RNA helicase DHX15 exemplifies a unique dependency in acute leukemia

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Supplemental Material

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Supplemental Methods

Cell culture

Human T-ALL cell lines were grown in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gemini), 1% penicillin/streptomycin (Hyclone), 1% non-essential amino acids (Gibco), 2mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma) and 55 μM β-mercaptoethanol (Sigma). 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FBS (Gemini) and 1% penicillin/streptomycin (Hyclone). Purified human $CD34^+$ HSPCs were cultured in SFEM II (StemSpan) containing $CD34^+$ Expansion Supplement (StemSpan). All cell lines were authenticated using short tandem repeat (STR) analysis, cultured for fewer than 3 months after resuscitation, and tested for mycoplasma contamination once a month using PCR assay.

Plasmid construction

The shRNA expression vector pLKO.1 and the overexpression vector pHAGE and pCDH were obtained from Addgene and subjected to modifications of adding epitope tags. For gene knockdown, the short hairpin RNAs (shRNAs) against target genes were cloned into the EcoRI and AgeI sites of the pLKO.1 vector. For gene overexpression, pHAGE-Flag-DHX15 and pCDH-HA-DHX15 were constructed by standard molecular cloning approaches. The retroviral vector MSCV-ICN1-IRES-GFP (MigR1-ICN1) and packaging plasmids pCgp and pHIT were kindly provided by Dr Warren Pear (University of Pennsylvania, USA). All primers for plasmid constructions were listed

in Supplemental Table 1.

Lentiviral and retroviral transduction

Lentiviral production and transduction were performed as described[1,2]. Briefly, lentiviral constructs (pLKO.1 for shRNA, pHAGE or pCDH for overexpression) were transfected into 293T cells together with helper plasmids (pMD2.G and psPAX2) using Lipofectamine 2000 (Thermo Fisher Scientific). Viral supernatants were generally collected 48 hr post transfection, and appropriate amount was added into two million cells suspended in 2 mL medium with the presence of 6 μg/mL polybrene (Sigma). After culture for 30 min, the mixture was subjected to centrifugation at 1000 × g for 90 min at 32°C. Cells were then supplemented with 1 mL fresh medium and continued culture for at least 48 hr to achieve efficient gene silencing or overexpression. Production of high-titer retroviruses was performed as described[2,3]. Briefly, retroviral vectors MigR1, MigR1-ICN1 were transfected into 293T cells simultaneously with packaging plasmids (pCgp and pHIT) using Lipofectamine 2000. Viral supernatants were generally collected 48 hr post transfection. Bone marrow hematopoietic progenitor cells were added with the appropriate amount of viral supernatant and 6 μg/mL polybrene in a final volume of 2 mL. The mixture was then centrifuged at 1000×g for 90 min at 32°C.[4] Transduction efficiency was measured 48 h post-transduction by flow cytometry.

Chromatin immunoprecipitation (ChIP)

Ten million cells were fixed with 1% formaldehyde for 10 min, quenched with 0.125 M glycine for 5 min at 37℃ and lysed in SDS Lysis Buffer. Cell lysate was sonicated by Pico Sonifier (Bioruptor) to shear chromatin DNA to a size range of 200-1000 bp. The supernatant was diluted 10 fold in ChIP Dilution Buffer and precleared with 60 μg agarose beads for 30 min. The supernatant fraction was immunoprecipitated with indicated antibodies (2 μg) against MYB (A13776, ABclonal) overnight at 4℃. Antibody-chromatin complexes were pulled down with protein A agarose/salmon sperm DNA beads (Sigma-Aldrich) for 1 hr at 4℃. De-crosslinked DNA was subjected to qPCR analysis using specific primers listed in Supplemental Table 1.

Luciferase reporter assay

0.5 μg pGL3 vector expressing MYB-RE (or indicated mutant), along with 0.5 μg pHAGE-MYB, 20 ng Renilla luciferase reporter was co-transfected in triplicates into 293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). Luciferase activities were measured 24 hr later using Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized to Renilla luciferase control values and shown as an average of triplicates. All primers for luciferase reporter assay were listed in Supplemental Table 1.

Human T-ALL xenografts

MOLT-3 xenografts were carried out as previously described[1,5]. Human T-ALL MOLT-

3 cells were first infected with lentiviruses expressing the green fluorescent protein (GFP) and luciferase (pWPXLd-Luciferase-GFP). Transduced GFP^+ cells were sorted by flow cytometry and then subjected to secondary transfection with lentivirus expressing control or *DHX15* shRNA. After puromycin selection (2 μg/mL) for 48 hr, two million infected cells were intravenously injected into 6-week-old irradiated (1.5 Gy) NPG mice. Disease progression was assessed by *in vivo* bioimaging (Ami HTX, Spectral Instruments Imaging) with the intraperitoneal injection of D-Luciferin (150 mg/kg).

Flow cytometry analysis

For analysis of apoptotic cell death, cells were washed with PBS and resuspended in Annexin V binding buffer, and stained with Annexin V-FITC and propidium iodide (PI) (BD pharmingen) at room temperature for 15 min. For cell cycle analysis, cells were washed with PBS and fixed with frozen 70% ethanol at 4°C overnight. Upon washing, cells were stained with PI/TritonX-100 solution (0.1% Triton X-100, 0.2 mg/mL RNase A, 0.02 mg/mL PI in PBS) at 4°C for 30 min before analysis.

For immunophenotypic analysis, single-cell suspensions were prepared and stained with indicated antibodies at 4°C for 30 min. Fluorochrome-conjugated antibodies against CD3e (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD25 (PC61), CD44 (IM7), CD45R/B220 (RA3-6B2), CD11b (M1/70), Ly-6C (RB6-8C5), TER-119 (TER-119), NK1.1 (PK136) were from BD pharmingen or Biolegend. Acquisition was performed

on Accuri C6 (BD Biosciences) and live cells were gated based on FSC and SSC characteristics.

RNA sequencing (RNA-Seq)

CUTLL1 cells were infected with lentiviruses expressing *DHX15* shRNA or treated with ciclopirox (MedChemExpress, HY-B0450). Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) and subjected to quality control for RNA integrity by Agilent Bioanalyzer 2100 system (Agilent Technologies). Total RNA was further purified by RNAClean XP Kit (Beckman Coulter) and RNase-Free DNase Set (QIAGEN). Poly(A)+ RNA libraries were constructed using VAHTS Universal V6 RNA-seq Library Prep Kit (Vazyme) and sequenced as 150 bp paired-end reads by Illumina NovaSeq 6000 (Annoroad Gene Technology Corporation). RNA-Seq reads quality was evaluated using FastQC (version 0.11.9) and mapped to the human genome reference assembly (hg19) using STAR 2.4.1[6]. Differential gene expression analysis was performed using DESeq2 (version 1.32.0) (p<0.05, Fold change>1.5). Differential alternative splicing (AS) events were quantified by using rMATS[7] and JUM[8] for five major types (SE, A5SS, A3SS, MXE, RI). AS events were considered as significant if |IncLevelDifference| or $|\Delta PSI| \geq 0.1$ and p-value < 0.05.

RNA immunoprecipitation sequencing (RIP-Seq)

Ten million CUTLL1 cells were lysed in RIP lysis buffer [20 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1% SDS, 100 U/ml RNasin Ribonuclease

Inhibitors (Promega)]. The resulting cell lysates were incubated with 5 μg of DHX15 antibody (Abcam) or control IgG at 4°C overnight. Protein A/G magnetic beads (Thermo Fisher Scientific) were then added and incubated for 2 h at 4°C to conjugate the protein-antibody complexes. After washing with RIP buffer, beads were resuspended in 80 μl PBS, followed by DNase treatment (Thermo Fisher Scientific) and proteinase K (50 μg) (Thermo Fisher Scientific) digestion at 37 °C for 15 min. Input and co-immunoprecipitated RNAs were isolated by TRIzol reagent (Thermo Fisher Scientific), and analyzed by qPCR or RNA-seq. NEBNext rRNA Depletion Kit v2 (New England BioLabs) was used to remove rRNA, and NEBNext Ultra II RNA Library Prep Kit (New England BioLabs) was then applied for library construction. RNAs were sequenced as 150 bp paired-end reads using Illumina NovaSeq 6000 (Annoroad Gene Technology Corporation, China). The reads quality was evaluated using FastQC (version 0.11.9) and mapped to the human genome reference assembly (hg38) using STAR software (version 2.7.5c). The resulting P values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). Genes with Fold change > 2 and FDR < 0.05 found by DESeq were considered to be significant enrichment.

