Supplementary Materials and Methods

Conventional anti-cancer drugs

Conventional anti-cancer drugs used in this study and their sources are 4-hydroxycyclophospamide, cytosine arabinoside (Wako Biochemicals, Osaka, Japan), dexamethasone, L-asparaginase (Sigma-Aldrich) and bortezomib (Millennium Pharmaceuticals, Cambridge, MA). All drugs were dissolved in dimethyl sulfoxide (DMSO) at appropriate concentrations and used at a final dilution of 1/1000.

Cell proliferation and cell death assays

Cell proliferation was monitored using a Cell Counting Kit (Wako Biochemicals), in which the absorbance of reduced MTT is proportional to viable cell numbers. Cell death and apoptosis were judged by annexin-V reactivity on flow cytometry (BD Biosciences, San Jose, CA) and TdT-mediated dUTP nick end-labeling (TUNEL) in tissue specimens using an Apoptosis *in situ* Detection Kit (Wako Biochemicals).

Drug combination study

We calculated the combination index of LSD1 inhibitors and other anti-leukemic drugs using the CompuSyn software and generated isobolograms according to the manufacturer's instructions. The overall effect of drug combination was analyzed by the method of Chou and Talalay.

Immunoblotting

Immunoblotting was carried out according to the standard method using specific antibodies against H3K4me2 (Millipore), H3K9me2 (Abcam, Cambridge, UK), histone H3, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), LSD1, c-Myb, Cyclin D1, Notch3 (Cell Signaling

Technology, Beverly, MA) and TAL1 (Millipore).

Immunofluorescence staining

Frozen sections of tissues were prepared for immunostaining as described previously (Ref. 15 in the main text). We used Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 594-conjugated anti-mouse IgG (Sigma-Aldrich) as secondary antibodies. Nuclei were counterstained with DAPI.

Supplementary Table S1. Primers for semi-quantitative RT-PCR

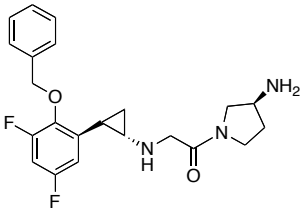
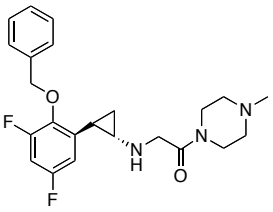
Gene	Sequence (corresponding nucleotide positions)*	Product size
ZBTB20	forward: 5'- gattaaagagcgcgaggaga -3' (44)	322 bp
	reverse: 5'- cttgggcctagtgcagatgt -3' (366)	
RHOB	forward: 5'- gcatgaacaggacttgacca -3' (1575)	187 bp
	reverse: 5'- gacaggcacaaagttcgctta -3' (1762)	
CD84	forward: 5'- cacatatatcatggcttcaaggaac -3' (865)	206 bp
	reverse: 5'- gcagcctagatcacaatttcatag -3' (1071)	
CD19	forward: 5'- ggacagcctgaaccagagc -3' (593)	345 bp
	reverse: 5'- gaccttccagccaccagtc -3' (938)	
HRK	forward: 5'- tactggccttggctgtgc -3' (322)	148 bp
	reverse: 5'- cacagggttttcaccaacct -3' (470)	
NOTCH3	forward: 5'- gccaagcggctaaaggta -3' (5261)	253 bp
	reverse: 5'- gcctcatcctcttcagttgg -3' (5514)	
SMC1A	forward: 5'- attaatgcgaggcccaaagt -3' (2095)	221 bp
	reverse: 5'- gccaaaatcttgtaatggatcg -3' (2316)	
CXCL2	forward: 5'- cgcccatggttaagaaaatc -3' (429)	150 bp
	reverse: 5'- gcctctgcagctgtgtctc -3' (579)	
TAL1	forward: 5'- catggtgcagctgagtcct -3' (928)	298 bp
	reverse: 5'- gtgtggggatcagcttgc -3' (1226)	
ETS2	forward: 5'- catcagaggcaacgtgaaga-3' (1053)	192 bp
	reverse: 5'- gcctgactctggagaccaag-3' (1244)	
H2A	forward: 5'- gcaatttgatgcgaggagat -3' (18)	358 bp
	reverse: 5'- gcctgaatgttaggcaggac -3' (376)	
DAB2IP	forward: 5'- cccacctccacaaagtacca -3' (1824)	116 bp
	reverse: 5'- gcctggcctgaaggttct -3' (1940)	
MYB	forward: 5'- gcagcctttccgagaaga -3' (152)	220 bp
	reverse: 5'- cttcagcctcggcatcag -3' (372)	
GAPDH	forward: 5'-ccacccatggcaaattccatggca-3' (211)	600 bp
	reverse: 5'-tctagacggcaggtcaggtccacc-3' (811)	

*The validity of each primer pair was verified using appropriate cell lines.

Supplementary Table S2. Primers for ChIP assays

Gene	Sequence (corresponding genomic region)	Product size
Notch3 enhancer	forward: 5'-TAGATGCCCATCAGGGAAAG-3' (GRCh38/hg38: 15,198,031)	170 bp
	reverse: 5'-GGAAGCTGCCAGAGCTAAGA-3' (GRCh38/hg38: 15,197,862)	
TAL1 enhancer	forward: 5'-GAATGCACATGCGCTTAAAA-3' (GRCh38/hg38: 47,239,435)	317 bp
	reverse: 5'-TCCTCCTCTCACCACTTGCT-3' (GRCh38/hg38: 47,239,119)	

Supplementary Table S3. Pharmacokinetic parameters in ICR mice*

	S2116	S2157
		
	(±)-*trans*	(±)-*trans*
C_{max} (μM)	12.7 ± 1.60	4.24 ± 0.12
T_{max} (h)	0.25	0.50
$T_{1/2}$ (h)	3.76	1.32
AUC (μM•h)	59.2	5.45
nAUC (μM•h)**	1.18	0.11
Plasma concentration at 0.5 h (μM)	10.8 ± 1.50	3.86 ± 1.42
Brain concentration at 0.5 h (μmol/kg)	1.21 ± 0.14	76.7 ± 41.0
Brain/Plasma ratio	0.11	19.9

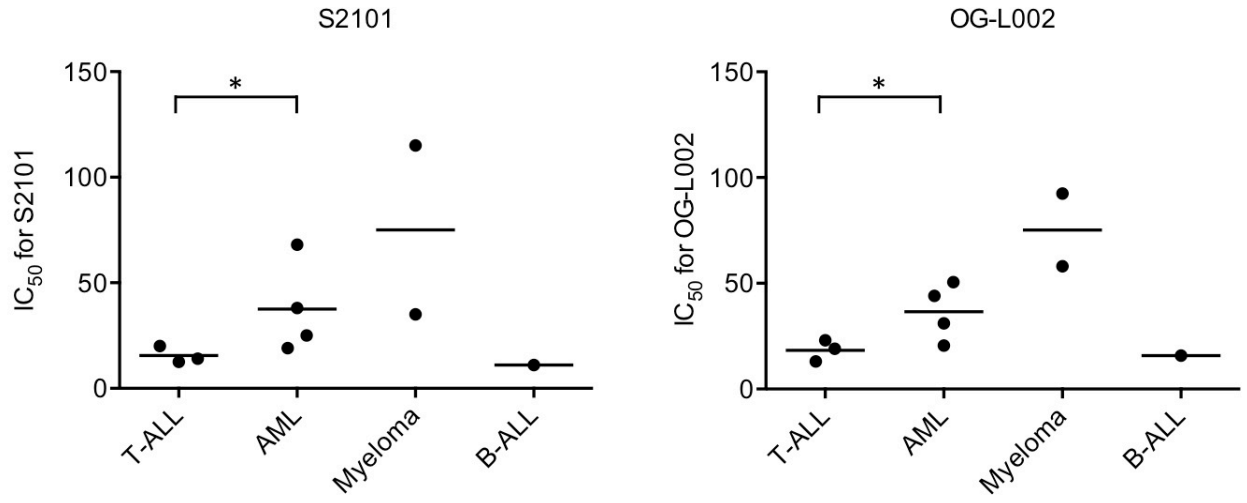
*Pharmacokinetic parameters were determined in 8-week-old ICR mice treated with a single intraperitoneal injection of either S2116 or S2157 at 50 mg/kg (n = 3).

**Dose-normalized AUC

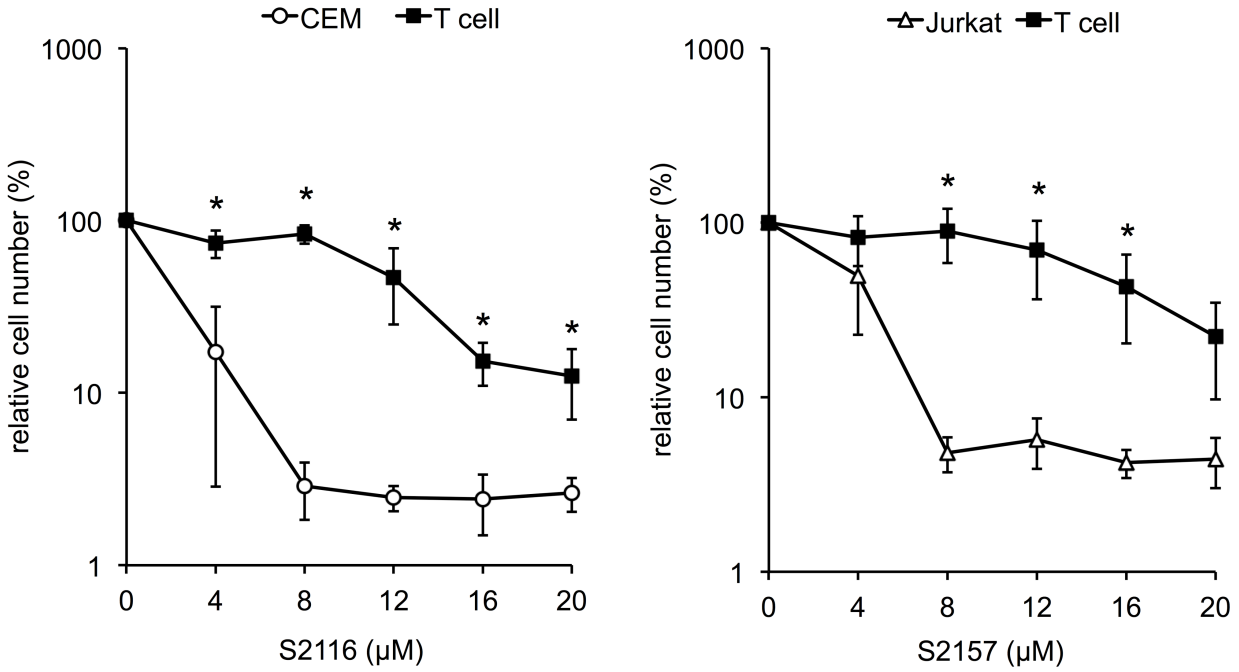
Supplementary Table S4. Expression of genes related to CNS invasion*

Gene	Vehicle-treated MOLT4 cells	S2157-treated MOLT4 cells	Fold increase by S2157
CCR7	1953.6	1564.1	0.80
CXCR4	25158	39805	1.58
CCL19	8.54	18.4	2.15
CXCL12	15.7	17.2	1.10
VEGF-A	20.0	117.5	5.88
VEGF-C	8.74	25.7	2.94
VEGFR1	10.6	5.08	0.48
CARMA1/CARD11	43.9	48.2	1.10
ITGA6	10.7	28.2	2.64

