CK2 inhibitor CX-4945 destabilizes NOTCH1 and synergizes with JQ1 against human T-acute lymphoblastic leukemic cells

Haiwei Lian,[1,2,3] Dun Li,[2,4] Yun Zhou,[2,5] Esther Landesman-Bollag,[4] Guanglan Zhang,[6] Nicole M. Anderson,[2] Kevin Charles Tang,[2] Justine E. Roderick,[7] Michelle A. Kelliher,[7] David C. Seldin,[4] Hui Fu[1] and Hui Feng[2,4]

[1]Department of Anatomy and Embryology, Wuhan University School of Basic Medical Sciences, Wuhan, Hubei, P.R. China; [2]Department of Pharmacology & Experimental Therapeutics, Boston University School of Medicine, MA, USA; [3]Department of Neurosurgery, Wuhan University Renmin Hospital, Wuhan, Hubei, P.R. China; [4]Department of Medicine, Section of Hematology and Medical Oncology, Boston University School of Medicine, MA, USA; [5]Department of Gynaecology, Wuhan University Renmin Hospital, Wuhan, Hubei, P.R. China; [6]Department of Computer Science, Metropolitan College, Boston University, MA, USA and [7]Department of Molecular, Cell and Cancer Biology, University of Massachusetts School of Medicine, Worcester, MA, USA

Correspondence: huifeng@bu.edu/hueyfu@hotmail.com
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SUPPLEMENTARY INFORMATION

Materials and Methods:

Gene Expression Analysis

Previously published gene expression datasets (GSE33470 and GSE42328) deposited in NCBI Gene Expression Omnibus (GEO) were re-analyzed in this study.[1] The GSE33470 dataset contains gene expression information on seven different subsets of T-cells isolated from three pediatric thymi, while GSE42328 contains 28 early immature T-ALL samples and 25 cortical/mature T-ALL samples. Both experiments were carried out on the Illumina HumanHT-12 V4.0 Expression BeadChip.

To compare the differential gene expression profiles between the normal T-cells and T-ALL patient samples, we merged data of the two series records and applied quantile normalization to the raw data to remove sources of variation between different experiments. Multiple methods have been proposed for normalizing probe signals when analyzing microarray data involving multiple experiments.[2] Quantile normalization is considered as one of the best-performing normalization methods.[3]

Protein Extraction and Western Blotting Analysis

Whole cell lysates were prepared in RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate and 1 mM EDTA) supplemented with Halt proteinase and phosphatase inhibitor cocktail (Thermo Scientific, Cambridge, MA, USA) for the following T-ALL cell lines: JURKAT, JURKAT overexpressing *BCL-2*,

ALL-SIL, RPMI-8402 and MOLT3. The primary antibodies included: anti-CK2α that recognizes both CK2α and CK2α’ at their respective molecular weights, anti-CK2β, anti-cleaved-NOTCH1 (Val1744), anti-MYC, anti-cleaved-PARP, anti-phospho-AKT serine 129, anti-AKT and anti-ACTIN antibodies, all of which were purchased from Cell Signaling (Danvers, MA, USA). Secondary antibodies included horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Pierce, Waltham, MA, USA). Autoradiographs were imaged with a G:BOX Chemi XT4 (Syngene, Frederick, MD, USA) and a CCD camera, and then subjected to quantification analysis with the Syngene GeneTools software (Syngene, Frederick, MD, USA).

Cell Lines and Cell Viability Assay

Cell lines were cultured in RPMI1640 (Corning, Corning, NY, USA) supplemented with 10% fetal bovine serum (Life Technology, Carlsbad, CA, USA). Peripheral blood monocytes (PBMC) were isolated from normal human peripheral blood of two healthy donors and cultured in RPMI 1640 medium supplemented with L-glutamine and 10% donor’s serum. For viability assay cells were plated at a density of 10,000 cells per well in a 96-well plate (Corning, Corning, NY, USA) and incubated for indicated times with DMSO (ATCC, Manassas, VA, USA), CX-4945 (MCE, Monmouth Junction, NJ, USA), JQ1 (a generous gift from Dr. James Bradner’s laboratory at the Dana-Farber Cancer Institute), or the two drugs in combination. Cell Titer Blue assay (Promega, Madison, WI, USA) was used to assess cell viability.