RNA extraction and quantitative real-time PCR

Total cellular RNA was extracted using TRIzol (Thermo Fisher Scientific). Complementary DNA (cDNA) synthesis was performed using the ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer's instructions. Quantitative PCR was

conducted using FAST SYBR Green Master Mix (Bio-Rad) on CFX Connect Real-Time PCR System (Bio-Rad). mRNA relative expression was calculated by $2^{-\Delta\Delta Ct}$ method and normalized to *ACTIN*. Specific PCR primer sequences were listed in Supplemental Table 1.

Immunoblot

Cells were lysed with RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2mM sodium pyrophosphate, 25mM β-glycerophosphate, 1mM EDTA, 1 mM Na_3VO_4, and 0.5 μg/mL leupeptin) containing protease inhibitor cocktail and protein concentrations were determined using BCA assay kit (Thermo Fisher Scientific). 30-50 μg total cellular proteins were subjected to SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After being blocked with 5% fat free milk for at least 2 hr, blots were generally incubated with primary antibodies at 4°C overnight. Appropriate horseradish peroxidase conjugated secondary antibodies were applied for 1 hr at room temperature before detection with Clarity Western ECL Substrate (Bio-Rad). Densitometric analyses of protein abundance were determined by ImageJ software. Antibodies used were as follows: DHX15 (ab70454, Abcam; sc-271686, Santa Cruz), β-ACTIN (AC026, ABclonal), p-S6K T389 (#9234, Cell Signaling Technology), p70S6 Kinase (#2708, Cell Signaling Technology), p-4EBP1 T37/46 (#2855, Cell Signaling Technology), 4EBP1 (#9644, Cell Signaling Technology), SLC7A6 (13823-1-AP, Proteintech), SLC38A5 (28102-1-AP, Proteintech), MYC (sc-764, Santa Cruz), Cleaved NOTCH1

(#4147, Cell Signaling Technology), Cleaved Caspase-3 (#9661, Cell Signaling Technology), LMO1 (A7561, ABclonal), LMO2 (A6832, ABclonal), GATA3 (A1638, ABclonal), TSC2 (A19540, ABclonal), TLX1 (A12861, ABclonal), TLX3 (17454-1-AP, Proteintech) and MYB (05-175, Millipore).

Immunohistochemistry (IHC)

Spleen or thymi were surgically removed from sacrificed mice to prepare paraffin-embedded sections. Tissue sections were deparaffinized, and then rehydrated through an alcohol series followed by antigen retrieval with sodium citrate buffer. IHC was carried out using specific antibodies against PCNA (sc-56, Santa Cruz), Ki67 (A16919, ABclonal) or Cleaved Caspase-3 (#9661, Cell Signaling Technology). These slides were then subjected to horseradish peroxidase-linked secondary antibodies for 1 hr at room temperature. Staining was visualized by the DAB substrate kit (Vector Labs).

References

1. Jiang J, Wang J, Yue M, et al. Direct Phosphorylation and Stabilization of MYC by Aurora B Kinase Promote T-cell Leukemogenesis. *Cancer Cell*. 2020;37(2):200-215.e205.
2. Su H, Hu J, Huang L, et al. SHQ1 regulation of RNA splicing is required for T-lymphoblastic leukemia cell survival. *Nat Commun*. 2018;9(1):4281.
3. Liu H, Chi AW, Arnett KL, et al. Notch dimerization is required for leukemogenesis and T-cell development. *Genes Dev*. 2010;24(21):2395-2407.
4. Gavrilescu LC, Van Etten RA. Murine retroviral bone marrow transplantation models for the study of human myeloproliferative disorders. *Curr Protoc Pharmacol*. 2008;Chapter 14:Unit14.10.
5. Hu J, Wang T, Xu J, et al. WEE1 inhibition induces glutamine addiction in T-cell acute lymphoblastic leukemia. *Haematologica*. 2021;106(7):1816-1827.
6. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.
7. Shen S, Park JW, Lu ZX, et al. rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc Natl Acad Sci U S A*. 2014;111(51):E5593-5601.
8. Wang Q, Rio DC. JUM is a computational method for comprehensive annotation-free analysis of alternative pre-mRNA splicing patterns. *Proc Natl Acad Sci U S A*. 2018;115(35):E8181-e8190.