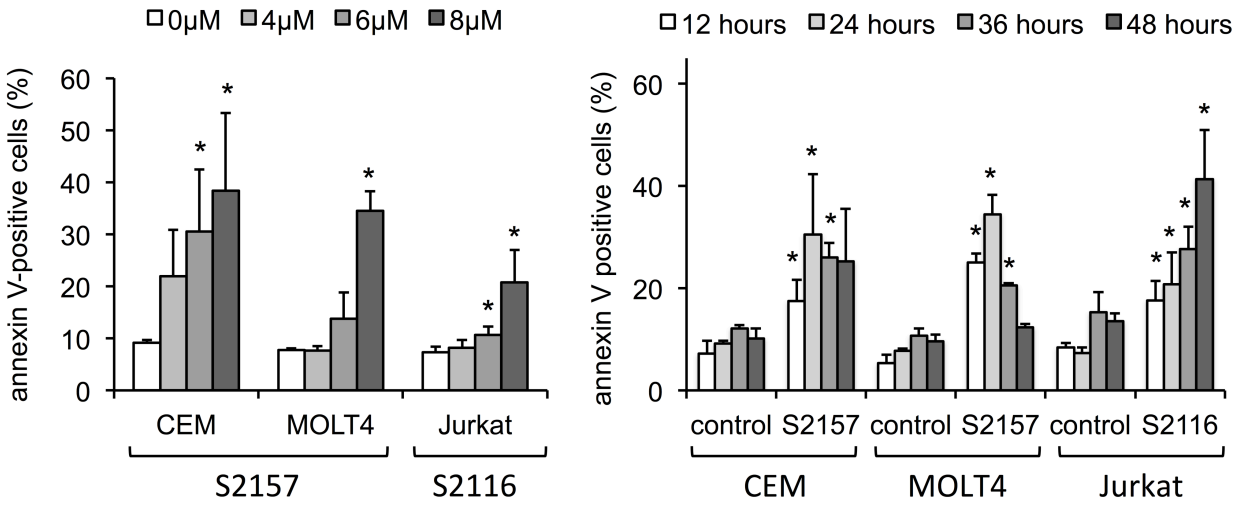
*Raw data from cDNA microarray analyses (deposited in the MIAME-compliant GEO database under accession number GSE85956).



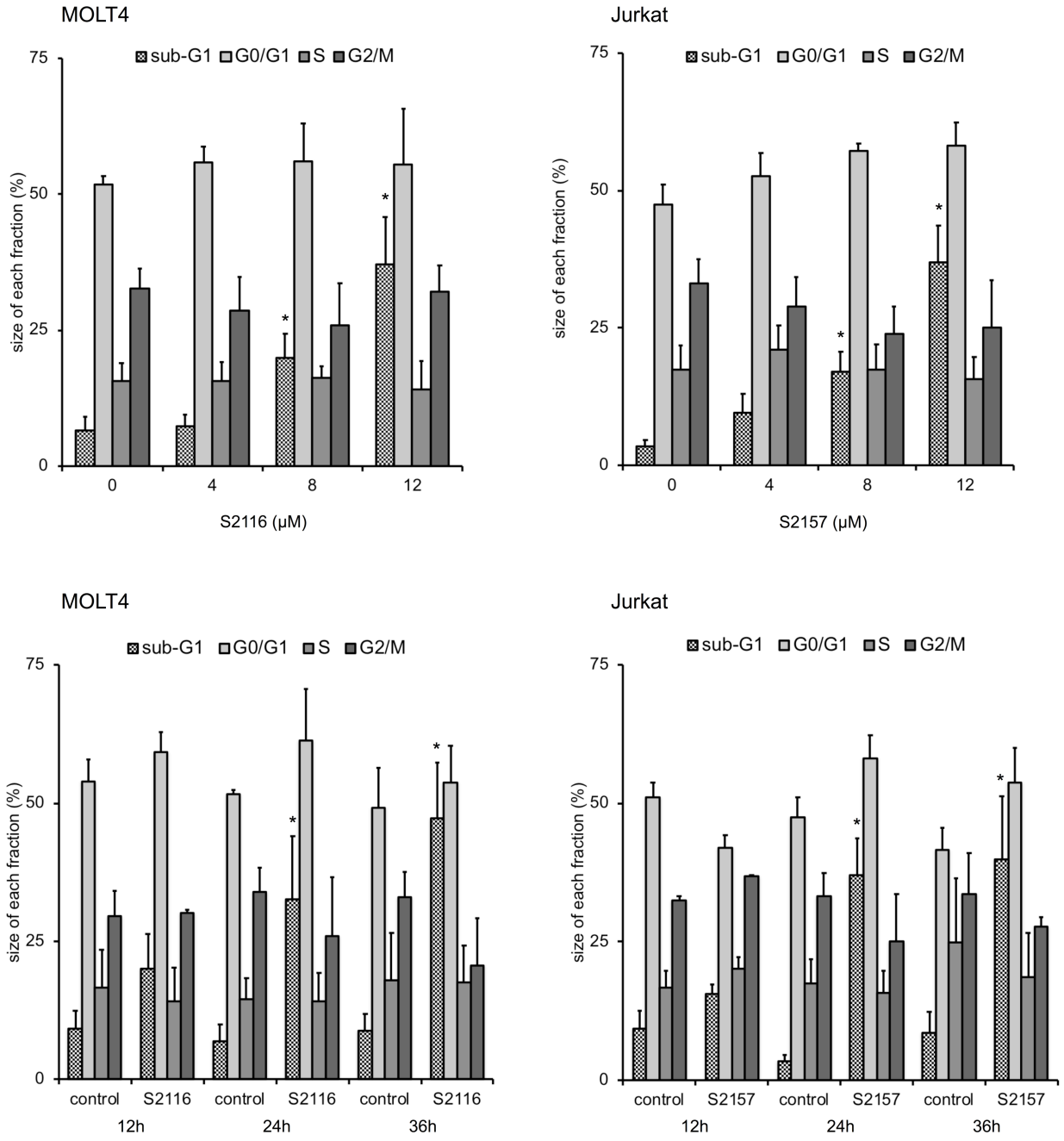
Supplementary Figure S1. LSD1 inhibitors induce apoptotic cell death in T-ALL cells *in vitro*. The IC_{50} values of S2101 (left) and OG-L002 (right) were calculated from dose-response curves of cell lines derived from T-ALL (CEM, Jurkat, and MOLT4), AML (HEL, MV4-11, K562, and KCL22), multiple myeloma (KMS12-BM and KMS28-BM) and B-ALL (PALL2) obtained at 72 hours of culture. Bars indicate the means of IC_{50} values of each group. *P <0.05 by Student's t test.



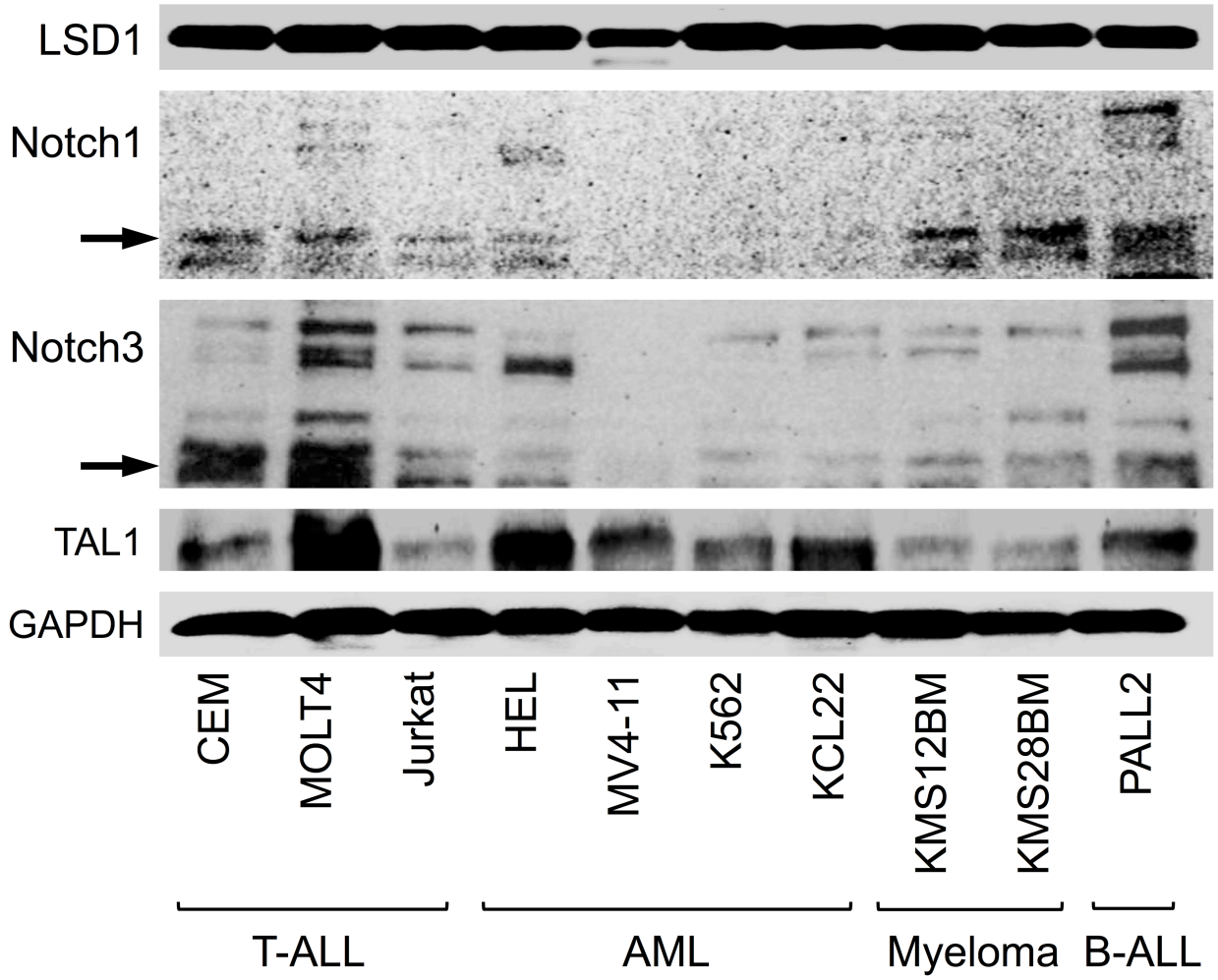
Supplementary Figure S2. Effects of S2116 and S2157 on normal T-lymphocytes. We cultured phytohemagglutinin-stimulated T-lymphocytes from healthy volunteers and CEM or Jurkat cells with various concentrations of S2116 (left panel) or S2157 (right panel) for 72 hours. Cell viability was determined by the MTT reduction assay and expressed as a percentage of the T-0 values. Each point represents the mean ± S.D. (bars) of three independent experiments. *P <0.05 by one-way ANOVA with the Bonferroni post-hoc test.