CK2 Kinase Assay

Protein lysate from each sample (2–5 μg) were incubated with the specific CK2 substrate peptide (RRREEETEEE, 1 mM, Sigma-Genosys, Woodlands, TX, USA) in kinase buffer (100 mM Tris pH 8.0, 20 mM $MgCl_2$, 100 mM NaCl, 50 mM KCl, 0.1 μg/μl BSA and 100 μM Na_3VO_4) and 5 μCi of [γ-32P]-GTP (6000 Ci/mM; PerkinElmer, Waltham, MA, USA) at 30ºC for 10 minutes as previously described.[4] The reaction was stopped by adding 25 μl of 100 mM ATP in 0.4 N HCl. Samples were spotted onto a P81 Whatman filter and washed in 150 mM H_3PO_4 to remove unincorporated [γ-32P]-GTP. Phosphorylated peptides were quantified in an automatic scintillation counter (PerkinElmer, Downers Grove, IL, USA).

Quantitative Real-time PCR and Pulse-chase Analysis

Total RNA was extracted from human JURKAT and ALL-SIL cell lines using the Aurum total RNA mini kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). About 50 ng of cDNA was used for each qRT-PCR reaction, performed with the SYBR green PCR master mix (Qiagen, Valencia, CA, USA) and a Step-One PCR instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. All reactions were performed in triplicate, using *GAPDH* as controls. The qRT-PCR primer sequences include: *MYC*-forward, 5'-AATGAAAAGGCCCCCAAGGTAGTTATCC-3' and reverse, 5'-

GTCGTTTCCGCAACAAGTCCTCTTC-3’; *GAPDH*-forward, 5’-GAGTCAACGGATTTGGTCGT-3’ and reverse, 5’- TTGATTTTGGAGGGATCTCG -3’.

For pulse-chase analysis, human T-ALL cells were treated with 10 μg/mL cycloheximide (Sigma, St. Louis, MO, USA). Cells were then collected at 0, 2, 4, 6 and 8 hours post treatment, and proteins were extracted for Western blotting analysis to detect cleaved-NOTCH1 and ACTIN levels.

Apoptosis and Cell Cycle Analysis

Apoptosis was assessed with Annexin V and propidium iodide (PI) staining, based on two populations: early apoptosis (PI^-, Annexin V^+) and late apoptosis (PI^+, Annexin V^+). Specifically, cells were incubated at room temperature for 20 minutes with Annexin V-APC (BD Bioscience, San Jose, CA, USA), PI (50 μg/ml) (Sigma, St. Louis, MO, USA) and 1x binding buffer (BD Pharmagen, San Jose, CA, USA). For cell cycle analysis, cells were fixed in ice cold 100% ethanol and incubated at -20°C for 30 minutes. Fixed cells were then stained in PI staining solution (PBS, 50 μg/ml PI and 100 μg/ml RNAse A) and incubated for 20 minutes at 4°C in the dark. Cells stained for apoptosis and cell cycle were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and data were processed with FlowJo (Ashland, OR, USA) and Modfit softwares (Verity Software House, Topsham, ME, USA), respectively.

Combination Treatment of Small Molecules

T-ALL cell lines were treated with a combination of CX-4945 and JQ1 with a range of doses around the IC50 of each drug alone. Cell viability was analyzed by CellTiter-Blue assay (Promega, Madison, WI, USA). CalcuSyn software (Biosoft, Cambridge, UK) was used to calculate the potency of drug combination and we used the Chou-Talalay method to calculate a combined index (CI).[5] Normalized isobolograms were also produced with CalcuSyn software. The CI has been interpreted as follows: strong synergism (0.1 to 0.4), moderate synergism (0.4 to 0.8), nearly additive (0.9 to 1.1) and antagonism (>1.1).

Patient Samples

Primary human T-ALL samples were obtained from children with T-ALL enrolled in clinical trials of the Dana-Farber Cancer Institute or University of Massachusetts Memorial Hospital. Samples were collected with informed consent and with approval of the Institutional Review Board. Leukemic blasts were isolated from peripheral blood or bone marrow by Ficoll-Hypaque centrifugation, and subsequently expanded in NSG (Nod-Scid-Gamma) mice by transplanting 0.5 to 5×10^6 viable leukemic cells via intravenous injection. Primary human T-ALL cells used in this research were isolated from the spleen and bone marrow of NSG mice. All animal procedures used in this study were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee. Human peripheral blood monocytes (PBMC) were collected from healthy donors with informed consent and with approval of the Boston

University Institutional Review Board. All samples were analyzed without linked identifiers.

