTGCTGGTGAGTGGAGACG
Il7r	For	GCGGACGATCACTCCTTCTG
	Rev	AGCCCCACATATTTGAAATTCCA
Hes1	For	AAGACGGCCTCTGAGCACA
	Rev	CCTTCGCCTCTTCTCCATGAT
Dtx1	For	TGCCTGGTGGCCATGTACTC
	Rev	GACACTGCAGGCTGCCATCC
Igf1r	For	CATGTGCTGGCAGTATAACCC
	Rev	CCACAGGATACAGGCAGCTAT
Tasp1	For	TGCTGCTAAAGTAACAGTCAAGG
	Rev	TGACAAGCTCGTTTGCATACA

Supplementary Table 4. shRNA clones used in the study

shRNA clone	Clone ID	Target gene
shRUNX1 #1	TRCN0000013659	*RUNX1*
shRUNX1 #2	TRCN0000013660	*RUNX1*
shRUNX3 #1	TRCN0000235675	*RUNX3*
shRUNX3 #2	TRCN0000235674	*RUNX3*
shCBFβ#1	TRCN0000016644	*CBFβ*
shCBFβ#2	TRCN0000016645	*CBFβ*

Supplementary Table 5. qPCR primers for human genes analyzed in Supplementary Figure 2F

Gene symbol	Direction	Primer sequence (5' to 3')
GAPDH	For	CTCCTCTGACTTCAACAGCGACAC
	Rev	TGCTGTAGCCAAATTCGTTGTCAT
RPS9	For	TGTCGCAAAACTTATGTGACCC
	Rev	TCCAGACCTCACGTTTGTTCC
RUNX1	For	CCAATACCTGGGATCCATTGC
	Rev	CTGGCACGTCCAGGTGAAA
RUNX3	For	AGGCAATGACGAGAACTACTCC
	Rev	CGAAGGTCGTTGAACCTGG
CXCR4	For	GGGCAATGGATTGGTCATCCT
	Rev	TGCAGCCTGTACTTGTCCG
CD4	For	GTCCCAAAGGCTTCTTCTTGAG
	Rev	GGAAATCAGGGCTCCTTCTTAAC
MYB	For	TGTTGCATGGATCCTGTGTT
	Rev	AGTTCAGTGCTGGCCATCTT
GATA3	For	TTCAGTTGGCCTAAGGTGGT
	Rev	CGCCGGACTCTTAGAAGCTA
CDK6	For	CAGATGGCTCTAACCTCAGTGG
	Rev	CACGAAAAAGAGGCTTTCTACGA
MYC	For	GCAGCTGCTTAGACGCTGGATTTT
	Rev	GCAGCAGCTCGAATTTCTTCCAGA
IL7R	For	CCTTCCCGATAGACGACACTC
	Rev	CCCTCGTGGAGGTAAAGTGC
HES1	For	GAGAGGCGGCTAAGGTGTTTG
	Rev	CTGGTGTAGACGGGGATGAC
DTX1	For	TGGTCACAGCATCAGGCTAC
	Rev	TGGTCTGGGTATCAGGGAAG
IGF1R	For	ATGCTGACCTCTGTTACCTCT
	Rev	GGCTTATTCCCCACAATGTAGTT
TASP1	For	AGGCACTTTGGACACGGTAG
	Rev	GCTGTGGAGTAGGGGTTATGAG

Supplementary Table 6. Primers for ChIP-qPCR

Target locus	Direction	Primer sequence (5' to 3')
ChIP-mNMe	For	AACCCTGAACCTGGTGATTG
	Rev	AGTGCTGGTGCCAAGAACTC
ChIP-mNMe H	For	CCCAACGTATTCCTCAACTGC
	Rev	AATGAAGTCACCTGCCCACT
ChIP-mMyb+15	For	CTGTGTCTGGGGAAGGGGGT
	Rev	TCTTGCCTCCAACAGCATCT
ChIP-mMyb-92	For	TGGTTTCCAGGGACCGTTAG
	Rev	GCAAACCACAGAGACTTGCA
ChIP-mMyb-92 H	For	TGGATCCACTGAGCAGAACA
	Rev	TGGCTTCCCTACTGAGCTGT
N.control (Gene desert)	For	AACCTCACACACAACAAGCTG
	Rev	TGTGATAGGGAGAATGCTTGC
ChIP-mHes1pro	For	GACCTTGTGCCTAGCGGCCA
	Rev	AGACAGGGGATTCCGCTGTT