Supplemental Figures

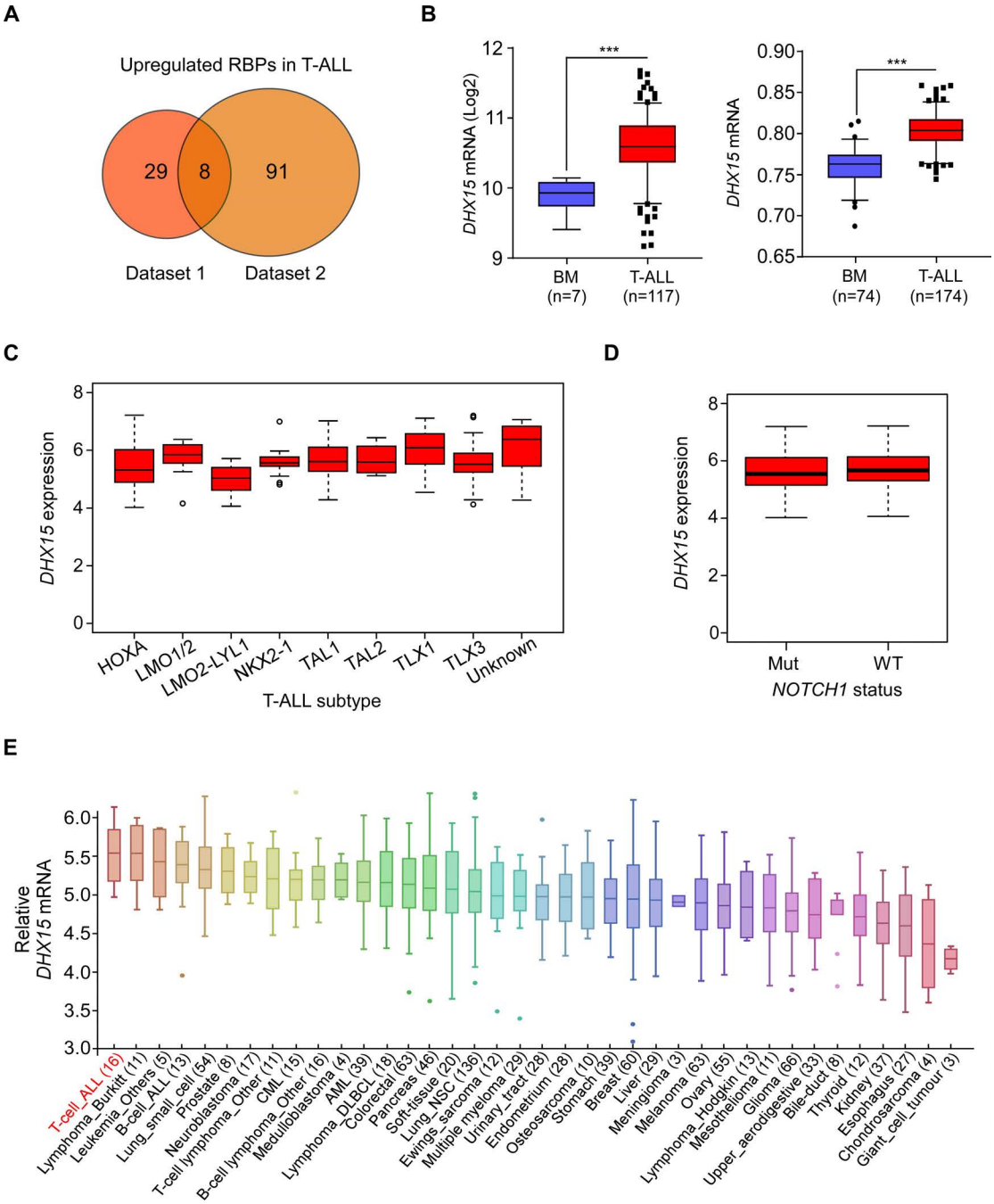

Figure S1. Elevated *DHX15* expression in T-ALL. (A) Venn diagram showing eight overlapping elevated genes encoding RNA-binding protein (RBP) in T-ALL patient samples from two independent datasets (FDR < 0.01). Dataset 1, 57 T-ALL patient samples (GSE33469) and 21 normal thymocyte samples (GSE33470). Dataset 2, 60 T-ALL patient samples (GSE110637) and 20 normal thymocyte samples (GSE151081). **(B)** *DHX15* mRNA expression was analyzed in primary T-ALLs and normal bone marrow (BM) (left, 7 normal BM and 117 T-ALL samples in GSE26713; right, 74 normal BM and 174 T-ALL samples in GSE13204). **(C-D)** Analysis of *DHX15* mRNA expression in 264 primary T-ALL samples (TARGET phs000218) that are categorized into various subgroups (C) or differential *NOTCH1* mutational status (D). **(E)** *DHX15* mRNA expression was analyzed among 1,062 human cancer cell lines in CCLE database (https://portals.broadinstitute.org/ccle). The distributions of *DHX15* mRNA expression are presented as log2 median-centered intensity. Box-and-whisker plots are shown with the median shown as a solid line inside the box and whiskers extending to 1.5 interquartile range from first and third quartiles. ***$p < 0.001$.

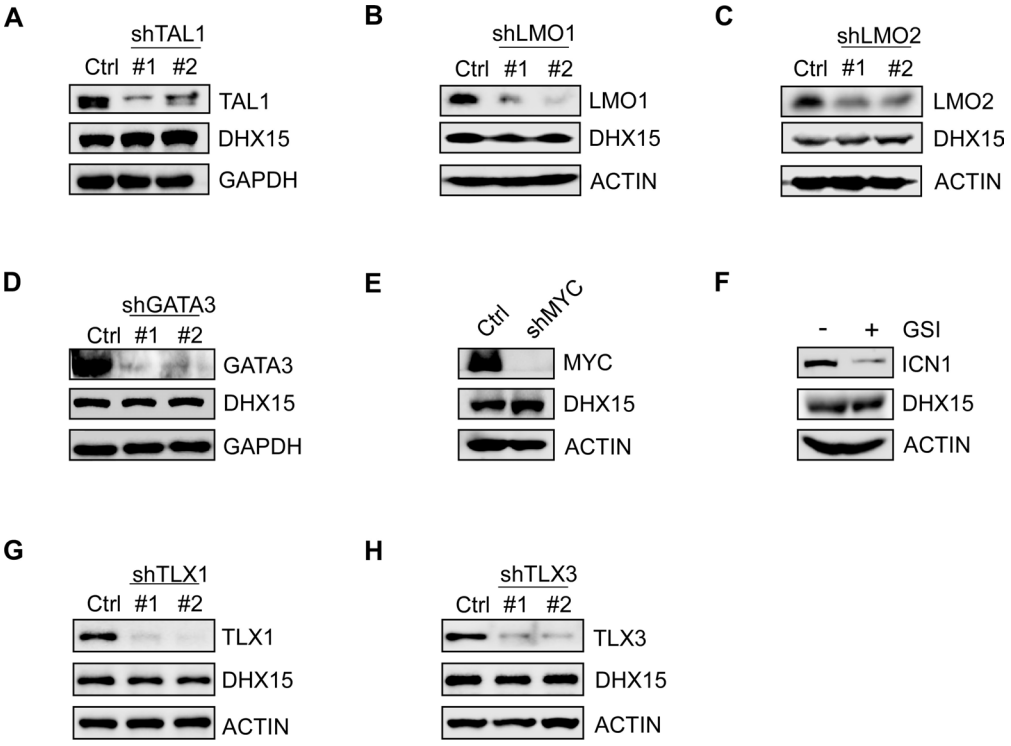

Figure S2. Evaluation of transcriptional dependency of *DHX15* on major transcription factors in T-ALL. (A-C) Jurkat cells were transduced with lentiviruses expressing indicated shRNA (#1, #2), DHX15 protein was analyzed by immunoblot. **(D)** DHX15 protein was analyzed by immunoblot in KOPTK1 cells expressing shRNA targeting *GATA3* (#1, #2). **(E-F)** Immunoblot of DHX15 protein in CUTLL1 cells expressing *MYC* shRNA (E), or undergoing gamma-secretase inhibitor (GSI) Compound E treatment (1 μM) for 48 h (F). **(G-H)** Immunoblot of DHX15 protein in CUTLL1 cells expressing shRNA targeting *TLX1* (#1, #2) or *TLX3* (#1, #2).

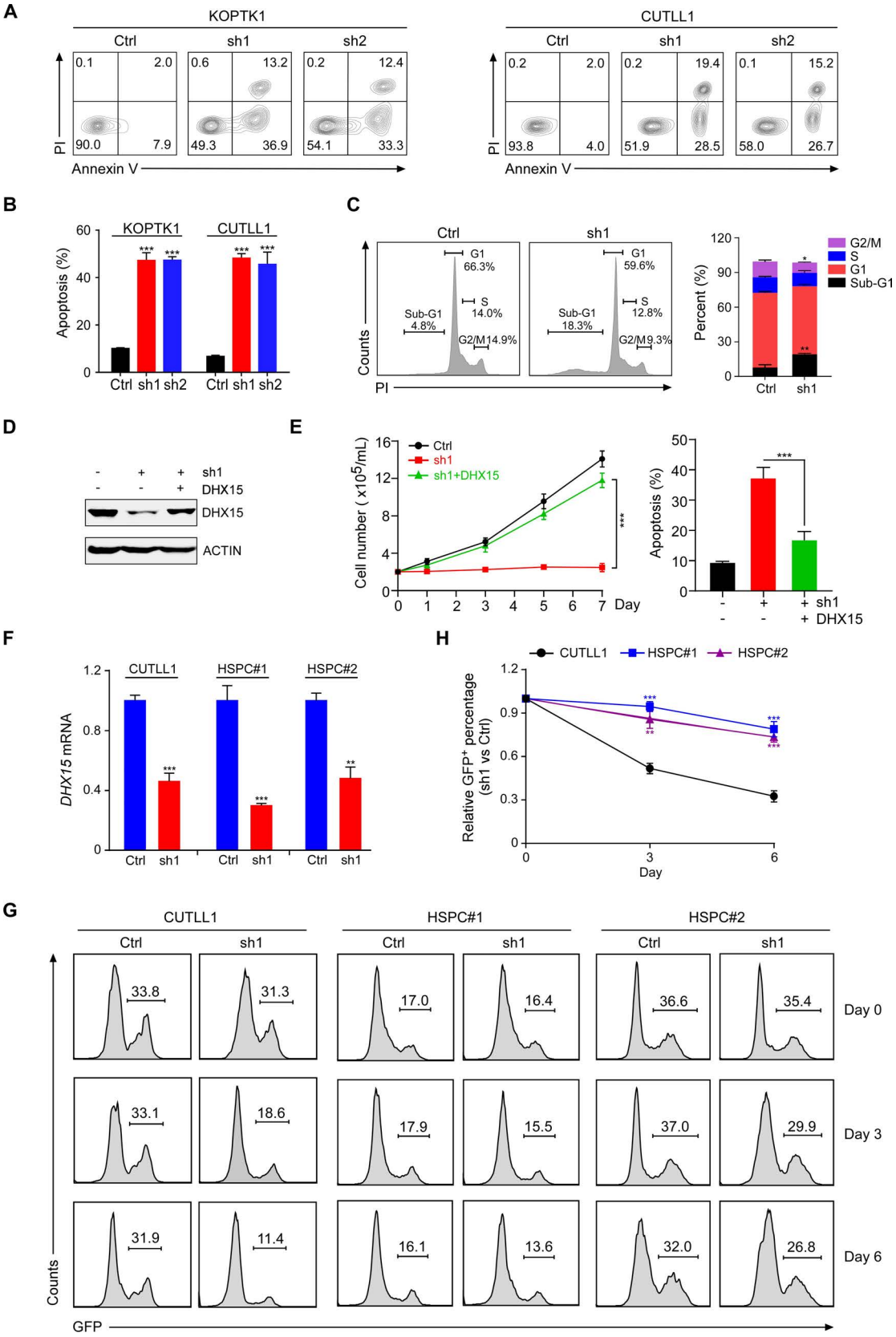
A
KOPTK1
Ctrl
sh1
sh2
0.1
2.0
90.0
7.9
0.6
13.2
49.3
36.9
0.2
12.4
54.1
33.3
PI
Annexin V
CUTLL1
0.2
2.0
93.8
4.0
0.2
19.4
51.9
28.5
0.1
15.2
58.0
26.7
B
Apoptosis (%)
KOPTK1
CUTLL1
Ctrl sh1 sh2 Ctrl sh1 sh2
C
Ctrl
sh1
G1
66.3%
S
14.0%
Sub-G1
4.8%
G2/M14.9%
G1
59.6%
S
12.8%
Sub-G1
18.3%
G2/M9.3%
Counts
PI
Percent (%)
G2/M
S
G1
Sub-G1
Ctrl
sh1
D
sh1
DHX15
DHX15
ACTIN
E
Ctrl
sh1
sh1+DHX15
Cell number (x10^5/mL)
Day
Apoptosis (%)
sh1
DHX15
F
CUTLL1
HSPC#1
HSPC#2
DHX15 mRNA
Ctrl sh1 Ctrl sh1 Ctrl sh1
H
CUTLL1
HSPC#1
HSPC#2
Relative GFP+ percentage (sh1 vs Ctrl)
Day
G
CUTLL1
HSPC#1
HSPC#2
Ctrl
sh1
33.8
31.3
17.0
16.4
36.6
35.4
Day 0
33.1
18.6
17.9
15.5
37.0
29.9
Day 3
31.9
11.4
16.1
13.6
32.0
26.8
Day 6
Counts
GFP