Statistical Analysis

GraphPad Prism software was used to calculate significant values *(p)* using a two-tailed Student's t test. *P*-values less than or equal to 0.05 were considered statistically significant.

Supplementary Figure Legends:

Supplementary Figure 1. The expression of *CK2* subunits in primary patient samples. (**A**) *CK2β* (probe ID: ILMN_1800461) transcripts are elevated in early immature and mature T-ALL patient samples, compared with different subsets of T-cells (mean ± SD: 2485 ± 146.3 for early immature T-ALL and 3041 ± 142.3 for cortical/mature T-ALL *vs.* 413.5 ± 79.75 for CD34+CD1a-, 370.2 ± 51.27 for CD34+CD1a+, 409.7 ± 51.94 for CD4ISP, 247.3 ± 39.38 for DPCD3-, 244.6 ± 60.39 for DPCD3+, 222.1 ± 23.64 for CD3+CD4+, 247.8 ± 42.72 for CD3+CD8+; P<0.0001 for all comparison between T-ALL patient samples versus subsets of T-cells; n=28 for early immature T-ALL, 25 for cortical/mature T-ALL and 3 for subsets of T-cells, respectively). (**B**) CK2α versus ACTIN protein ratios demonstrating that CK2α levels are slightly but not significantly higher in primary T-ALL patient cells, compared with control thymocytes (P=0.92; n=10 and 3, respectively). AU: arbitrary unit.

Supplementary Figure 2. CK2 expression is elevated in human T-ALL cell lines. (**A**) Western blotting analysis of CK2α, CK2α', CK2β, cleaved-NOTCH1 (clv-NOTCH1) and MYC in a panel of human T-ALL cell lines, compared to normal thymus. ACTIN serves as a loading control. CK2α (**B**), CK2α' (**C**), CK2β (**D**) clv-NOTCH1 (**E**) and MYC (**F**) versus ACTIN protein ratios demonstrating that CK2α, CK2α', CK2β, clv-NOTCH1 and MYC levels are significantly or nearly significantly higher in T-ALL cells, compared with control thymocytes (mean ± SD of CK2α to ACTIN ratio: 1.05 ± 0.26 *vs.* 0.26 ±

0.07, *P*=0.058; CK2α' to ACTIN ratio: 1.85 ± 0.17 *vs.* 1.26 ± 0.09, *P*=0.04*;* CK2β to ACTIN ratio: 0.37 ± 0.13 *vs.* 0.03 ± 0.01, *P*=0.07*;* clv-NOTCH to ACTIN ratio: 1.08 ± 0.18 *vs.* 0.04 ± 0.02, *P*=0.0095; and MYC to ACTIN ratio: 2.56 ± 0.14 *vs.* 0.07 ± 0.05, *P<0.001;* n=4 and 3, respectively).

Supplementary Figure 3. CK2 inhibition by CX-4945 treatment leads to decreased kinase activity in T-ALL cells and a moderate decrease of cleaved-NOTCH1 levels in RPMI-8402 cells. (**A**) The radioactive counts per minute (cpm) for JURKAT, ALL-SIL and RPMI-8402 T-ALL cells revealed that CX-4945 treatment (5 μM for 8 hours) effectively decreased CK2 kinase activity, compared to control DMSO-treated cells. (**B**) Western blotting analysis of cleaved-NOTCH1 (clv-NOTCH1), phospho-AKT at Serine 129 site (P-AKT ser129) and total AKT in RPMI-8402 T-ALL cells upon CX-4945 treatment for 8 hours. ACTIN serves as a loading control. Clv-NOTCH1 protein amounts (relative to ACTIN) are shown in the bottom of panel **B**.