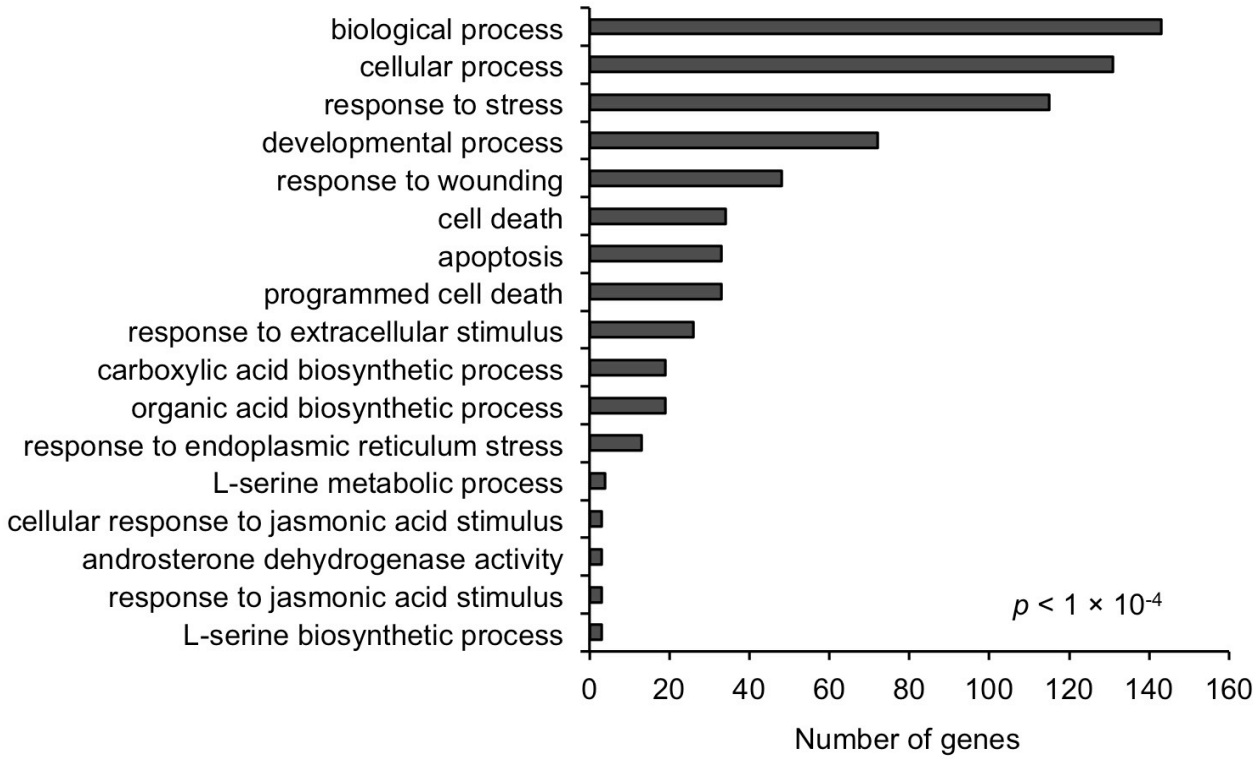
Supplementary Figure S3. LSD1 inhibitors induce apoptotic cell death in T-ALL cells *in vitro*. Left panel: T-ALL cells were cultured with various concentrations of S2116 or S2157 for 24 hours. Right panel: CEM and MOLT4 cells were cultured in the absence (control) or presence of 8 μM S2157, and Jurkat cells were cultured in the absence (control) or presence of 6 μM S2116 for the indicated periods. Cells were stained with annexin-V for the assessment of apoptosis on flow cytometry. The means ± S.D. (bars) of three independent experiments. **P* <0.05 *vs*. untreated control by one-way ANOVA with Tukey's multiple comparison test.



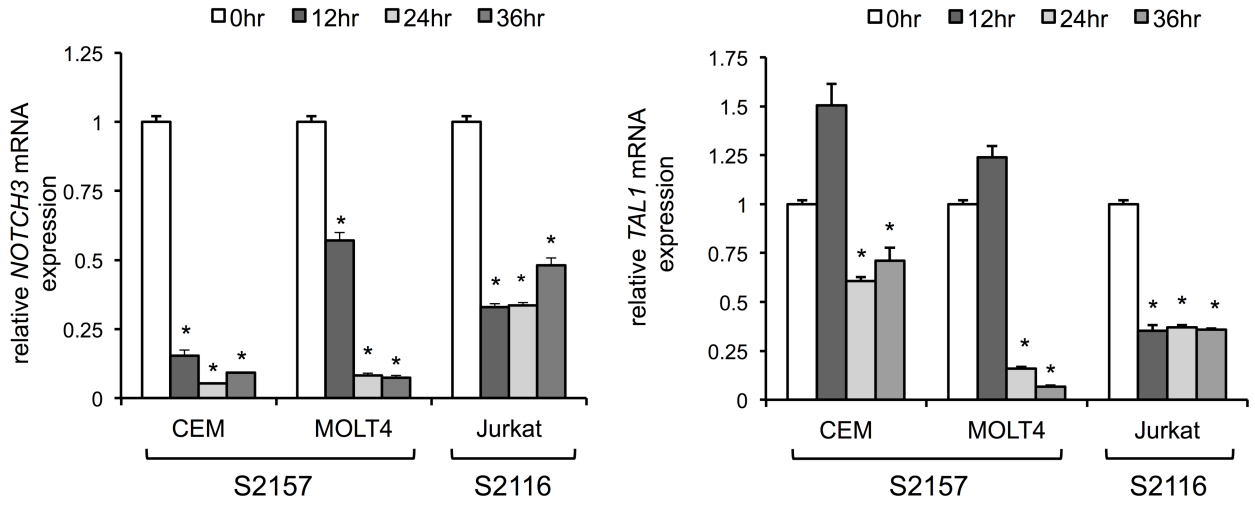
Supplementary Figure S4. Effects of novel LSD1 inhibitors on the cell cycle distribution of T-ALL cells. Upper panel: We cultured MOLT4 and Jurkat cells with S2116 and S2157, respectively, at the indicated concentrations for 24 hours. Lower panel: We cultured MOLT4 and Jurkat cells with S2116 and S2157, respectively, at 12 μM for the indicated periods. The cell cycle profile was obtained by staining cellular DNA with Vindelov's solution (0.04 mg/ml propidium iodide in 5 mM Tris-HCl, 5 mM NaCl, and 0.005% nonidet P-40) in preparation for flow cytometry. The size of the sub-G1, G0/G1 and S+G2/M fractions was calculated as a percentage by analyzing DNA histograms with the ModFitLT 2.0 program (Verity Software, Topsham, ME). Each point represents the mean ± S.D. (bars) of three independent experiments. *P <0.05 *vs*. the corresponding untreated controls by Student's t test.



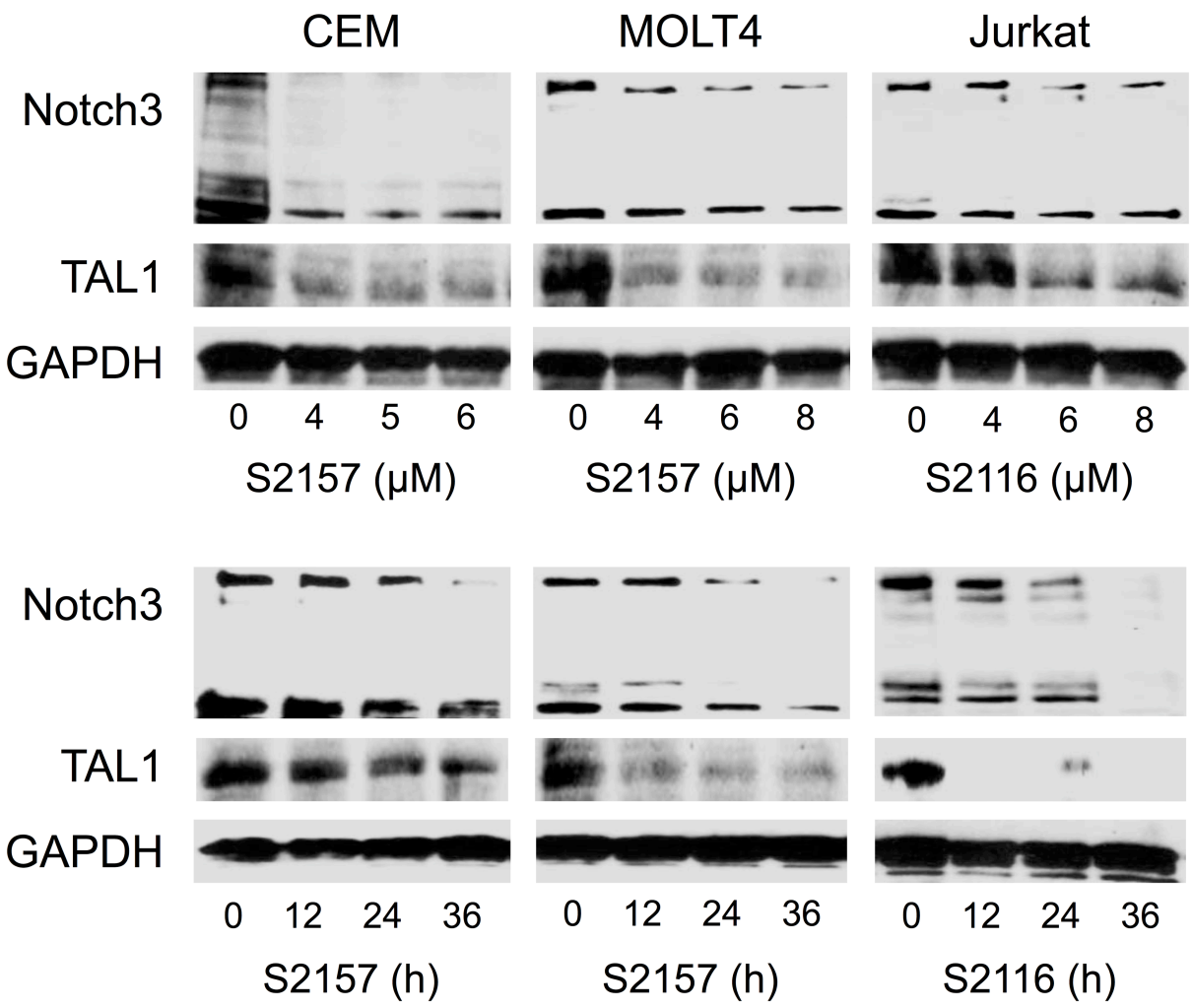
Supplementary Figure S5. The expression of T-ALL oncogenes in various hematopoietic cell lines. Whole cell lysates were prepared from cell lines derived from T-ALL (CEM, Jurkat and MOLT4), AML (HEL, MV4-11, K562 and KCL22), multiple myeloma (KMS12-BM and KMS28-BM) and B-ALL (PALL2), and subjected to immunoblot analysis for molecules related to T-cell leukemogenesis; LSD1, Notch1, Notch3 and TAL1. Arrows indicate the active/cleaved forms of Notch1 and Notch3.



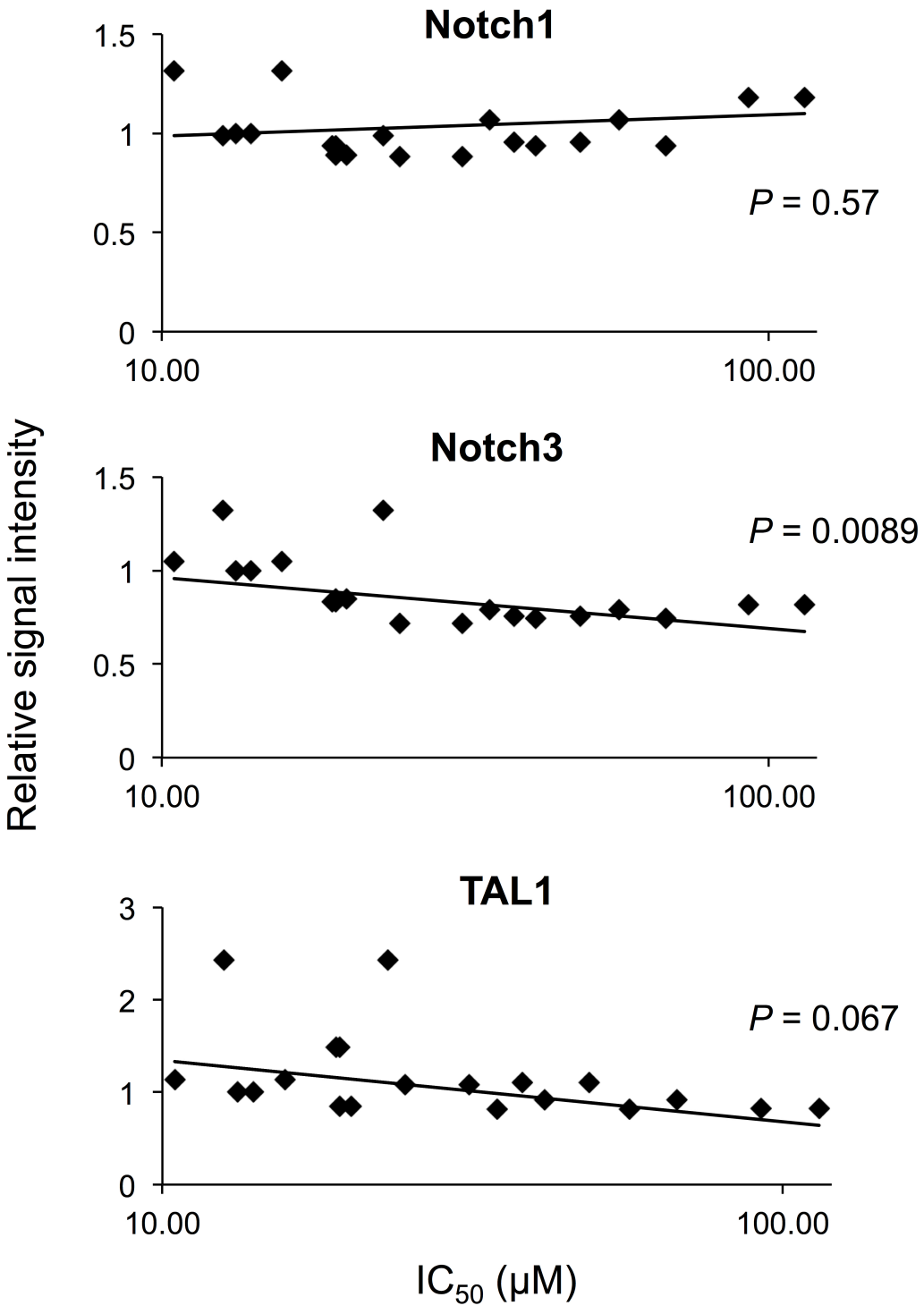
Supplementary Figure S6. LSD1 inhibitors modify the gene expression program in T-ALL cells. MOLT4 cells were cultured with either vehicle alone (0.1% DMSO) or 12 μM S2157 for 24 hours. RNA samples were subjected to microarray analysis for 32,078 genes including microRNAs. Gene ontology enrichment analysis for genes whose expression was altered more than 2-fold by S2157 treatment (P <0.0001 with an FDR threshold of 0.05).