Figure S3. DHX15 is required to maintain T-ALL cell survival *in vitro*. (A-B) Flow cytometry analysis of apoptotic subpopulations by Annexin V-PI staining in *DHX15*-depleted KOPTK1 and CUTLL1 (sh1 and sh2) and control (Ctrl) cells. **(C)** Cell cycle was analyzed in CUTLL1 cells upon *DHX15* withdrawal. Mean percentages of cells in sub-G1, G1, S and G2/M phase are presented on the right. **(D-E)** KOPTK1 cells were infected with lentiviruses expressing shRNA targeting *DHX15* mRNA 3′UTR alone (sh1) or in combination with ectopic expression of *DHX15* coding region. Immunoblot analysis of DHX15 expression (D). Cell growth (left) and death (right) were assessed as presented (E). **(F)** CUTLL1 cells and bone marrow $CD34^+$ hematopoietic stem and progenitor cells (HSPC #1 and #2) from two healthy donors were transduced with lentiviruses expressing *DHX15* shRNA (sh1) with GFP as a surrogate marker. *DHX15* mRNA in GFP^+ cells was analyzed by qPCR. **(G-H)** Percentages of GFP^+ cell populations of CUTLL1 cells or HSPCs were analyzed by flow cytometry at the indicated time points post infection (G). Relative GFP^+ percentage was normalized to vector control (H). Data shown represent the means (±SD), $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

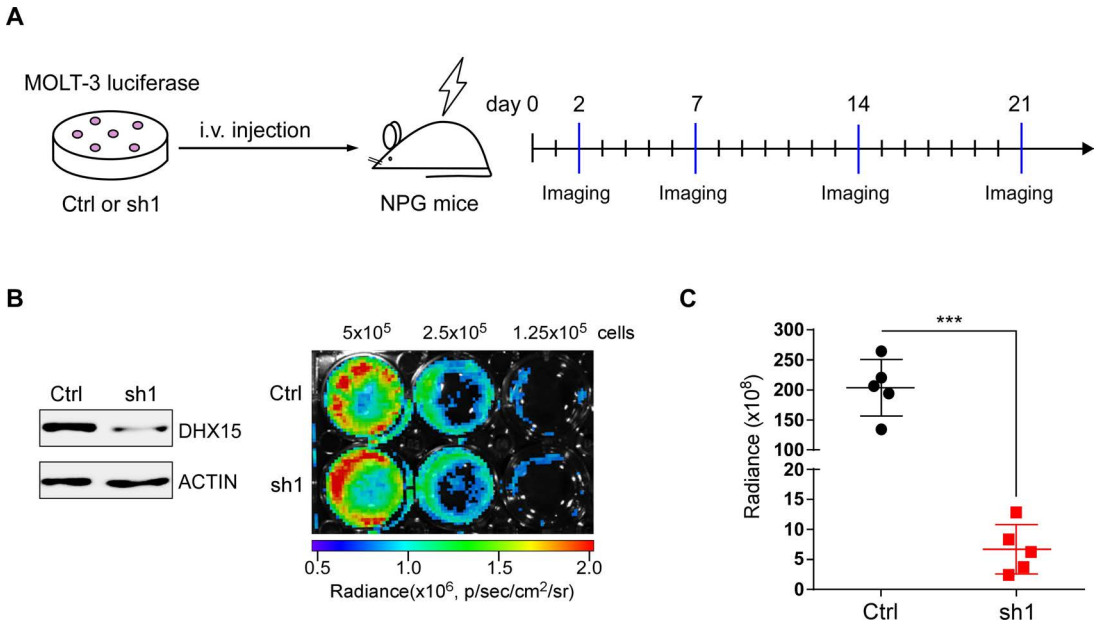

Figure S4. DHX15 is required to maintain T-ALL progression *in vivo*. (A) Graphic illustration of *in vivo* imaging of T-ALL xenografts. Human T-ALL MOLT-3 cells, expressing both luciferase and GFP markers, were infected with shRNA targeting *DHX15* mRNA (sh1) or control. Two million GFP^+ cells were injected into irradiated NPG mice, followed by *in vivo* bioimaging to assess leukemia progression. **(B)** DHX15 knockdown efficiency was confirmed by immunoblot (left). Luciferase signals of MOLT-3-Luc-GFP cells with or without *DHX15* depletion was determined by bioluminescence imaging before intravenous injection to NPG mice (right). **(C)** Quantification of luciferase signals in NPG mice engrafted by MOLT-3-Luc-GFP cells with or without *DHX15* depletion at day 21 (n = 5 per group). Data shown represent the means (±SD), ***$p < 0.001$.