Supplementary Figure 4. Flow cytometry analysis revealed enhanced apoptosis in a panel of T-ALL cell lines induced by synergistic effects of JQ1 and CX-4945. Apoptosis analysis was performed by flow cytometry to determine early apoptosis (PI^-, Annexin V^+) and late apoptosis (PI^+, Annexin V^+). JURKAT, ALL-SIL, RPMI-8402 and MOLT-3 cells were stained with Annexin V and PI after 48 hours of treatment. The T-ALL cells were treated with DMSO (control) (far left panel), CX-4945 2.5 μM (middle left

panel), JQ1 1 μM (middle right panel) and both drugs in combination (CX-4945: 2.5 μM;JQ1: 1 μM) (far right panel).

Supplementary Figure 5. Western blotting analysis demonstrated that JQ1 and CX-4945 synergizes to promote apoptosis in a panel of T-ALL cell lines. Western blotting analysis of cleaved-PARP (clv-PARP) in JURKAT, ALL-SIL, RPMI-8402 and MOLT3 cells that were treated with DMSO, CX-4945 (2.5 μM), JQ1 (1 μM) and both drugs in combination (CX-4945: 2.5 μM; JQ1: 1 μM) for 8, 16 and 30 hours. ACTIN was used as loading controls.

Supplementary Figure 6. JQ1 and CX-4945 confer minimal sensitivity to normal PBMC and does not synergize in these normal cells. (**A**) Cell viability upon combination treatment with CX-4945 (5 μM) and JQ1 (2 μM) is significantly reduced in ALL-SIL T-ALL but not in PBMC cells, compared with cells treated with a single agent. Cell viability was determined with CellTiter-Blue after 48 hours of treatment for ALL-SIL and freshly isolated PBMC cells treated with DMSO, CX-4945 (5 μM), JQ1 (2 μM) and both drugs in combination (CX-4945: 5 μM; JQ1: 2 μM). (**B**) Combination treatment of CX-4945 and JQ1 depicted as normalized isobolograms shows antagonism effect (CI = 1.11) in PBMC cells.

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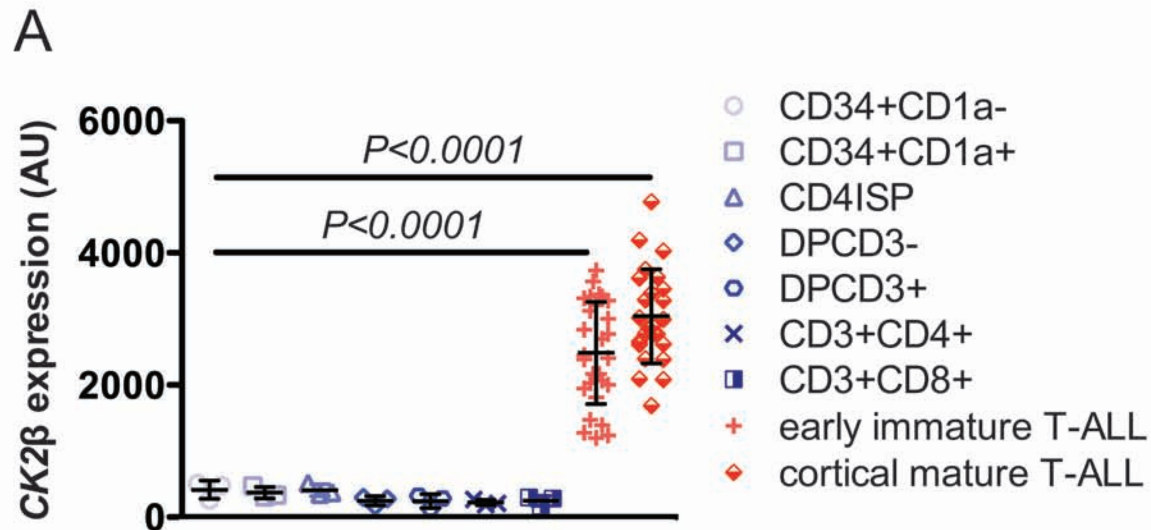
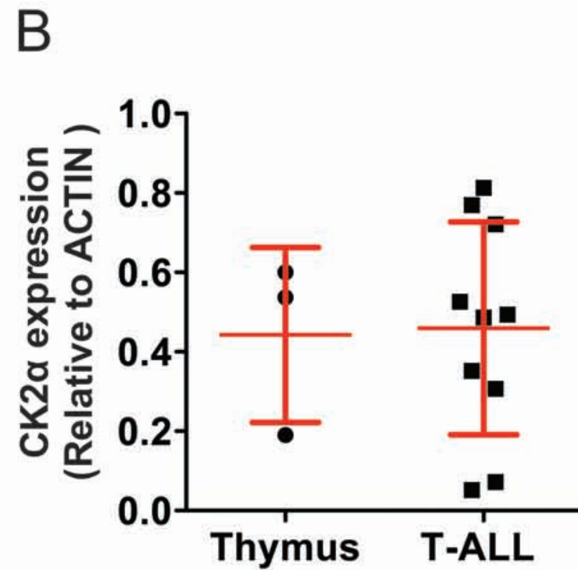
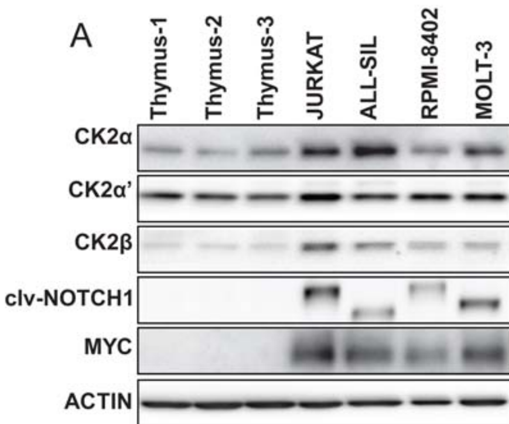
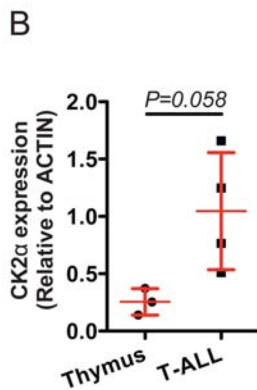
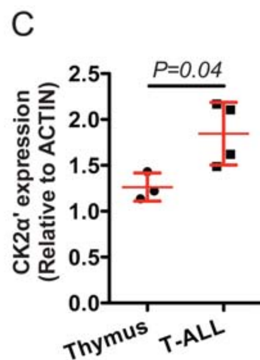