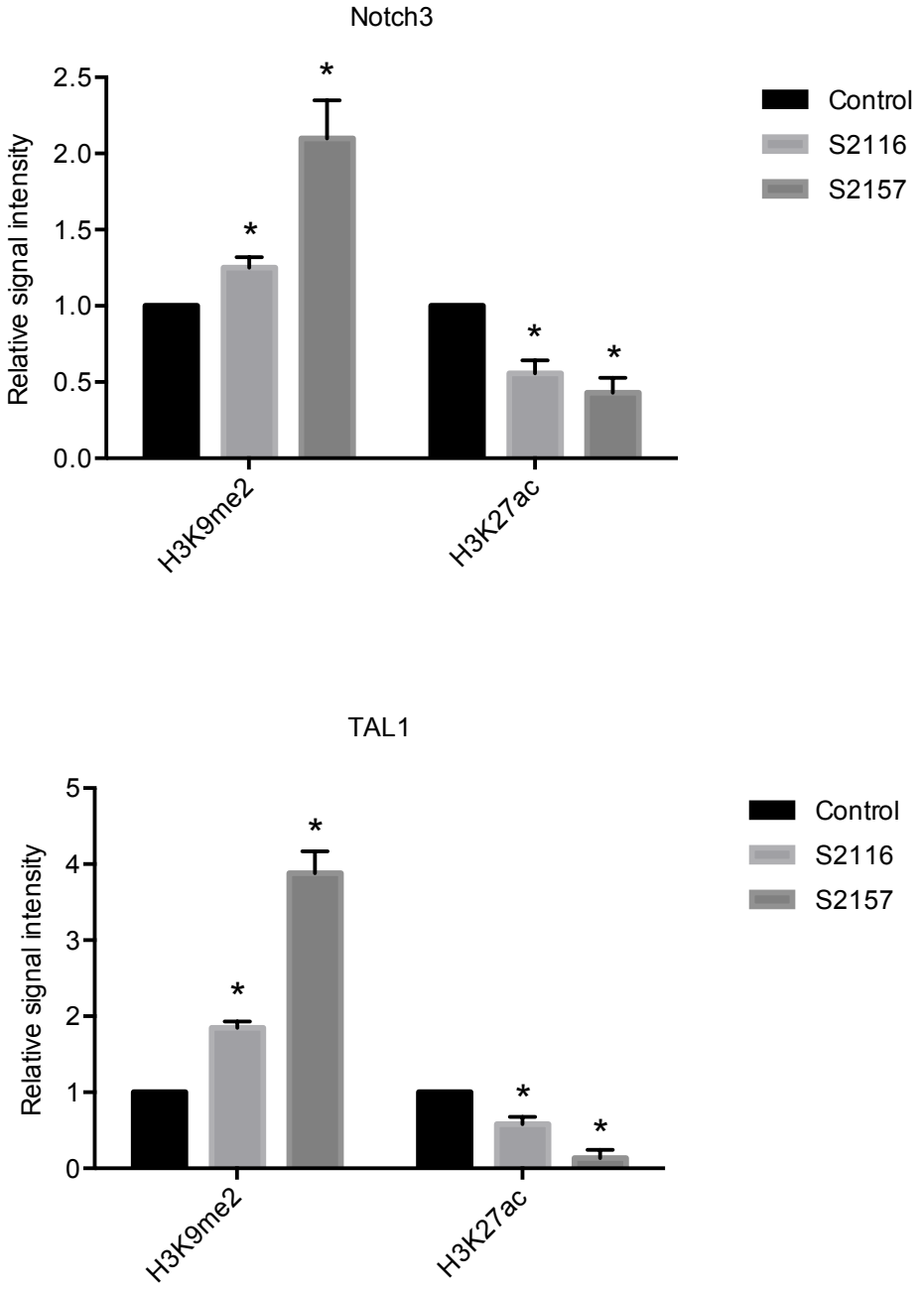
Supplementary Figure S7. LSD1 inhibitors down-regulate the expression of *NOTCH3* and *TAL1* transcripts in T-ALL cells. CEM and MOLT4 cells were cultured with 6 μM S2157, and Jurkat cells were cultured with 8 μM S2116 for the indicated periods. The expression levels of *NOTCH3* (left panel) and *TAL1* (right panel) were determined by RQ-PCR, normalized to that of *GAPDH*, and quantified by the $2^{-\Delta\Delta Ct}$ method with the T-0 values setting at 1.0. The means ± S.D. (bars) of three independent experiments are shown. **P* <0.05 *vs*. T-0 control by one-way ANOVA with Tukey's multiple comparison test.



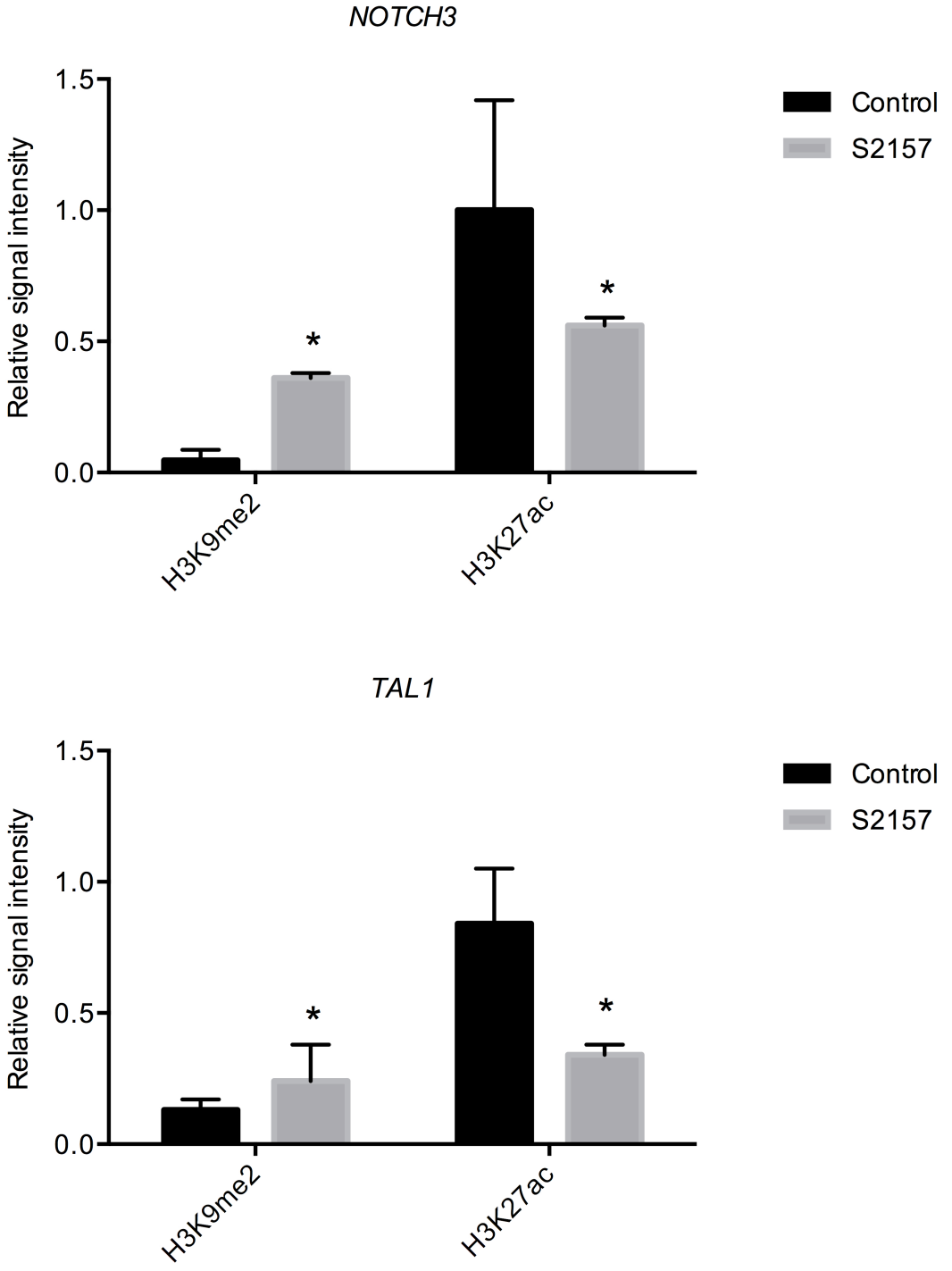
Supplementary Figure S8. LSD1 inhibitors down-regulate the expression of NOTCH3 and TAL1 proteins in T-ALL cells. Upper panel: T-ALL cells were cultured with various concentrations of S2116 or S2157 for 24 hours. Lower panel: CEM and MOLT4 cells were cultured with 6 μM S2157, and Jurkat cells were cultured with 8 μM S2116 for the indicated periods. Whole cell lysates were subjected to immunoblotting for Notch3, TAL1 and GAPDH.