Supplementary Table 7. Primers for ATAC-qPCR

Target Locus	Direction	Primer sequence (5' to 3')
mNMe	For	AGAGGAGTTCTTGGCACCAG
	Rev	TTAGGCAGACTGCAGGGAAC
Gene Desert 1	For	AACCTCACACACAACAAGCTG
	Rev	TGTGATAGGGAGAATGCTTGC
Gene Desert 2	For	GCTACAAAAGAGTGAGGTCGT
	Rev	TTCCTACCCAGAAGTGTGCC

Supplementary Figure Legends

Supplementary Figure 1. Mice that develop disease derive from T-ALL subclones that retain the floxed *Runx1* allele and Cre activation has no significant effects on mouse T-ALL growth *in vivo* or *in vitro*. (A) For the control samples, genomic DNA was isolated from tail biopsies of wild type, *Runx1*$^{f/+}$ and *Runx1*$^{f/f}$ mice (designated WT, F/+ and F/F). For the deleted control, DNA was isolated from mouse T-ALL cell line 1143 derived from a leukemic *Tal1/Lmo2/Rosa26-CreER*^{T2}*Runx1*$^{f/f}$ mouse that was treated with 4-OHT *in vitro* for 48h (designated F/Δ). Analysis of primary mouse T-ALL 7714 reveals a *Tal1/Lmo2/Rosa26-CreER*^{T2}*Runx1*$^{f/\Delta}$ genotype likely due to leaky Cre expression in the primary tumor. DNA was isolated from untreated mouse T-ALL 7714 cells (UN) and from tumor tissue isolated from transplanted mice at time of sacrifice. V1 and V2 refers to tumor DNA isolated from 2 independent vehicle treated mice transplanted with mouse T-ALL 7714 cells. WT band likely reflects presence of normal cells in tumor specimen. T1 and T2: Tumor DNA isolated from 2 independent tamoxifen treated mice transplanted with mouse T-ALL 7714 cells. These tamoxifen treated mice succumb to disease and select for leukemic clones that retain the floxed *Runx1* allele. M indicates DNA ladder used to estimate fragment size. (B) Mouse T-ALL cells from *Tal1/Lmo2/Rosa26-CreER*^{T2} mice were transplanted into mice and one a week later tamoxifen was administered for 3 days. The survival curve for 3 mouse T-ALLs (1353, 4456 and 2716) was estimated using the Kaplan-Meier method. (C) The 2 independent *Tal1/Lmo2/Rosa26-CreER*^{T2} mouse T-ALL cells were treated with vehicle (EtOH) or 4-OHT and the apoptotic cells determined by Annexin V-FITC and 7AAD staining followed by flow cytometry. The averages of 3 to 4 independent experiments are shown as means ± SD.

Supplementary Figure 2. RUNX protein expression in mouse T-ALL cells and T-ALL patient samples and *RUNX1* knockdown in human T-ALL cell lines alters the expression of a subset of RUNX1-, TAL1-, and NOTCH1-regulated genes resulting in cell apoptosis. (A) RUNX1 and RUNX3 expression levels in mouse thymus and *Tal1/Lmo2* mouse T-ALL cell lines were analyzed by immunoblotting. ERK1/2 was used as a loading control. (B) RUNX1 and RUNX3 expression in Jurkat, KOPTK1 and eight T-ALL patient samples were analyzed by immunoblotting. ERK1/2 was used as a loading control. (C) Quantification of apoptotic leukemia cells upon *RUNX1* knockdown in KOPTK1, PF382, and RPMI8402 human T-ALL cells. 3-4 independent experiments were performed and shown as mean ± SEM. (D) *RUNX1* mRNA levels in KOPTK1, PF382, and RPMI8402 cells transduced with control (GFP) or *RUNX1*-specific shRNAs was measured by qRT-PCR. *RPS9* or *GAPDH* were used for normalization. Three independent experiments were performed. (E) CD4 cell surface expression was determined by flow cytometry after staining with CD4 antibody. Representative flow data is shown and data from 3 independent experiments are shown as MFI ± SD (right). (F) Jurkat cells were infected with lentiviruses expressing shRNAs against *RUNX1* or GFP. RNA was isolated 4 days after infection. Gene expression changes upon *RUNX1* knockdown were determined by qRT-PCR. *GAPDH* was used for normalization of qPCR values. 3-4 independent experiments were performed and data are shown as mean ± SEM (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001, One-way ANOVA multiple comparisons test).