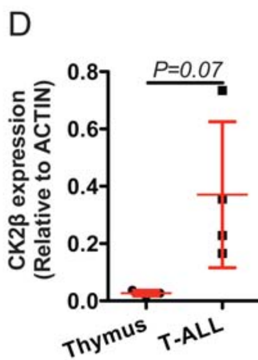


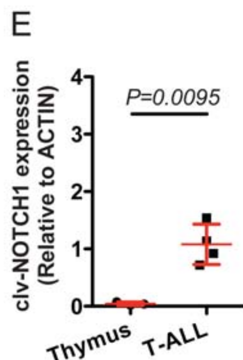
Supplementary Figure 1

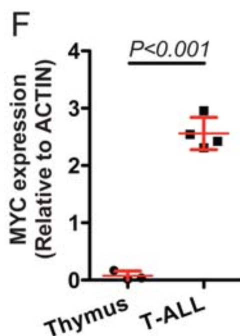












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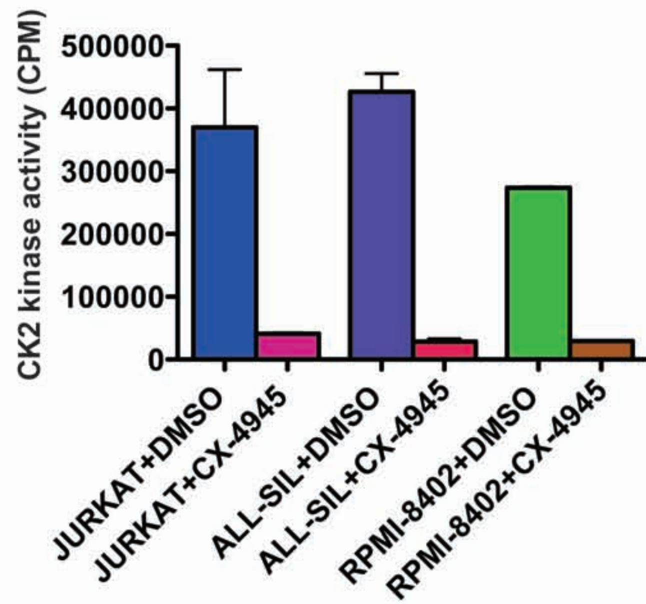