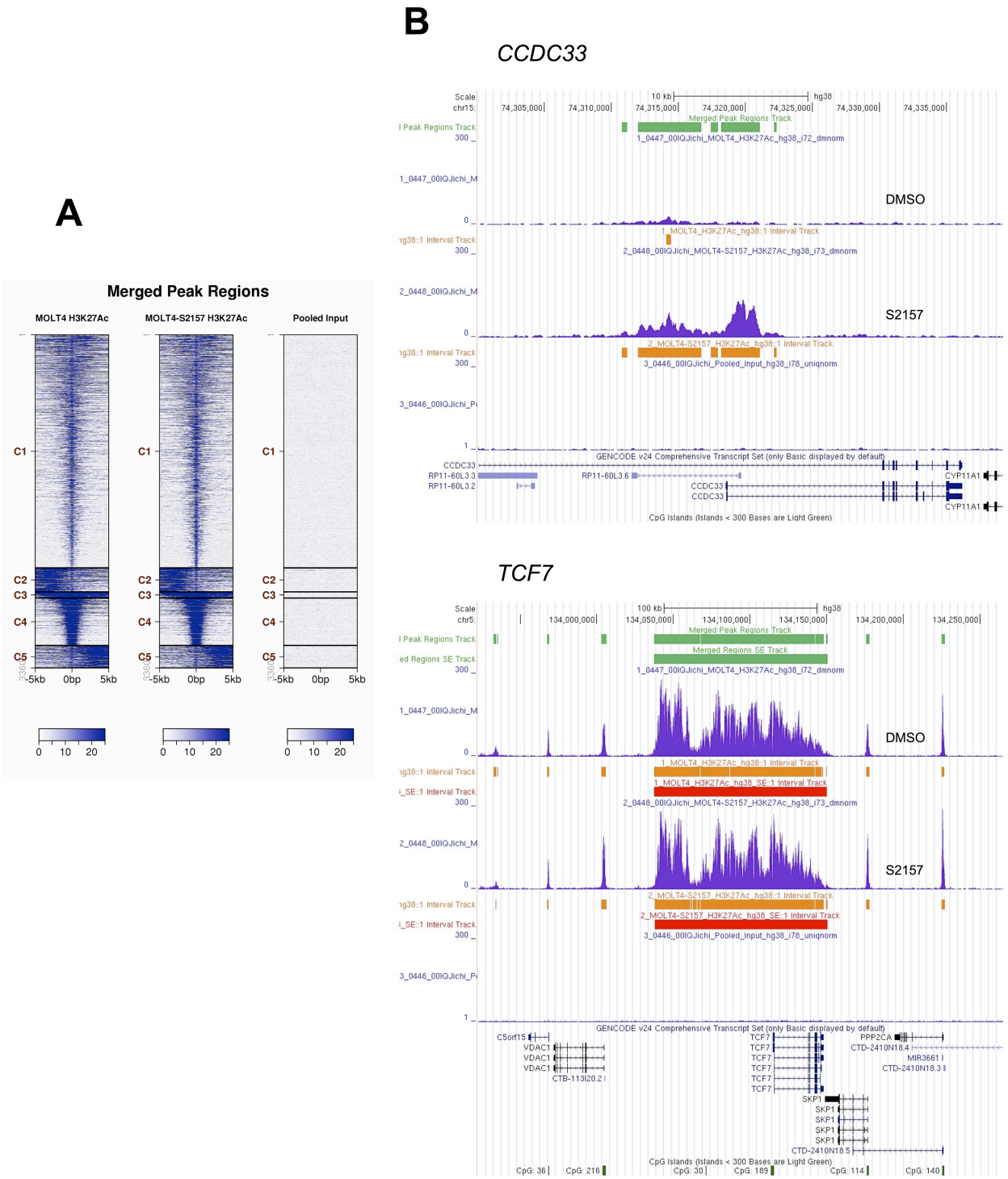
Supplementary Figure S9. The inverse correlation between Notch3/TAL1 expression levels and IC_{50} values to LSD1 inhibitors. The signal intensities of each band in Supplementary Figure S5 were quantified, normalized to those of the corresponding GAPDH, and shown as relative values with those of CEM at 1.0. The correlation between the expression level of Notch1, Notch3 or TAL1 and the IC_{50} values to LSD1 inhibitors (Figure 1A) was determined by Pearson's correlation coefficient.



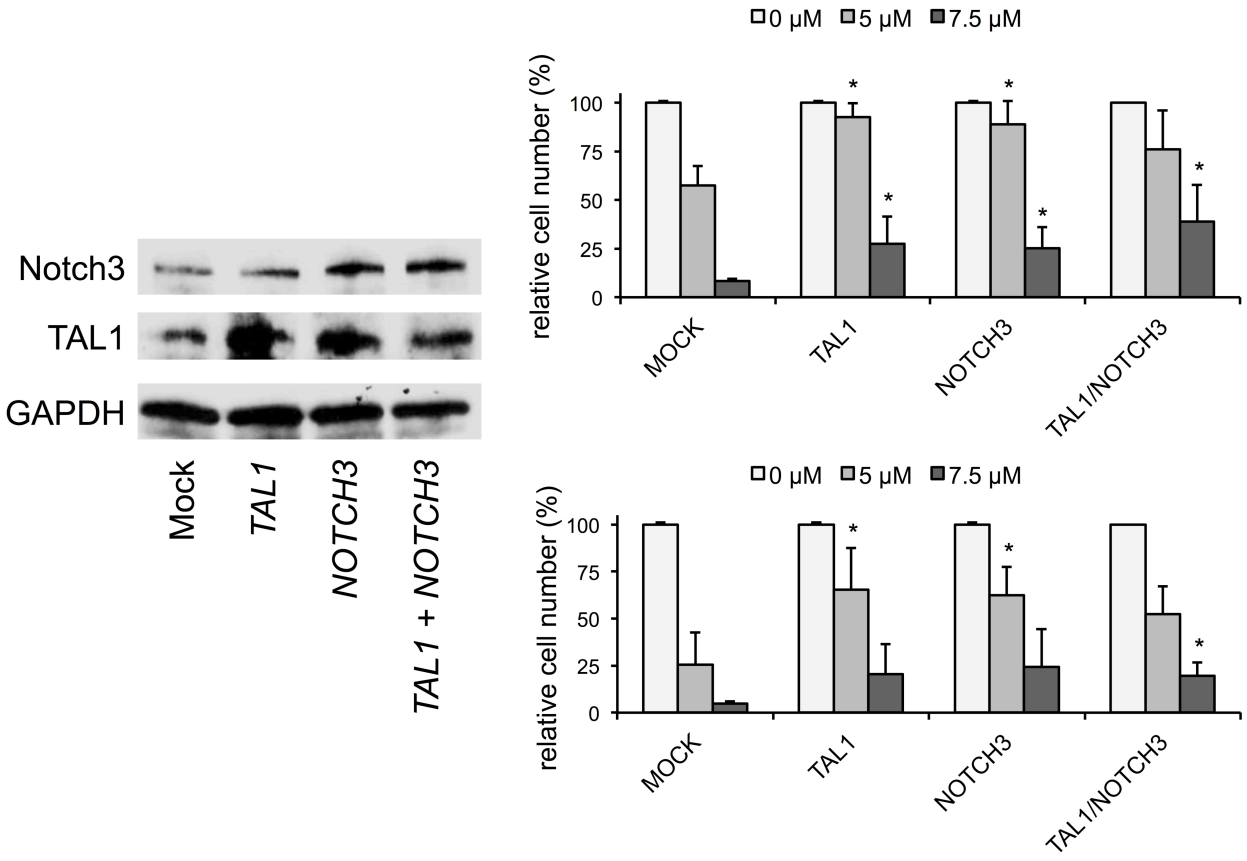
Supplementary Figure S10. Quantification of the results of ChIP assays. Using ChIP assays, we analyzed the status of H3K9 dimethylation and H3K27 acetylation at super-enhancer regions of the *NOTCH3* and *TAL1* genes (GRCh38/hg38: 15,198,031-15,197,862 and GRCh38/hg38: 47,239,435-47,239,119, respectively) in MOLT4 cells treated with either S2116 or S2157 for 24 hours. The data shown in Figure 2E were quantified and shown as the means ± S.D. (bars) of three independent experiments. *P <0.05 *vs*. untreated control by one-way ANOVA with Tukey's multiple comparison test.



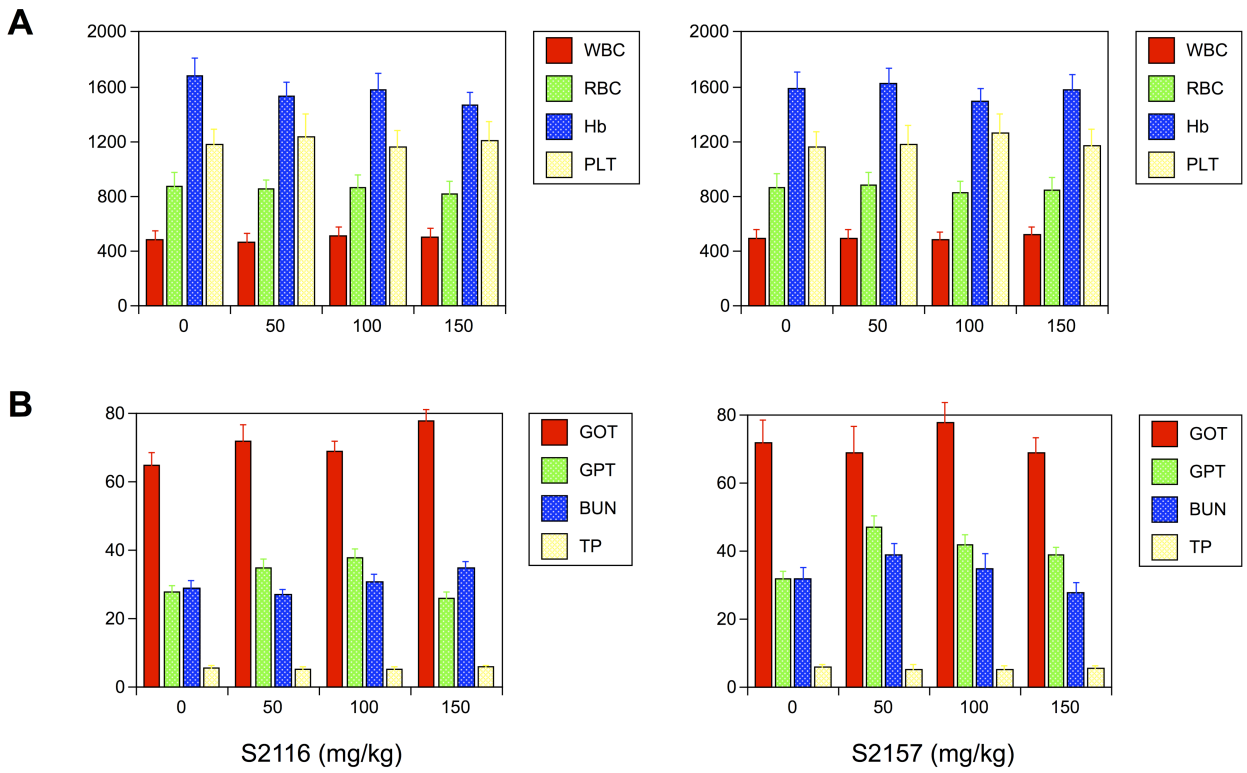
Supplementary Figure S11. ChIP-quantitative PCR analyses of S2157-induced enhancer modifications. Chromatin immunoprecipitation was performed as described in the Materials and Methods as well as the Figure 2E legend, followed by quantitative PCR using the primers corresponding to super-enhancer regions of the *NOTCH3* and *TAL1* genes (Supplementary Table S2) in MOLT4 cells treated with 0.1% DMSO (Control) 12 μM S2157 for 24 hours. The signal intensity was normalized to that of ß-actin and shown as % Input. The means ± S.D. (bars) of three independent experiments are shown. **P* <0.05 *vs*. untreated control by Student's *t* test.