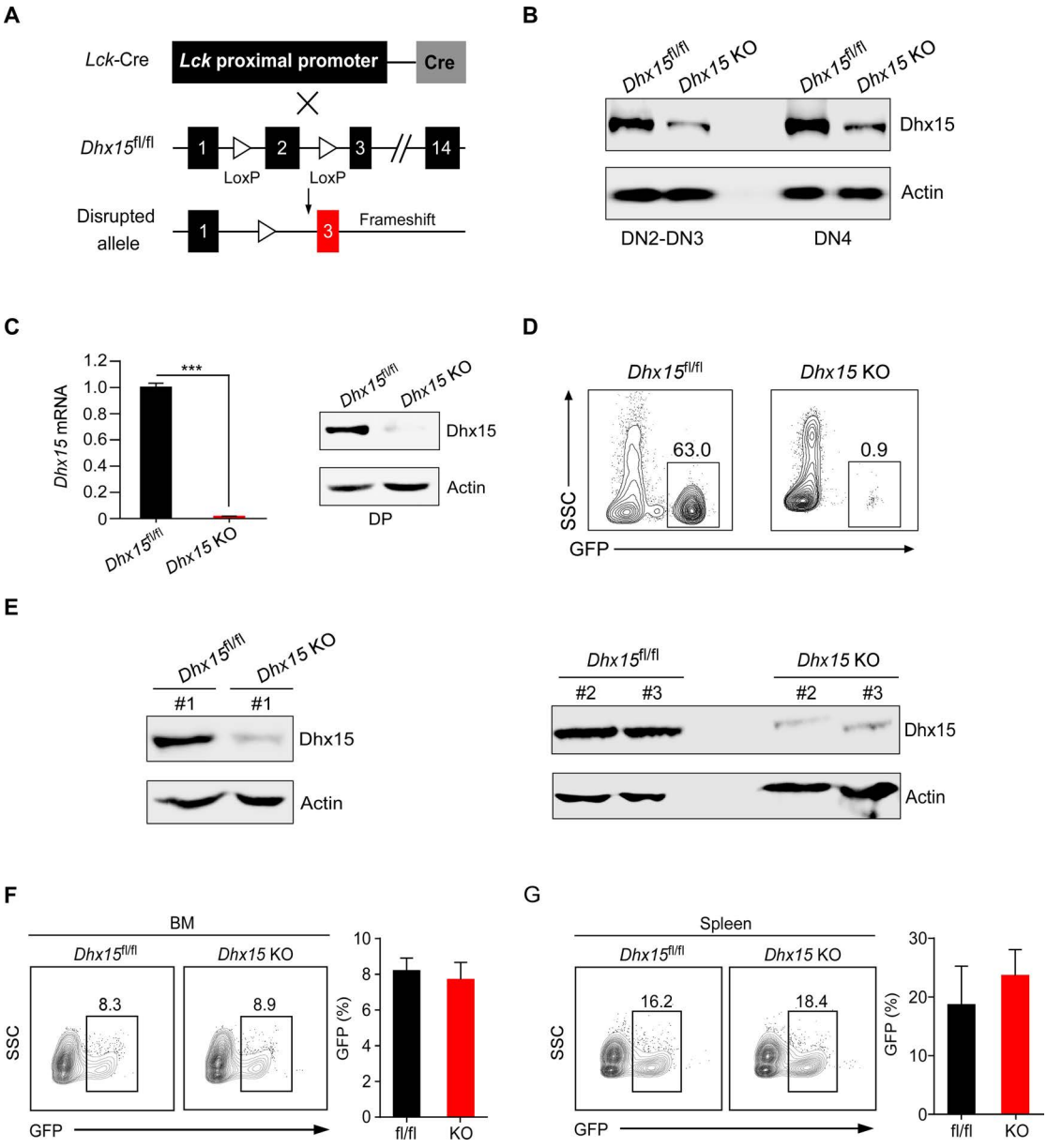

Figure S5. *Dhx15* deficiency impedes T-cell leukemogenesis *in vivo*. (A) Diagram showing the targeting strategy used to generate conditional *Dhx15* knockout strain. The exon 2 of *Dhx15* flanked by LoxP sites was removed upon activation of the Cre recombinase expression driven by the *Lck* promoter. **(B-C)** Analysis of Dhx15 expression in sorted $CD4^{-}CD8^{-}$ double negative (DN2-3 and DN4) (B) and $CD4^{+}CD8^{+}$ double positive (DP) (C) thymocytes from *Dhx15*$^{fl/fl}$ and *Dhx15* KO mice. **(D)** Representative flow cytometry analysis of GFP^{+} leukemia cells 6 weeks post transplantation. **(E)** Immunoblots of Dhx15 in primary murine T-ALL cells derived from *Dhx15*$^{fl/fl}$ and *Dhx15* KO donors. **(F-G)** Lin^{-} HSPC cells from *Dhx15*$^{fl/fl}$ or *Dhx15* KO mice were transduced with MigR1-ICN1 retroviruses and subjected to transplantation. Recipient mice were sacrificed within 36 hr and cells retrieved from the bone marrow (F) and spleen (G) were analyzed for GFP percentage by flow cytometry (n=4 per group). Data shown represent the means (±SD). ***$p < 0.001$.

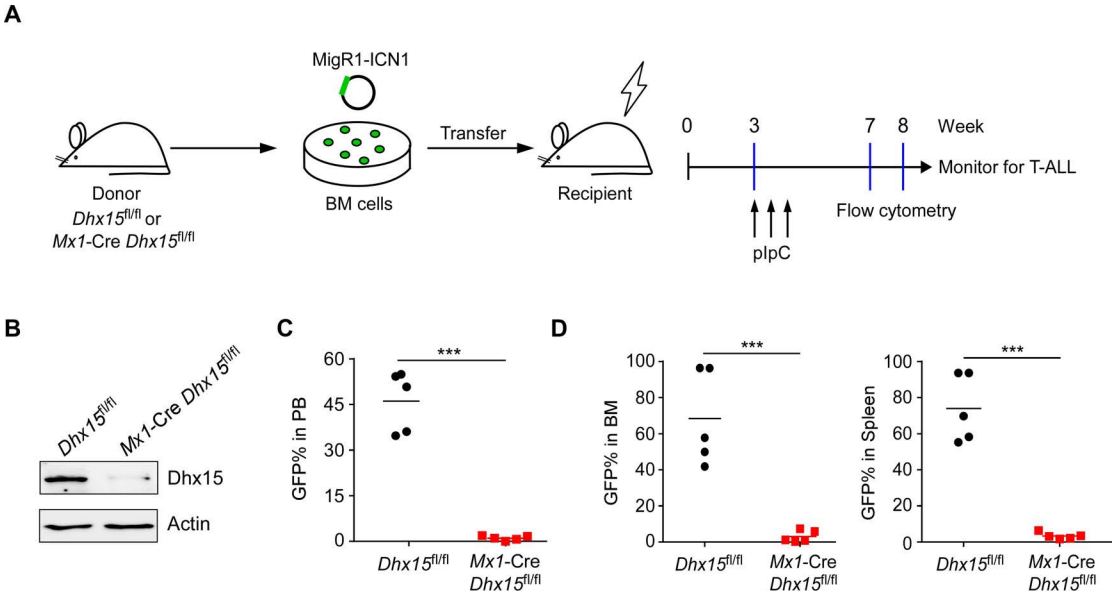

Figure S6. Dhx15 is required for T-ALL maintenance *in vivo*. (A) Graphical illustration of NOTCH1-induced T-ALL mouse model using *Dhx15*$^{fl/fl}$ or *Mx1*-Cre *Dhx15*$^{fl/fl}$ mice. pIpC (poly I:C) was injected 3 weeks post transplantation and GFP^{+} leukemia cells were analyzed at the 7th and 8th week. **(B)** Analysis of Dhx15 expression in Lin- HSPCs from *Dhx15*$^{fl/fl}$ and *Mx1*-Cre *Dhx15*$^{fl/fl}$ mice 2 weeks post pIpC injection by immunoblot. **(C-D)** Percentages of GFP^{+} cells in peripheral blood (PB) at the 7th week (C), and in BM and spleen at the 8th week (n=5 per group) (D). Data shown represent the means (±SD), ***$p < 0.001$.