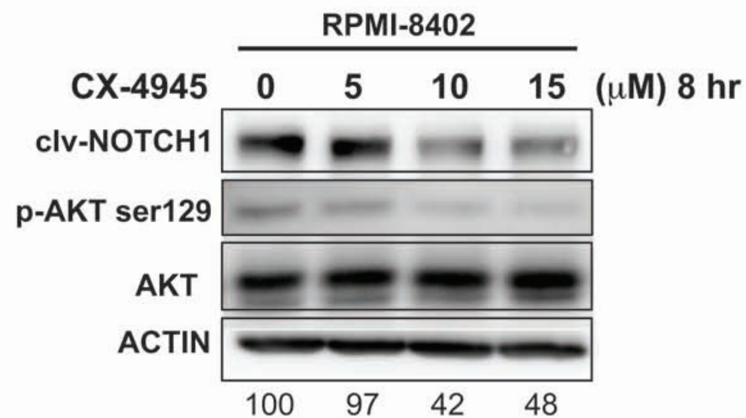

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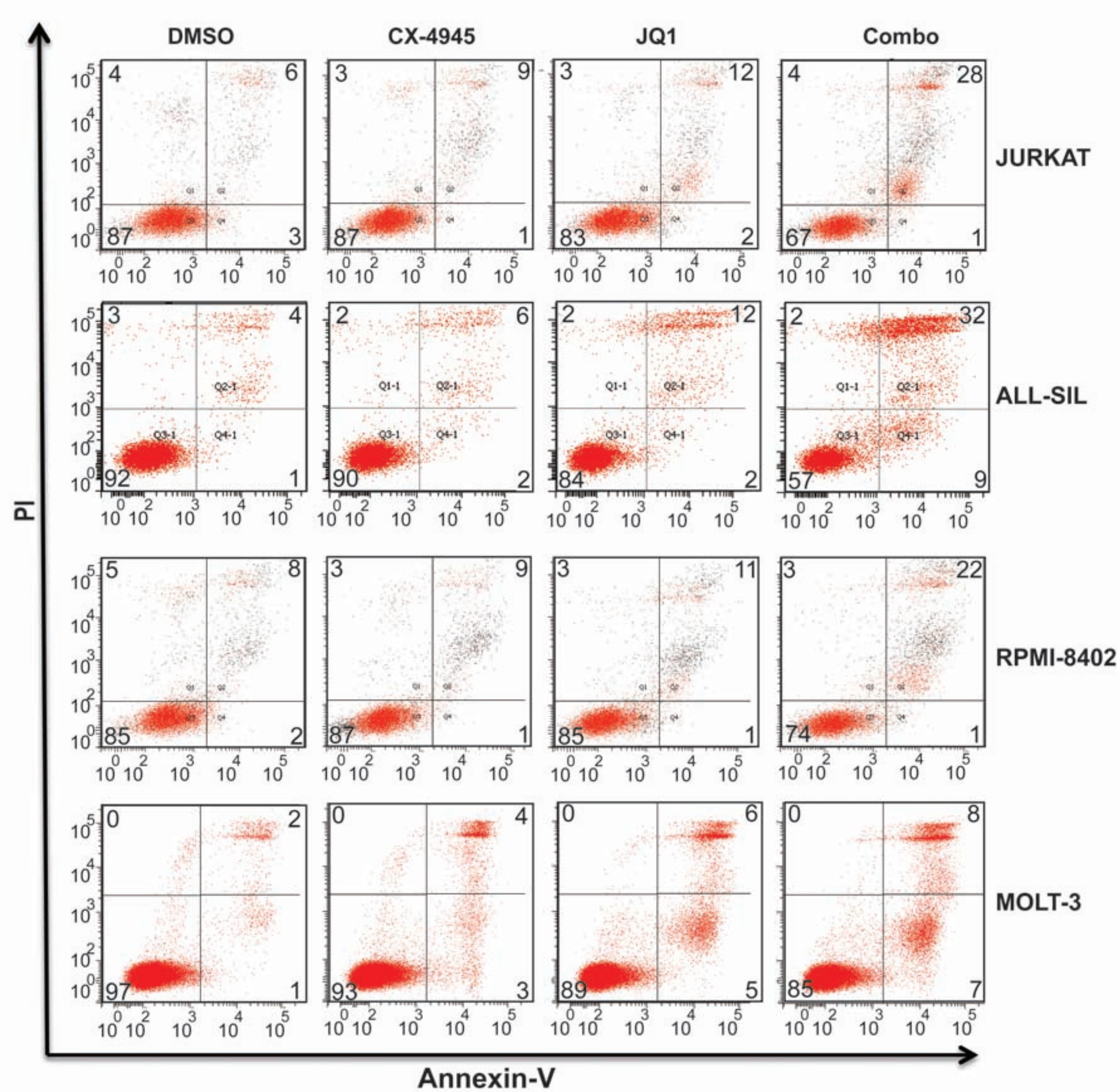