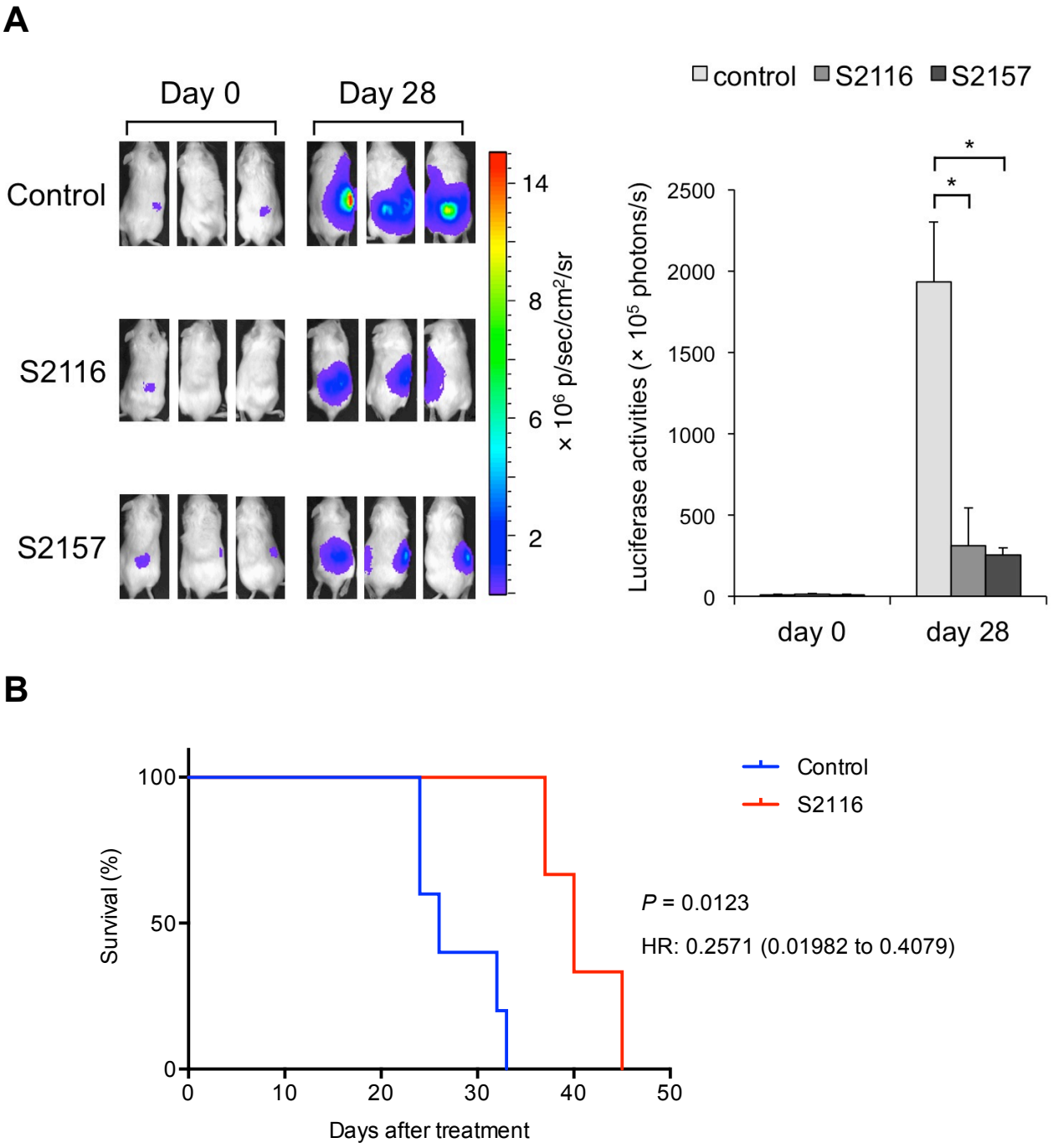
Supplementary Figure S12. ChIP-seq analyses of S2157-induced enhancer modifications. Chromatin immunoprecipitation was performed as described in the Materials and Methods, followed by high-throughput sequencing in MOLT4 cells treated with either vehicle alone (DMSO) or 12 μM S2157 for 24 hours. (A) Heatmap showing the acetylation level of histone H3K27 in the entire genome. (B) ChIP seq tracks of normalized tags showing enrichment of histone H3K27 acetylation (purple) in the enhancer regions of the *CCDC33* (upper panel) and *TCF7* (lower panel) genes visualized on the UCSC genome browser.



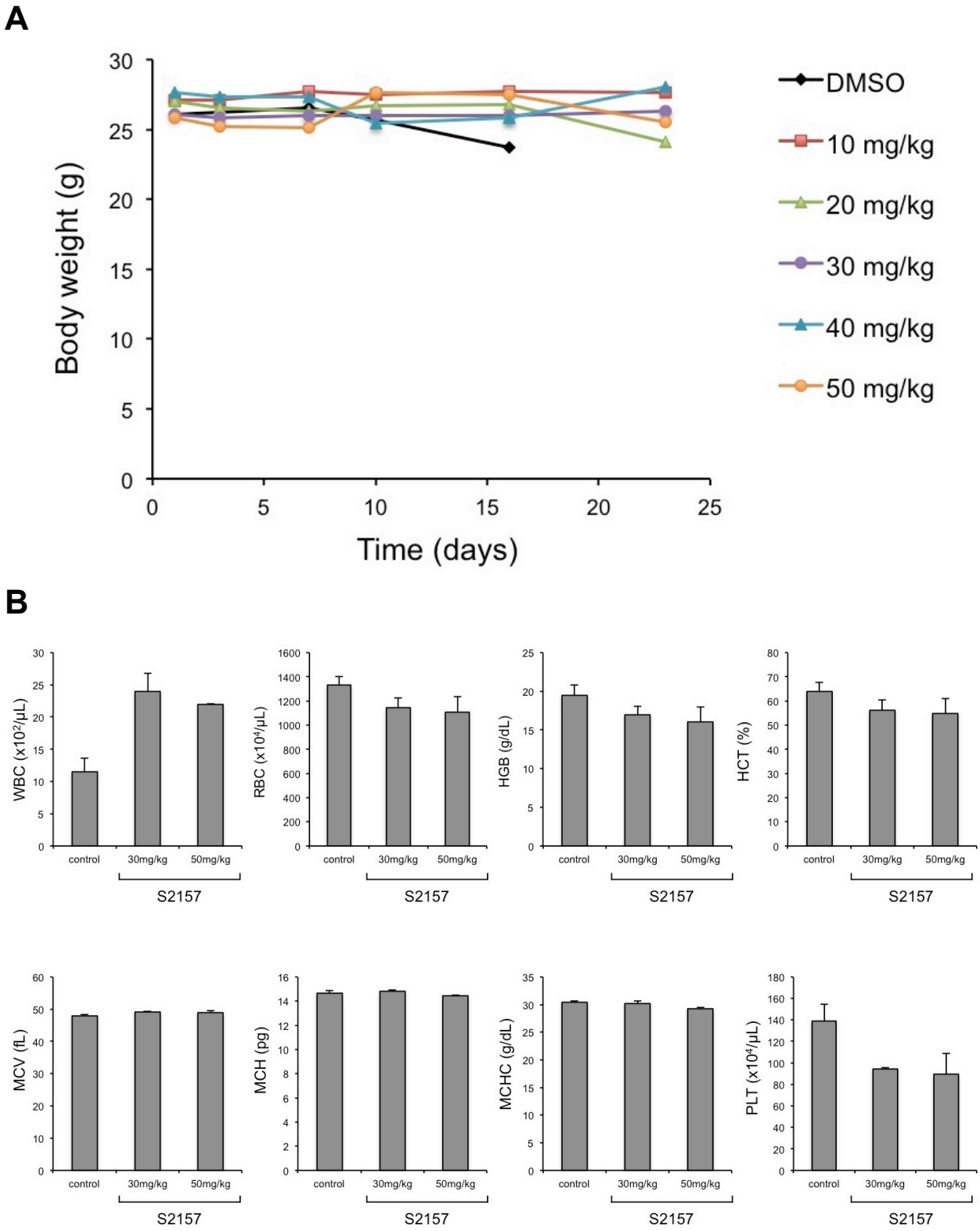
Supplementary Figure S13. Effects of dual expression of NOTCH3 and TAL1 on LSD1 inhibitor-mediated cell death in T-ALL cells. Left panel: Whole cell lysates were prepared from CEM cells transduced with an empty vector (Mock), *TAL1*-expression vector, *NOTCH3*-expression vector, or both *TAL1*- and *NOTCH3*-expression vectors and subjected to immunoblotting for Notch3, TAL1 and GAPDH. Right panel: We cultured CEM (Upper panel) and Jurkat (Lower panel) transformants with S2116 at the indicated concentrations for 72 hours. Cell viability was determined by the MTT reduction assay and is expressed as a percentage of the values for corresponding untreated cells. The means ± S.D. (bars) of three independent experiments are shown. **P* <0.05 *vs*. Mock-transfected cells by Student's *t* test.



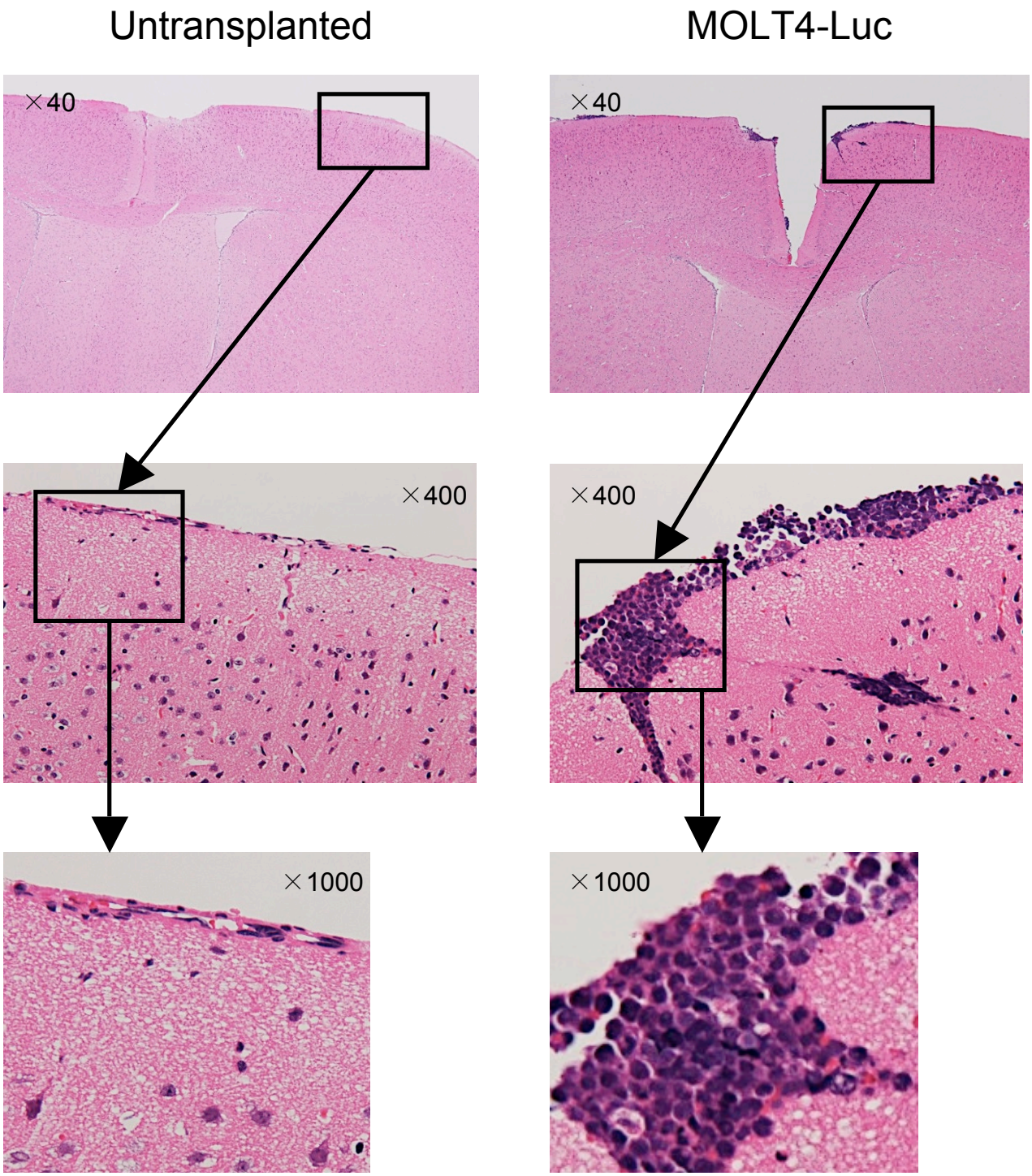
Supplementary Figure S14. Determination of the maximum tolerated doses of S2116 and S2157 in NOD/SCID mice. We carried out intraperitoneal injection of either S2116 (Left panels) or S2157 (Right panels) at the indicated doses into NOD/SCID mice for five consecutive days. At day 5, we examined complete blood count (A), liver and kidney functions (B), and histopathology of brain, bone marrow, liver, and spleen (data not shown). No significant difference was detected against vehicle-treated mice (0 mg/kg groups) by one-way ANOVA with the Bonferroni post-hoc test (n=3). The units for each parameter are WBC in cells/μL, RBC in $\times 10^4$ cells/μL, hemoglobin (Hb) in g/L, PLT in $\times 10^3$ cells/μL, GOT in IU/L, GPT in IU/L, BUN in mg/dL, and total protein (TP) in g/dL.