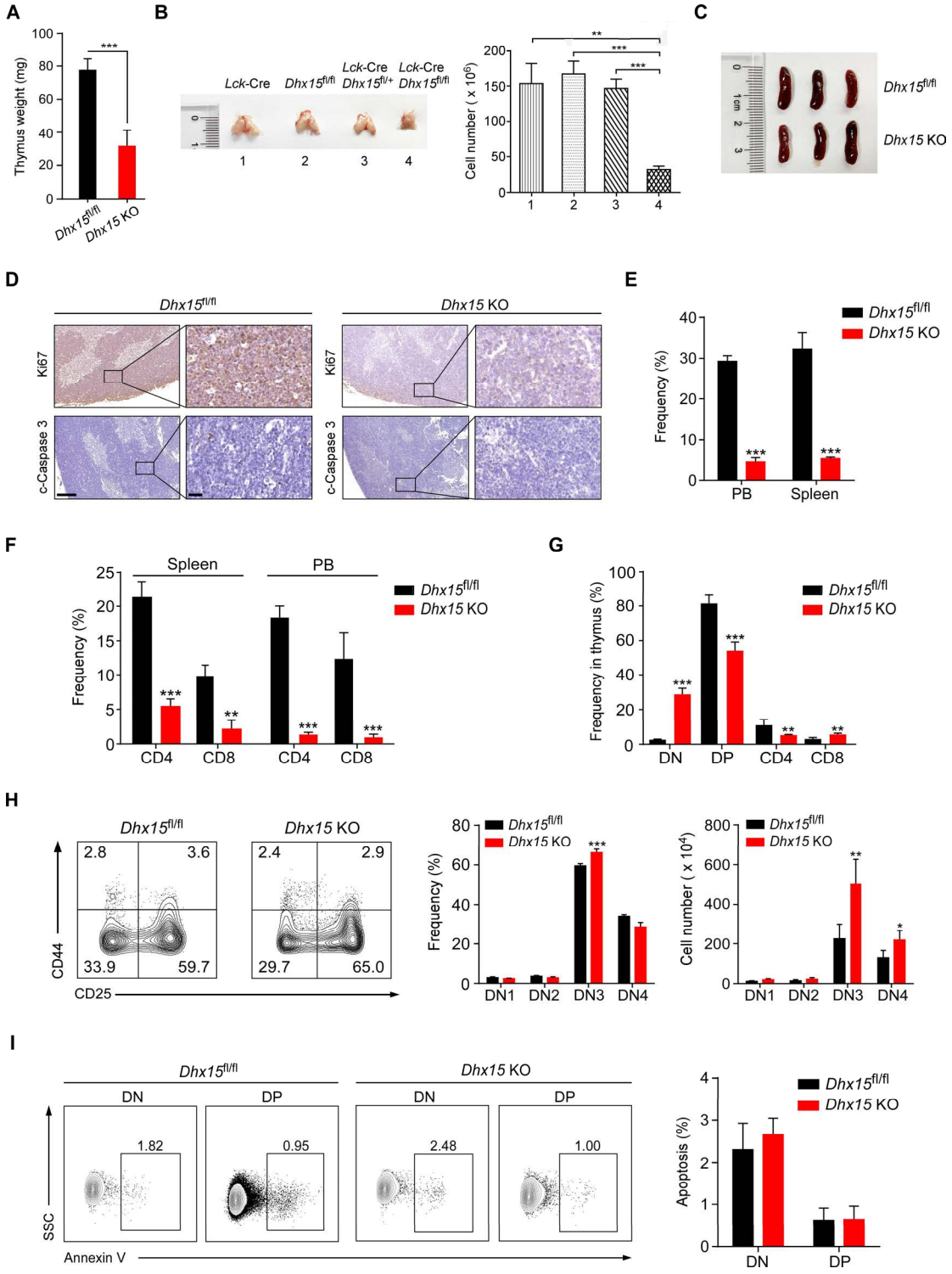
A
Thymus weight (mg)
Dhx15fl/fl
Dhx15 KO
B
Lck-Cre
Dhx15fl/fl
Lck-Cre Dhx15fl/+
Lck-Cre Dhx15fl/fl
Cell number (x 10^6)
C
Dhx15fl/fl
Dhx15 KO
D
Dhx15fl/fl
Dhx15 KO
Ki67
c-Caspase 3
E
Frequency (%)
PB
Spleen
F
Spleen
PB
Frequency (%)
CD4
CD8
G
Frequency in thymus (%)
DN
DP
CD4
CD8
H
CD44
CD25
2.8
3.6
33.9
59.7
2.4
2.9
29.7
65.0
Frequency (%)
Cell number (x 10^4)
DN1
DN2
DN3
DN4
I
DN
DP
1.82
0.95
2.48
1.00
SSC
Annexin V
Apoptosis (%)

Figure S7. T-cell lineage specific knockout of DHX15 hampers thymocyte development. (A) Thymus weights of 6-week-old *Dhx15*$^{fl/fl}$ and *Dhx15* KO mice (n=5 per group). **(B)** Thymus morphology (left) and cellularity (right) of 6-8 week old control (*Lck*-Cre or *Dhx15*$^{fl/fl}$), heterozygous (*Lck*-Cre *Dhx15*$^{fl/+}$) and homozygous (*Lck*-Cre *Dhx15*$^{fl/fl}$) knockout mice (n=3 per group). **(C)** Spleen morphology of 6- to 8-week-old *Dhx15*$^{fl/fl}$ and *Dhx15* KO mice. **(D)** Immunohistochemical staining of Ki67 and cleaved Caspase-3 of thymi from 6-week-old *Dhx15*$^{fl/fl}$ (left) or *Dhx15* KO (right) mice. Scale bar, 200 μm. Scale bar in the magnified image is 20 μm. **(E)** Flow cytometry analysis of CD3$^+$ T cells in peripheral blood (PB) and spleens from *Dhx15*$^{fl/fl}$ and *Dhx15* KO mice (n=5 per group). **(F)** Flow cytometry analysis of CD4$^+$ and CD8$^+$ T cells in spleen and PB from *Dhx15*$^{fl/fl}$ and *Dhx15* KO mice (n=5 per group). **(G)** Quantification of CD4 and CD8 T-cell populations in thymus from each group (n=5) in frequencies. DN, CD4$^-$CD8$^-$ double negative. DP, CD4$^+$CD8$^+$ double positive. **(H)** Flow cytometry analysis of DN populations (gated on Lin$^-$ events) based on CD44 and CD25 expression (left and middle). Absolute cell numbers are shown on the right (n=6 per group). DN1, CD44$^+$CD25$^-$; DN2, CD44$^+$CD25$^+$; DN3, CD44$^-$CD25$^+$; DN4, CD44$^-$CD25$^-$. **(I)** Apoptosis was analyzed by Annexin V staining in DN and DP populations from *Dhx15*$^{fl/fl}$ and *Dhx15* KO mice (left). Quantifications are shown in the bottom (right). Data shown represent the means (±SD), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