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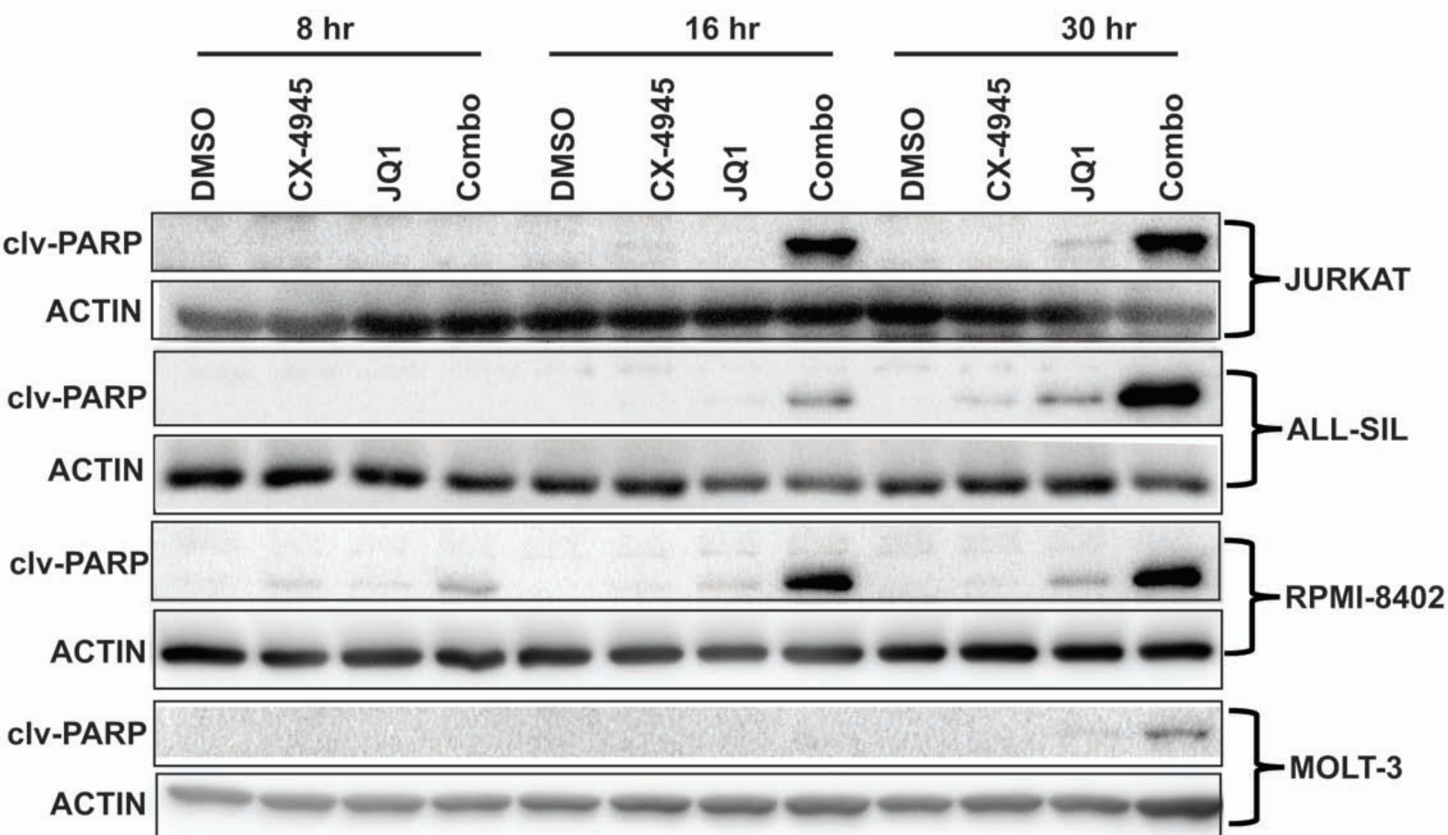