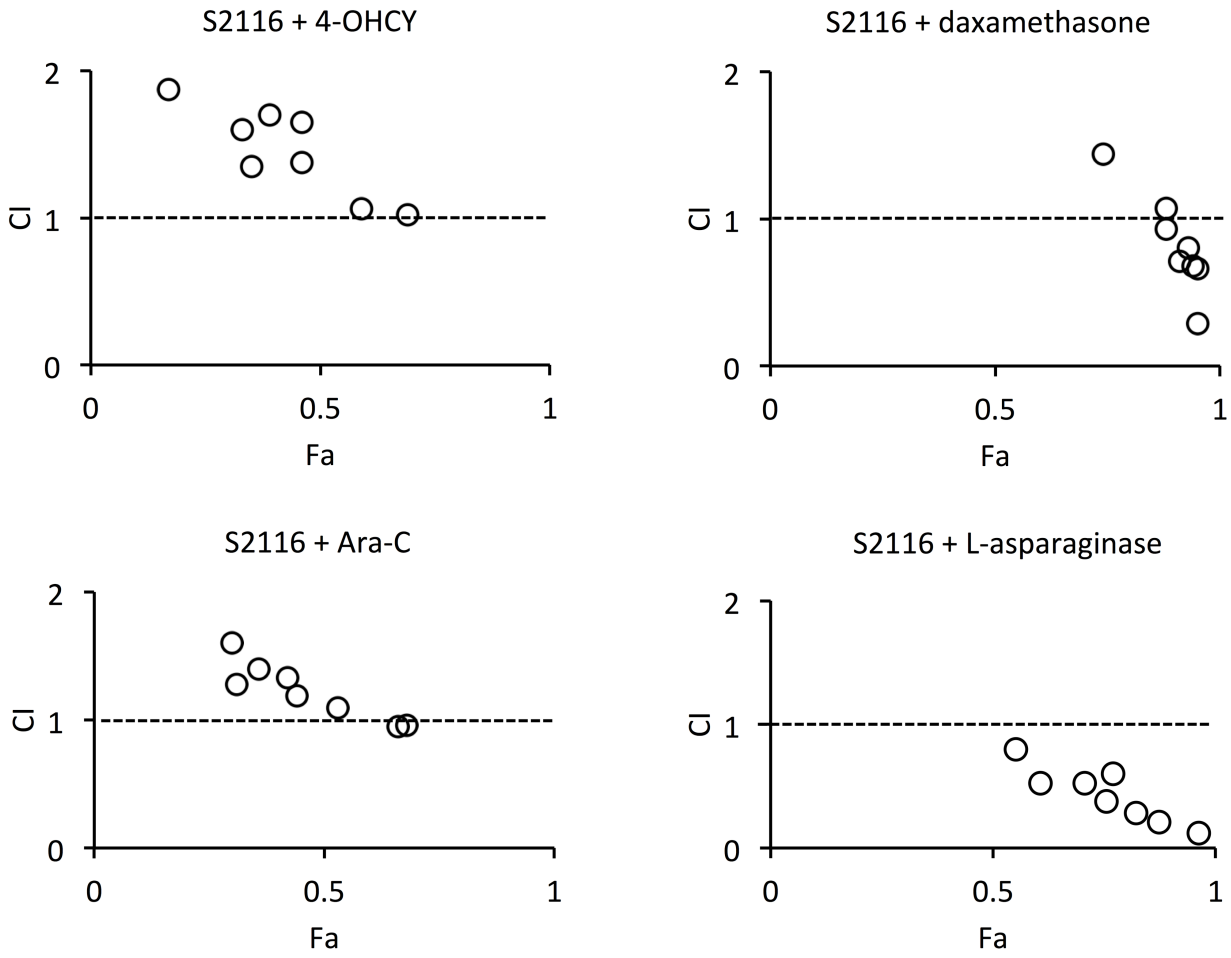
Supplementary Figure S15. Effects of *N*-alkylated LSD1 inhibitors on T-ALL cells *in vivo*. (A) We subcutaneously inoculated 5 × 10^6 luciferase-expressing MOLT4 cells into the right thigh of NOD/SCID mice. Mice were intraperitoneally administered with vehicle alone (Control), S2116 or S2157 at 50 mg/kg for 3 times a week. Tumor-derived luciferase activity was measured on days 0 and 28. *P <0.05 *vs*. the control determined by one-way ANOVA with Tukey's multiple comparison test (n=3). (B) Kaplan-Meier survival curves were generated and statistically analyzed by the log-rank test using the Prism software (GraphPad Software, La Jolla, CA).



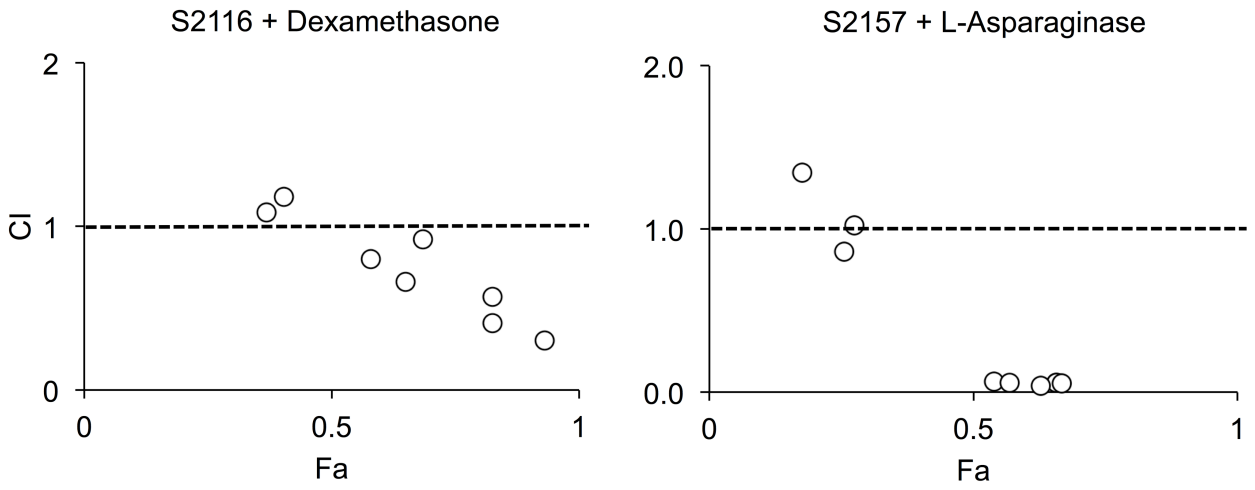
Supplementary Figure S16. Side effects of S2157 in NOD/SCID mice. During the experiments shown in Supplementary Figure S15, we monitored body weight (A) and complete blood count (B) in each mouse. No significant difference was detected *vs*. Controls by one-way ANOVA with Tukey's multiple comparison test (n=3).



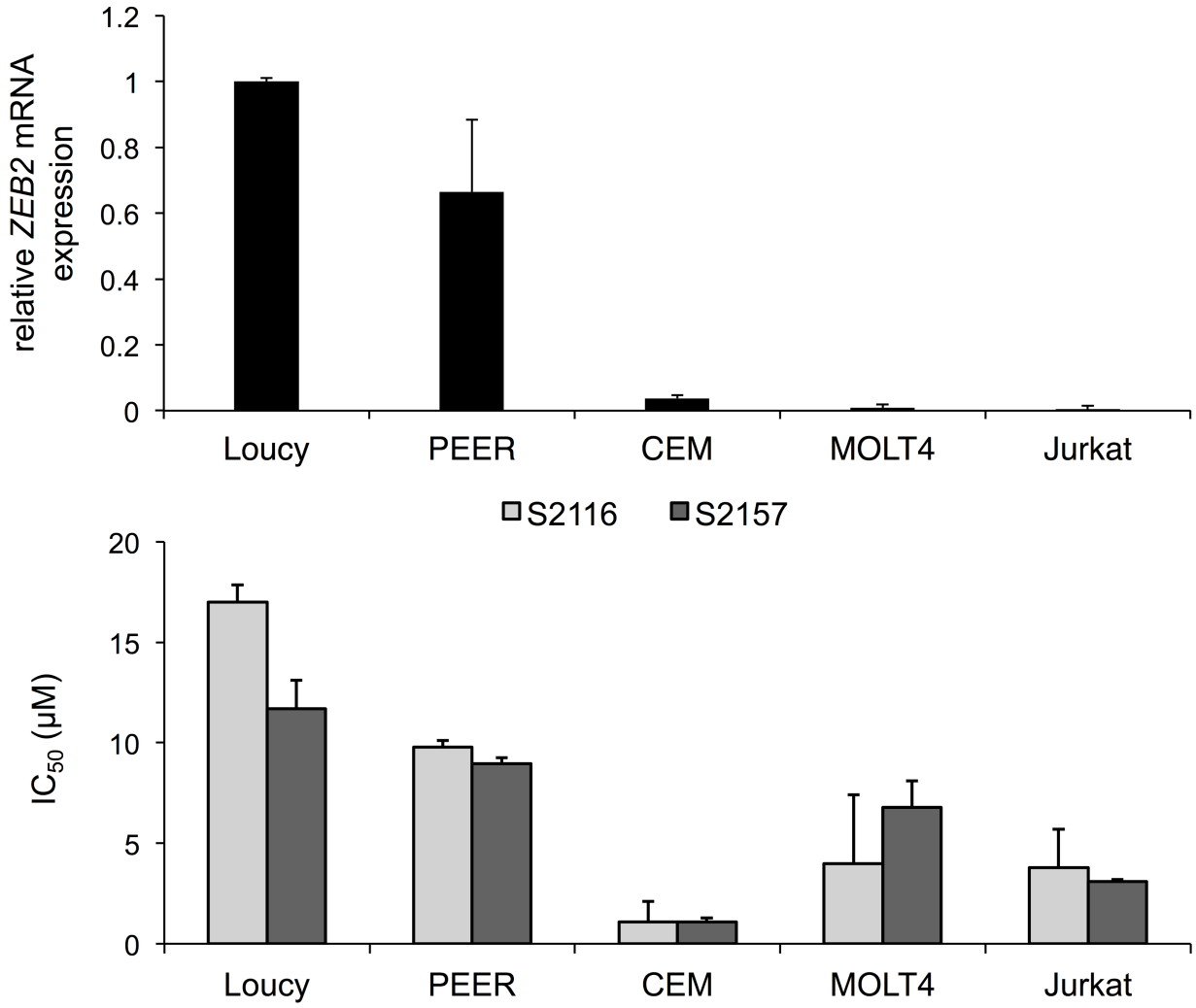
Supplementary Figure S17. Reproduction of CNS leukemia in NOD/SCID mice. Right panel: We injected 5 × 10^6 MOLT4 cells into NOD/SCID mice via a tail vein and resected the brain for histopathological analyses on day 29. Left panel: Corresponding brain tissues of untransplanted NOD/SCID mice.