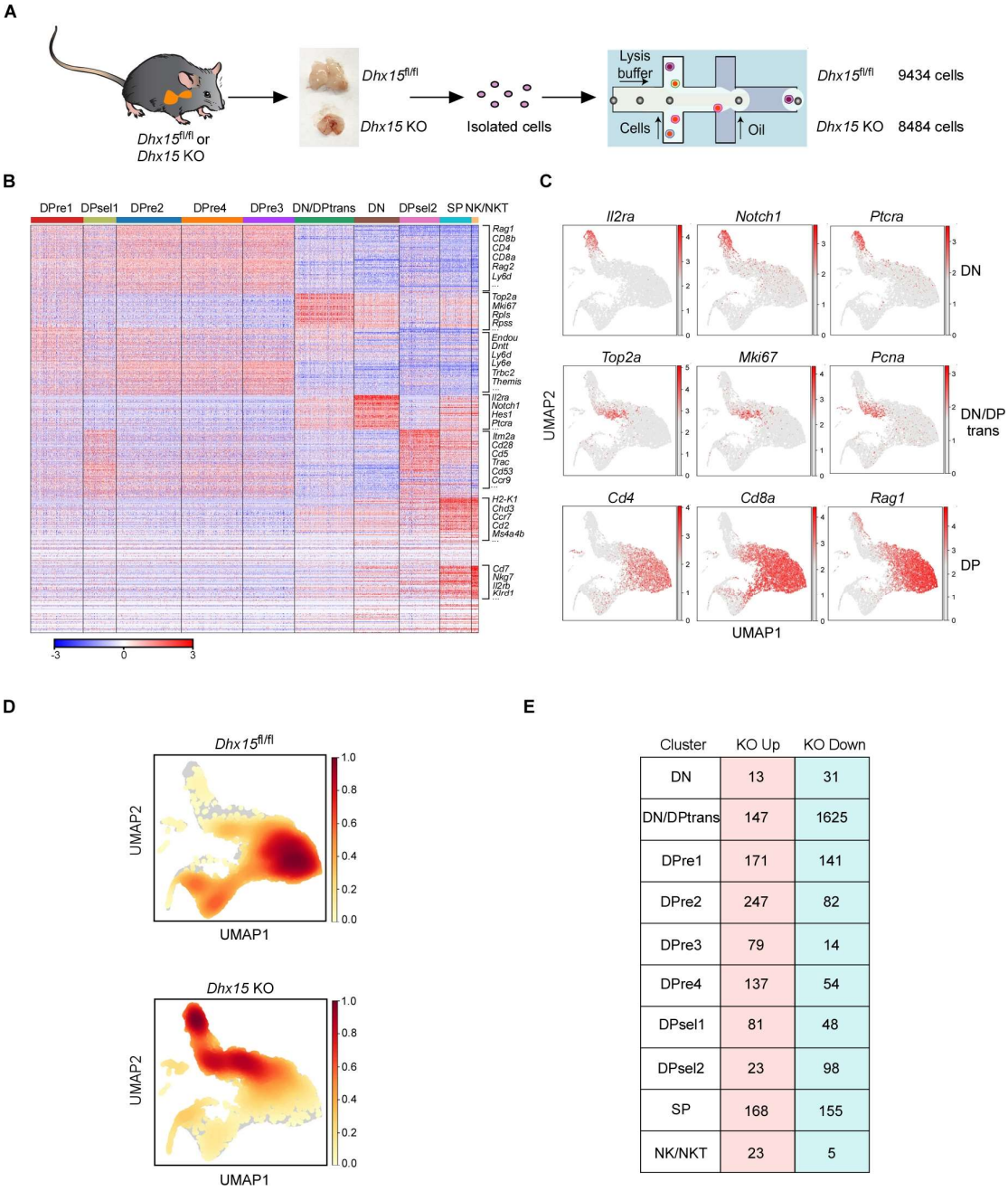

Figure S8. Single-cell RNA sequencing of thymocytes from *Dhx15*$^{fl/fl}$ and *Dhx15* KO mice. (A) Schematic diagram of procedures for the single-cell isolation and sequencing of *Dhx15*$^{fl/fl}$ and *Dhx15* KO thymocytes. **(B)** Heatmap presentation of cluster marker genes (color-coded by clusters), with representative genes displayed on the right. **(C)** Representative marker genes for DN (*Il2ra*, *Notch1* and *Ptcra*), DN-to-DP transitional cell (*Top2a*, *Mki67* and *Pcna*) and DP (*Cd4*, *Cd8a*, *Rag1*) clusters projected onto UMAP plots. **(D)** Cell type composition heatmap of single cells from *Dhx15*$^{fl/fl}$ and *Dhx15* KO thymus. High relative cell density is marked as dark red. **(E)** Differentially up- or down-regulated gene numbers in each cluster of *Dhx15* KO thymus cells as compared to control (*Dhx15*$^{fl/fl}$) (FDR<0.05, Fold change<-2).

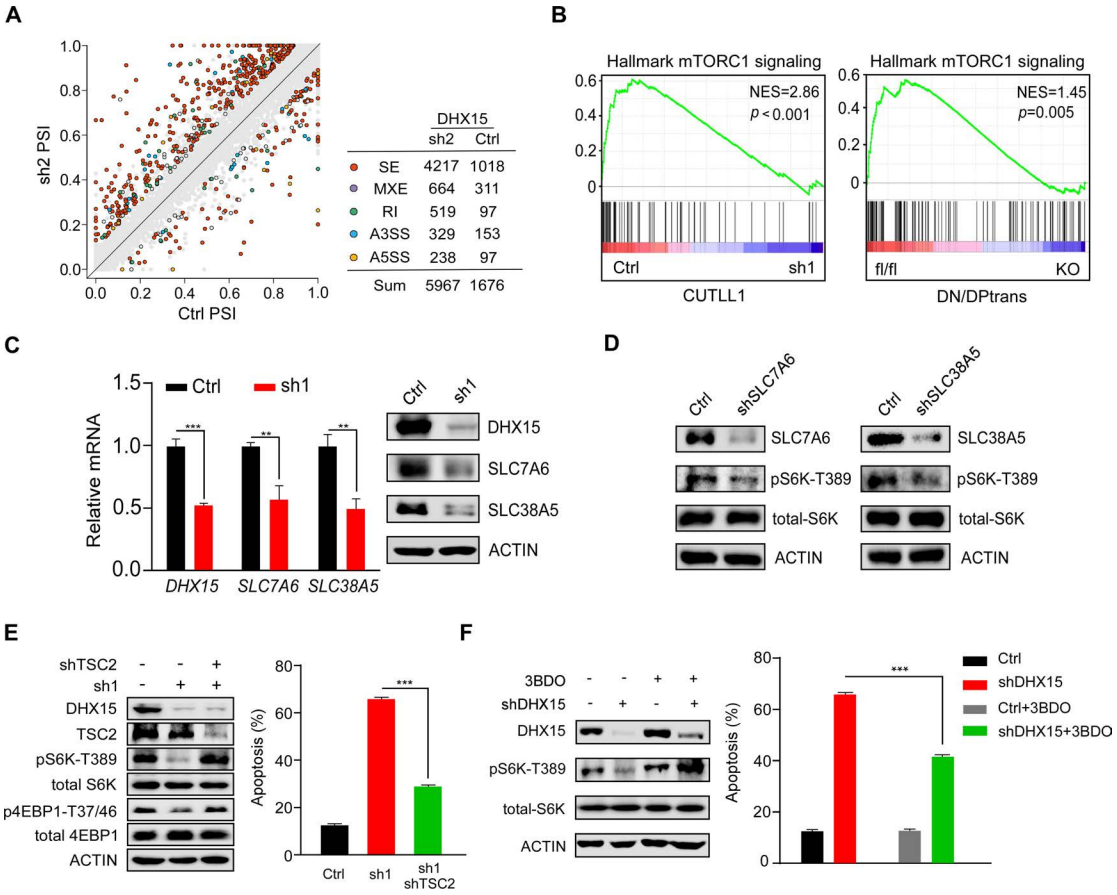

Figure S9. *DHX15* deficiency inhibits mTORC1 activity. (A) Scatterplot showing significantly changes in alternative splicing (|ΔPSI|≥0.1) (sh2 vs Ctrl). **(B)** GSEA of mTORC1 signaling in CUTLL1 cells (left) and DN/DPtrans cells (right) with or without *DHX15* depletion. **(C)** *DHX15* was depleted by shRNA (sh1) in KOPTK1 cells. *SLC7A6*, *SLC38A5* mRNA and protein were analyzed by qPCR (left) and immunoblot (right). **(D)** *SLC7A6* or *SLC38A5* was depleted by shRNA in CUTLL1 cells. S6K T389 phosphorylation (pS6K T389) was analyzed by immunoblot. **(E)** KOPTK1 cells were transduced with lentiviruses expressing *DHX15* shRNA (sh1) alone or in combination with *TSC2* shRNA. DHX15 and TSC2 proteins were analyzed by immunoblot (left); apoptotic cell death was assessed by Annexin V-PI staining 4 days post infection (right). **(F)** KOPTK1 cells were transduced with lentiviruses expressing *DHX15* shRNA (sh1) or in combination with mTORC1 agonist 3BDO (75μM for 48 hr). DHX15 and pS6K T389 were analyzed by immunoblot (left). Apoptotic cell death was analyzed by Annexin V-PI staining 4 days post infection (right). Data shown represent the means (±SD), $^{**}p < 0.01$; $^{***}p < 0.001$.

A

Drug	Connectivity Score	Adjusted P value	Mechanism of Action
Ciclopirox	0.6426	0.000070	Membrane integrity inhibitor
TW-37	0.5990	0.000220	BCL inhibitor
Pralatrexate	0.5916	0.000246	DHFR inhibitor
Rapamycin	0.5863	0.000264	mTOR inhibitor
NVP-BHG712	0.5862	0.000264	Ephrin inhibitor
VU-0418946-1	0.5832	0.000275	HIF activator