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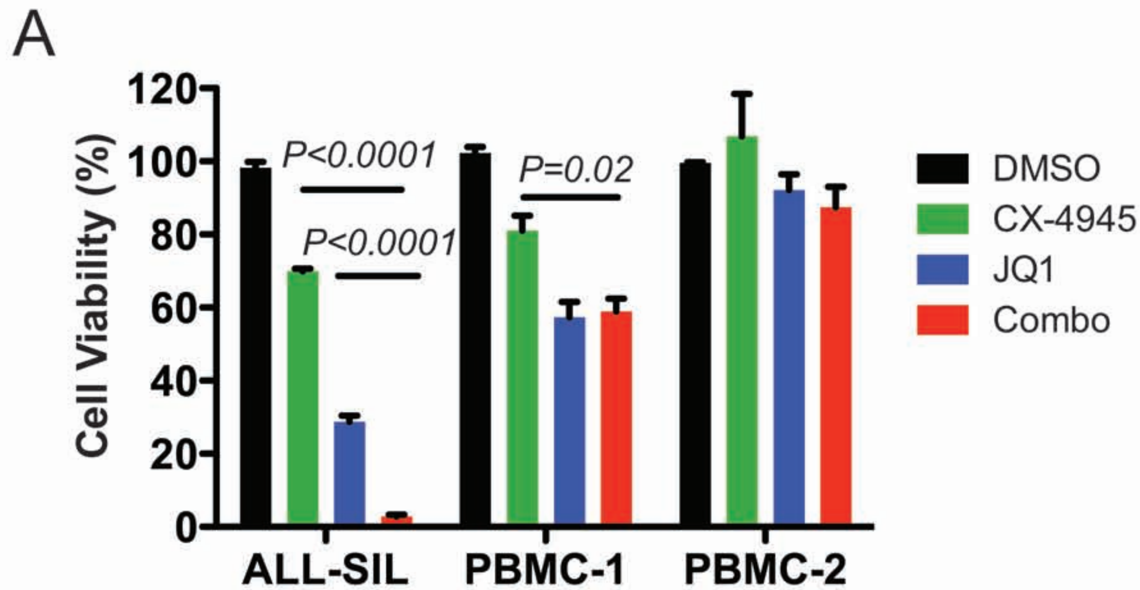

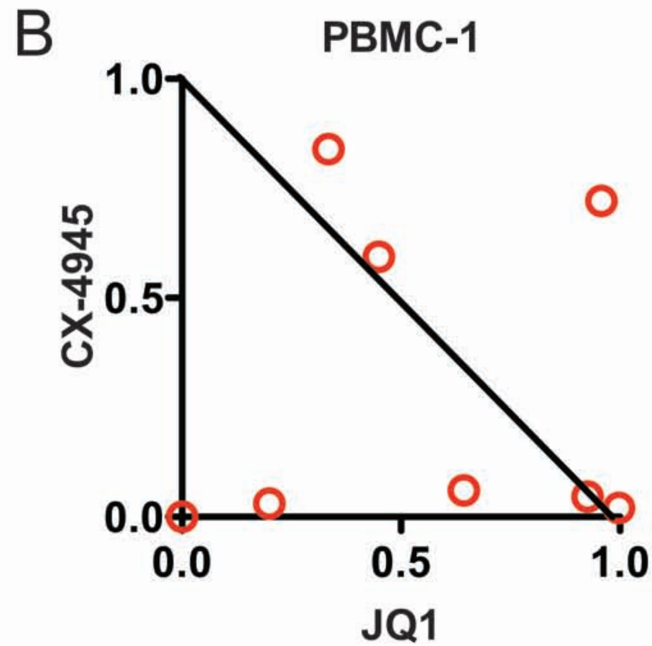

Supplementary Figure 6