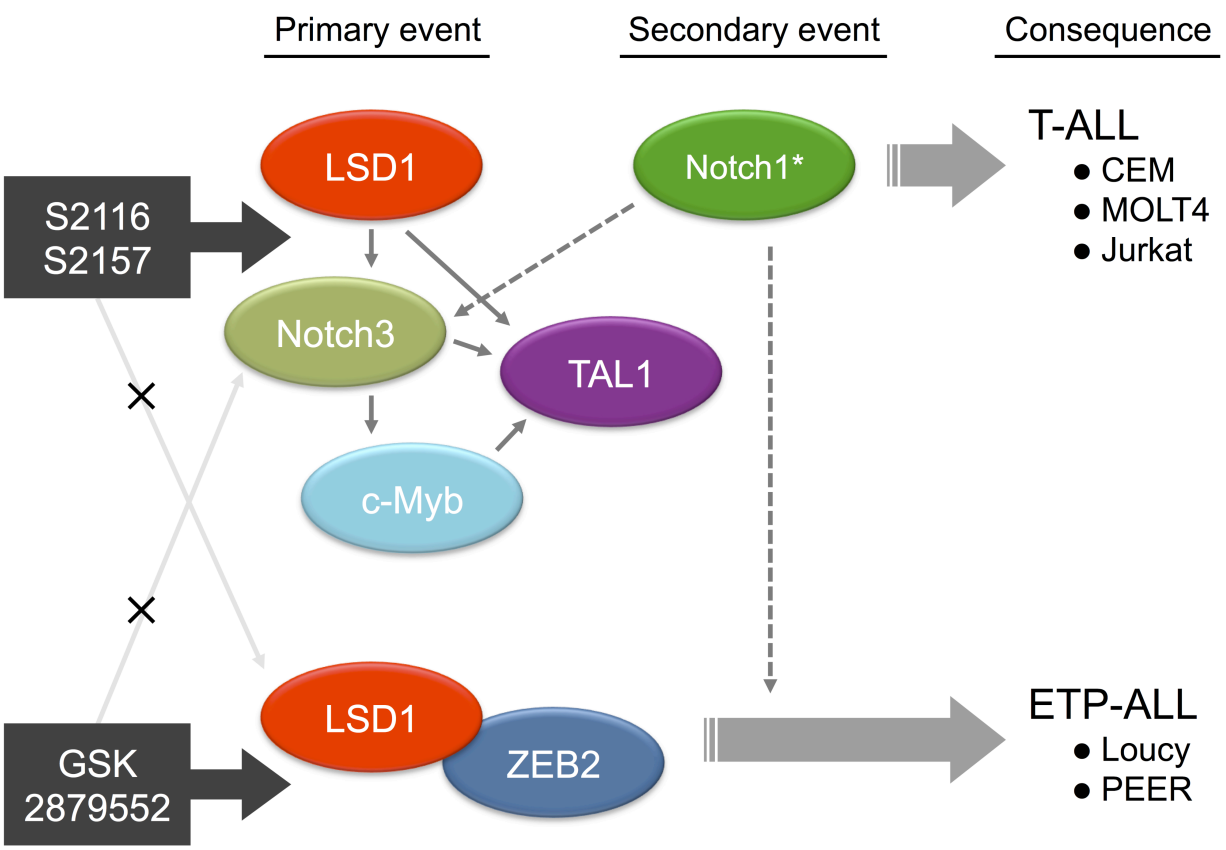
Supplementary Figure S18. Isobologram analysis of the interaction between S2116 and conventional anti-ALL drugs. MOLT4 cells were cultured with four anti-leukemia drugs in the absence or presence of S2116 for 72 hours to obtain dose-response curves of each combination. The combination index plots were generated by the CompuSyn software according to the method of Chou and Talalay. A combination index (CI) <1.0 indicates that the two drugs exhibit synergism.



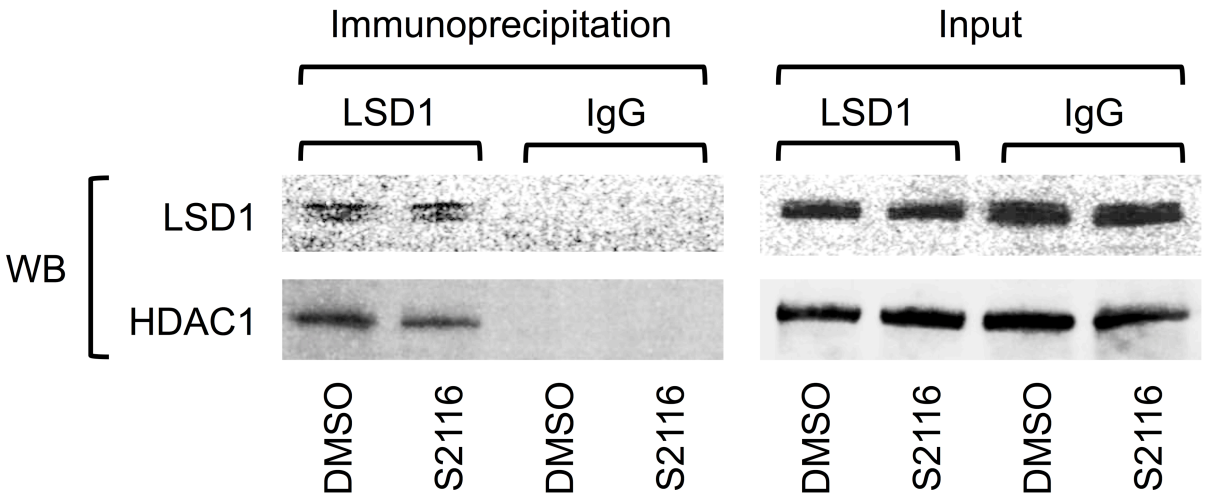
Supplementary Figure S19. Isobologram analysis of the interaction between novel LSD1 inhibitors and conventional anti-ALL drugs in primary T-ALL cells. Patient-derived T-ALL cells were cultured with S2116 and dexamethasone or S2157 and L-asparaginase for 72 hours to obtain dose-response curves of each combination. The combination index plots were generated by the CompuSyn software according to the method of Chou and Talalay. A combination index (CI) <1.0 indicates that the two drugs exhibit synergism.



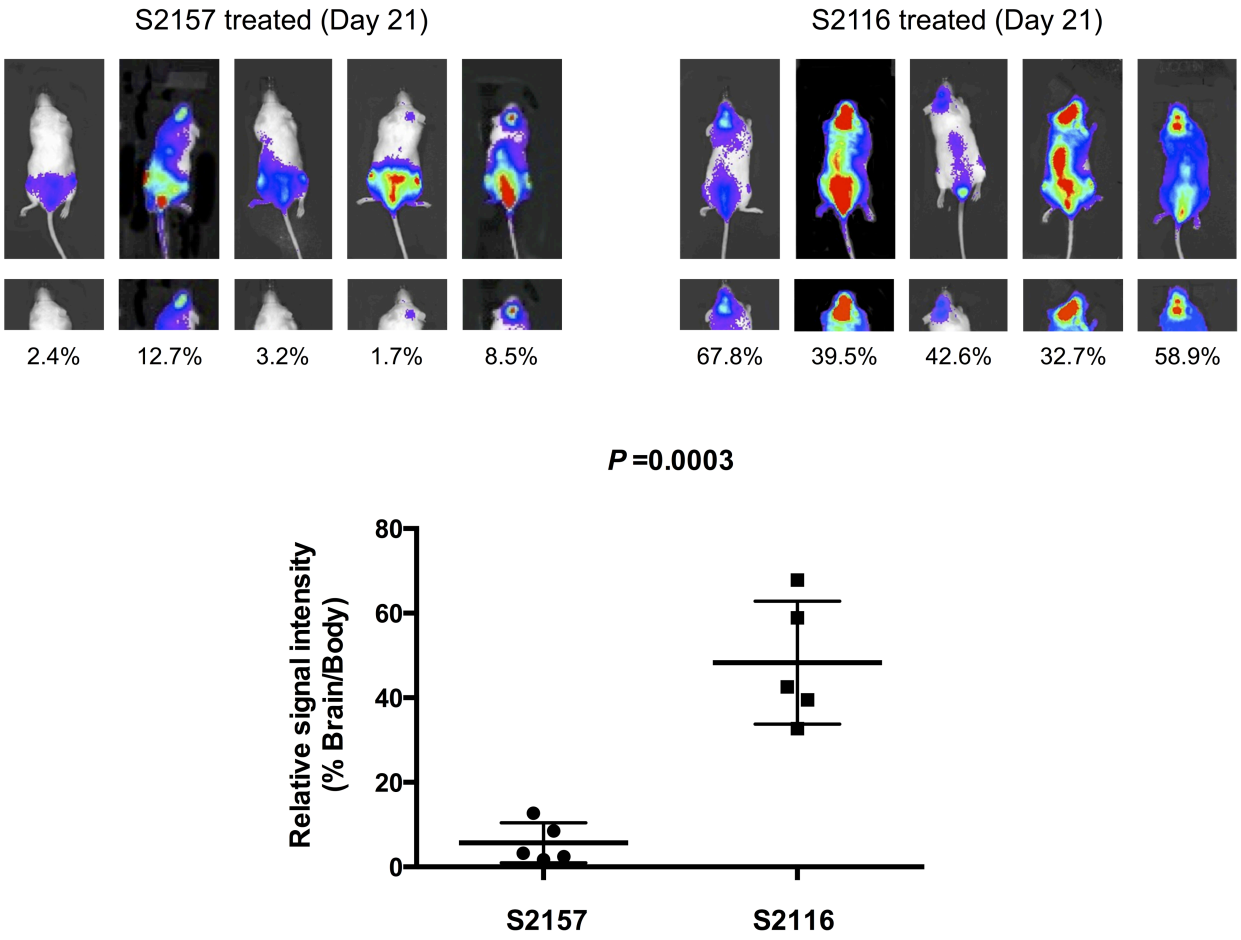
Supplementary Figure S20. ZEB2 expression and the sensitivity to *N*-alkylated LSD1 inhibitors in T-ALL cell lines. Upper panel: The expression levels of *ZEB2* were determined by RQ-PCR, normalized to that of *GAPDH*, and quantified by the $2^{-\Delta\Delta Ct}$ method with the value of Loucy setting at 1.0. Lower panel: The IC_{50} values of S2116 and S2157 were calculated from the dose-response curves of each cell line obtained 72 hours after culture. The means ± S.D. (bars) of three independent experiments are shown.



Supplementary Figure S21. Molecular mechanisms underlying differential sensitivity of T-ALL and ETP-ALL cells to LSD1 inhibitors. The novel *N*-alkylated LSD1 inhibitors S2116 and S2157 are effective for T-ALL cells with Notch3/TAL1 overexpression but lacking ZEB2 expression (CEM, MOLT4 and Jurkat), whereas conventional LSD1 inhibitors such as GSK2879552 are effective for T-ALL cells with ZEB2 expression but lacking Notch3/TAL1 overexpression, mostly early T-cell progenitor ALL (ETP-ALL) cells (Loucy and PEER), regardless of the presence of Notch1 mutations (Notch1*). Gray arrows indicate transcriptional activation; dotted arrows denote direct or indirect effects. Note that this illustration does not represent the entire genomic landscape of T-ALL or ETP-ALL.



Supplementary Figure S22. Effects of S2116 on complex formation between LSD1 and HDAC1. We prepared whole cell lysates from CEM cells cultured with the vehicle (0.1% DMSO) or 10 μM S2116 for 24 hours, and subjected them to immunoprecipitation with rabbit anti-LSD1 antibody or isotype-matched control (IgG). The precipitated complexes were separated on SDS–PAGE, followed by immunoblotting with mouse monoclonal antibodies against LSD1 or HDAC1. Input: direct immunoblotting with immunoprecipitation supernatants.



Supplementary Figure S23. Effects of S2157 and S2116 on CNS lesions in murine xenograft T-ALL models. We injected 5×10^6 luciferase-expressing MOLT4 cells into NOD/SCID mice via a tail vein and started treatments at day 10 after transplantation with either S2157 or S2116 at 30 mg/kg twice a week for 2 weeks. Tumor-derived luciferase activity was measured *ex vivo* by the IVIS Imaging System after D-luciferin injection on day 21 (Upper panel). The relative signal intensity of brain lesion per whole body was calculated and subjected to unpaired Student's *t* test (Lower panel).