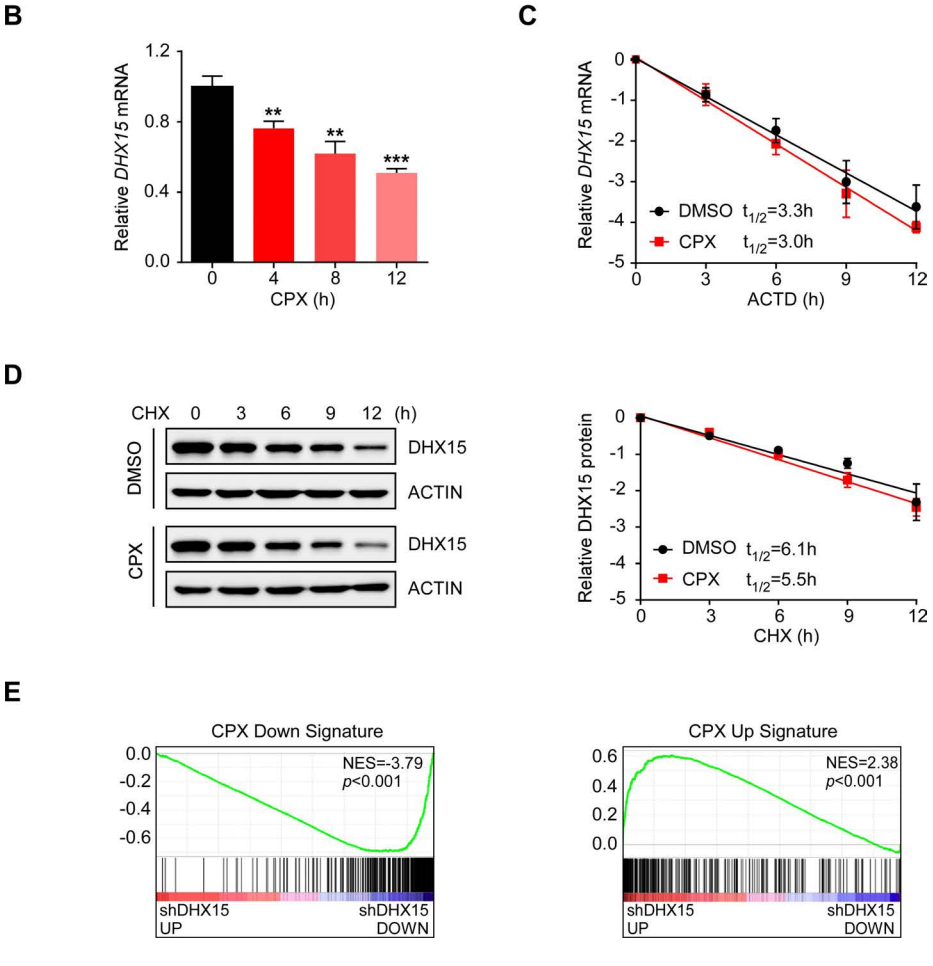

Figure S10. Identification of ciclopirox (CPX) as a *DHX15* signature modulator drug. (A) Top hits from Connectivity Map analysis for drugs with differential gene signatures overlapping with those induced by *DHX15* depletion. **(B-C)** CUTLL1 cells were treated with 10 μM CPX for indicated time points, *DHX15* mRNA was analyzed by qPCR (B). *DHX15* mRNA half-life (normalized to 18s rRNA) was assessed post actinomycin D (ACTD, 10 μg/mL) treatment (C). **(D)** CUTLL1 cells were treated with 10 μM CPX followed by 50 μg/mL cycloheximide (CHX) for indicated time points, DHX15 protein was analyzed by immunoblots (left) and its half-life was quantified (right). **(E)** GSEA plots of upregulated and downregulated genes upon *DHX15* depletion in the expression signature induced by CPX treatment (5μM, 12 hr) (p<0.001, Fold change >1.4; top up- and down-regulated 300 genes). **$p < 0.01$; ***$p < 0.001$.

Supplemental Table 1. List of primers used in this study.

Primers for real-time PCR		
DHX15 forward	5′-TCTACACTTCCACCTCAGCAGCA-3′	Human
DHX15 reverse	5′-CCAGGATCAATCACAAACACCACAC-3′	Human
MYB forward	5′-ATCTCCCGAATCGAACAGATGT-3′	Human
MYB reverse	5′-TGCTTGGCAATAACAGACCAAC-3′	Human
SLC7A6 forward	5′-CCACCTACCATCTTGTCCCTA-3′	Human
SLC7A6 reverse	5′-GCATAGTTTCGGAGGACCTTTG -3′	Human
SLC38A5 forward	5′-GCTACAGGCAAGAACGTGAGG-3′	Human
SLC38A5 reverse	5′-ATTCCAAACGATGTCTTCCCC -3′	Human
ACTIN forward	5′-CACCATTGGCAATGAGCGGTTC-3′	Human
ACTIN reverse	5′-AGGTCTTTGCGGATGTCCACGT-3′	Human
18S rRNA forward	5′-CGGCGACGACCCATTCGAAC -3′	Human
18S rRNA reverse	5′-GAATCGAACCCTGATTCCCCGTC -3′	Human
Dhx15-Exon2 forward	5′-CGGGAACGAGATCGGGATAGAGAGG -3′	Mouse
Dhx15-Exon2 reverse	5′-CAGACCCAGTCTCACCAACAAGTAC -3′	Mouse
Actin forward	5′-GGCTGTATTCCCCTCCATCG -3′	Mouse
Actin reverse	5′-CCAGTTGGTAACAATGCCATGT-3′	Mouse
Primers for ChIP assay		
ACTIN promoter forward	5'-GACTTCTAAGTGGCCGCAAG-3'	
ACTIN promoter reverse	5'-TTGCCGACTTCAGAGCAAC-3'	
DHX15 promoter forward	5'-CACCGTGTGGGCTGTAGTAG-3'	
DHX15 promoter reverse	5'-CCTAGGTCCAACCGGTGCC -3'	
Primers for luciferase reporter constructs		
DHX15 promoter WT forward	5′-TGCAACTGAGGGTAGCGCA -3′	
DHX15 promoter WT reverse	5′-TCGAACGGGCAGTTATTAAGGAA-3′	
DHX15 promoter Mut forward	5′-TGCGCCTGAGGGTAGCGCAGTGG -3′	
DHX15 promoter Mut reverse	5′-TCGGCCGGGCAGGCATTAAGGAAGAAAGCTGGCTGC-3′	
shRNA sequences		
shDHX15#1	5′-GTTGGTTCGATAATGGCCTTT-3′	Targeting 3′-UTR
shDHX15#2	5′-ACTGTTCTAATGAGGTCCTAT-3′	Targeting CDS
shMYB#1	5′-CAGATGACATCTTCCAGTCAA-3′	
shMYB#2	5′-ACTATTCCTATTACCACATTT-3′	
shTSC2	5′-GAGGGTAAACAGACGGAGTTT-3′	

shTAL1#1	5′-CCTGCTGAACGGCGTCGCCAA-3′	
shTAL1#2	5′-ACCAAAGTTGTGCGGCGTATC-3′	
shGATA3#1	5′-GCCTACATGCTTTGTGAACAA-3′	
shGATA3#2	5′-CATCCAGACCAGAAACCGAAA-3′	
shLMO1#1	5′-CAGCTCAATGGCACCTTTGAA-3′	
shLMO1#2	5′-GCCACATTAGAACTTCTCCGT-3′	
shLMO2#1	5′-TCTGACATAGTGTGCGAACAG-3′	
shLMO2#2	5′-CCATAGTAACTGACATGATTA-3′	
shTLX1#1	5’-CGCACCTGTCTGAACTGTTAA-3′	
shTLX1#2	5’-CGGCTCCTACAACGTGAACAT-3′	
shTLX3#1	5’-CCTGACAAGAAAGCGCCTTAC-3′	
shTLX3#2	5’-CGGCTCATGCTGCAGCTGCAA-3′	
Primers for cloning		
pCDH-HA-DHX15-WT forward	5′-CATGGAATTCATGTCCAAGCGGCACCGGTTGGACCTAGG-3′	
pCDH-HA-DHX15-WT reverse	5′-TGACGCTAGCTCAGTACTGTGAATATTCCTTGGATTGAAGTTTG-3′	
pHAGE-Flag-DHX15-WT forward	5′-AAGGAAAAAAGCGGCCGCATGTCCAAGCGGCACCGGTTG-3′	
pHAGE-Flag-DHX15-WT reverse	5′-CCGCTCGAGGTACTGTGAATATTCCTTGGATTGAAG -3′	
Primers for genotyping		
Dhx15 5′ *loxp* forward	5′-AGAGAGAGAGTGCTCTTTCTTGCTCTG-3′	WT: 425bp
Dhx15 5′ *loxp* reverse	5′-CCTAGACCAATTGAAATGAAATGTAGTC-3′	Flox: 524bp
Lck-Cre forward	5′-TGTGAACTTGGTGCTTGAGG-3′	~250bp
Lck-Cre reverse	5′-CAGGTTCTTGCGAACCTCAT-3′	

Supplemental Table 2. Clinical information for primary T-ALL samples used in this study.

Sample ID	Age	Gender	Immunophenotype (positive)	Classification
#1	13	Male	CD99, CD7, CD38, cCD3, TdT, CD34, CD4	Pro-T
#2	19	Female	CD4, CD5, CD99, CD7, TdT, CD1a, CD8, CD2, cCD3	Cortical-T
#3	15	Male	CD8, CD5, CD99, CD7, CD38, cCD3, CD2, CD3, HLA-DR, TdT	Mature-T
#4	57	Male	CD5, CD34, TdT, cCD3, CD7, CD99	Pro-T
#5	69	Female	TdT, CD99, CD7, cCD3, CD33	Pro-T
#6	29	Male	CD5dim, CD34, CD99, CD7bri, CD38, TdT, CD13, cCD3, CD2, CD117	ETP-ALL
#7	50	Female	CD7,CD2,CD5, CD10,cCD3,CD4,TdT	Cortical-T