Supplementary Figure 3. MYB is required for *Tal1/Lmo2* mouse T-ALL growth *in vivo* and *in vitro*. (A) Four independent murine *Tal1/Lmo2* T-ALL cells (5059, 5109, 4673 and 6124) were infected with retroviruses encoding an shRNA to *Myb* or *Renilla luciferase*. Cells were sorted for GFP expression, transplanted into syngeneic recipients and monitored for evidence of disease. The survival curve for each group of mice was estimated using the Kaplan-Meier method and the difference in overall survival between the two groups assessed by the log-rank test (5059: n=12 mice, 5109: n=9 mice, 4673: n=9, 6125: n=9). (B) MYB suppression impairs

leukemic growth *in vitro* but not all mouse T-ALLs. Cell viability of the mouse T-ALL cell lines (5059, 5109 and 6124) transduced with *Renilla luciferase* or *Myb* shRNAs was calculated via a trypan blue exclusion assay. *Myb* suppression in murine *Tal1/Lmo2* 6124 T-ALL cells failed to suppress leukemic growth *in vitro*. (C) MYB protein level in leukemic cells was determined by immunoblotting. ERK1/2 protein levels were used for loading control.

Supplementary Figure 4. *Runx1* depletion has no effect on intracellular NOTCH1 binding to the *Hes1* promoter or to gene desert regions. (A) *Runx1* deletion did not change the recruitment of intracellular NOTCH1 or H3K27ac to mouse *Hes1* promoter. (B) *Runx1* deletion has no effect on TAL1, NOTCH1 or H3K27ac binding to gene desert region. 2-4 independent experiments were performed and data is shown as mean ± SEM.

Supplementary Figure 5. RUNX3 does not bind to *MYC* and *MYB* enhancer loci and either support cell survival in Jurkat cells. (A) Apoptotic leukemic cells upon *RUNX3* knockdown in Jurkat, RPMI8402 human T-ALL cells were determined by Annexin V-FITC/7-AAD staining followed by flow cytometry. (B) *RUNX3* mRNA levels in Jurkat and RPMI8402 cells transduced with control (GFP) or *RUNX3*-specific shRNA were measured by qRT-PCR. RPS9 was used for normalization. (C) RUNX1 and RUNX3 binding at *N-Me*, *MYB* +14-kb and *MYB* -93-kb loci in Jurkat cells was determined using ChIP-qPCR. 3-5 independent experiments were performed and shown as mean ± SEM (**p*<0.05, ****p*<0.0005, *****p*<0.0001, Two-way ANOVA multiple comparisons test).

Supplementary Figure 6. Treatment with the RUNX-CBFβ inhibitor but not the inactive analogue induces apoptosis of human T-ALL cell lines while maintaining the viability of human bone marrow cells. (A) Protein was isolated from AI-10-104 treated KOPTK1 cells and RUNX1, RUNX3, and CBFβ expression levels were determined by immunoblotting. GAPDH was used as loading control. (B) Seven human T-ALL cell lines were treated with increasing concentrations of AI-10-104 (2.5-10μM) or 10μM of the inactive analogue AI-4-88 for 6 days and apoptotic cells were determined by Annexin V-FITC and 7AAD staining followed by flow cytometry. The results are the means of 2 to 3 independent replicates and error bars represent SEM (**p*<0.05, ***p*<0.005, ****p*<0.0005, Two-way ANOVA multiple comparisons test). **(**C) Human T-ALL cell lines and T-ALL patient samples were treated with increasing concentrations of the inactive analogue AI-4-88 (1-20μM) for 3 days. Cell growth/metabolism were analyzed by MTS assay. (D) *MYC* and *MYB* gene expression changes were determined in RPMI8402 cells treated with 10μM of AI-4-88 or AI-10-104 for 12 hours. The results are the means of 3 independent replicates and error bars represent SEM (**p<0.005, Student *t* test). (E) Randomly selected patient samples (n=3) were treated with 10μM of AI-4-88 or 5, 10μM of AI-10-104 for 6 days. Apoptotic cells were determined by Annexin V-FITC and 7AAD staining followed by flow cytometry. Three independent replicates are shown as mean ± SEM (**p*<0.05, ***p<0.0005, ANOVA multiple comparisons test). (F) G-CSF mobilized normal human BM cells were treated with increasing concentrations of AI-10-104 for 3 days and effects on cell growth/metabolism were determined by MTS assay. GI_{50} of each donor cells was calculated using Graph Pad Prism 7 software.