Supplementary Material:

Supplementary Figures and Legends

Figure S1

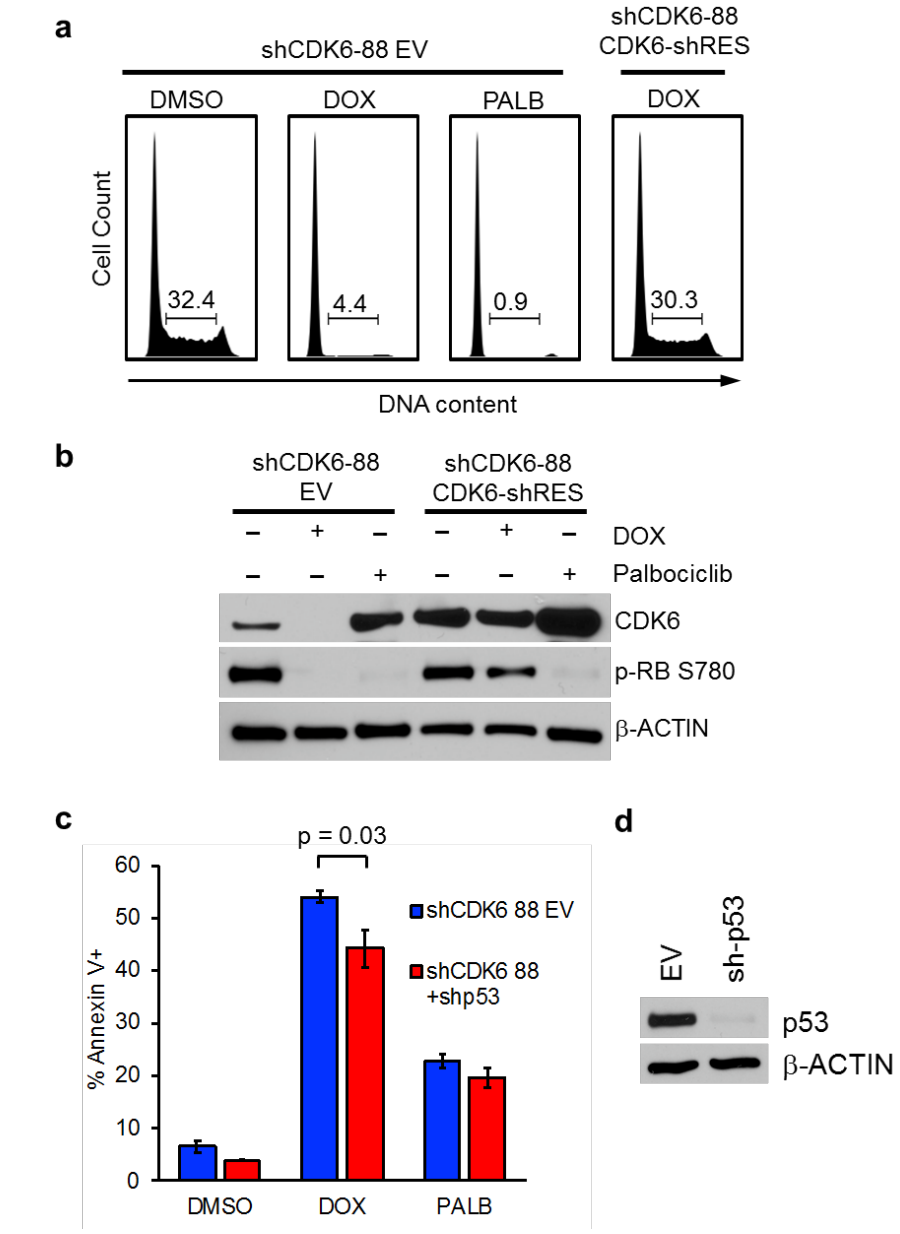

Figure S1**. Specific effects of CDK6 silencing on the cell cycle and apoptosis of BV173 cells.** a) Cell cycle analysis of BV173 cells transduced with TET-ON shCDK6-88 or the empty vector and treated with DMSO, DOX (1µg/ml) or Palbociclib (1 µM) for 2 days or BV173 shCDK6-88 expressing a shRNA resistant form of CDK6 (CDK6-shRES) treated with DOX (1µM) for 2 days; b) CDK6 and phospho-RB levels in BV173 shCDK6-88 EV or CDK6-shRES

cells treated with DMSO, DOX (1µM) or Palbociclib (1 µM) for 3 days; c) Apoptosis monitored by Annexin V staining of BV173 cells transduced with TET-ON shCDK6-88, empty (EV) vector, or a p53-targeting shRNA (sh-p53) and treated with DOX (1µg/ml) or Palbociclib (1 µM) for 10 days; d) p53 levels in EV or sh-p53-transduced TET-ON shCDK6-88 BV173 cells.

Figure S2

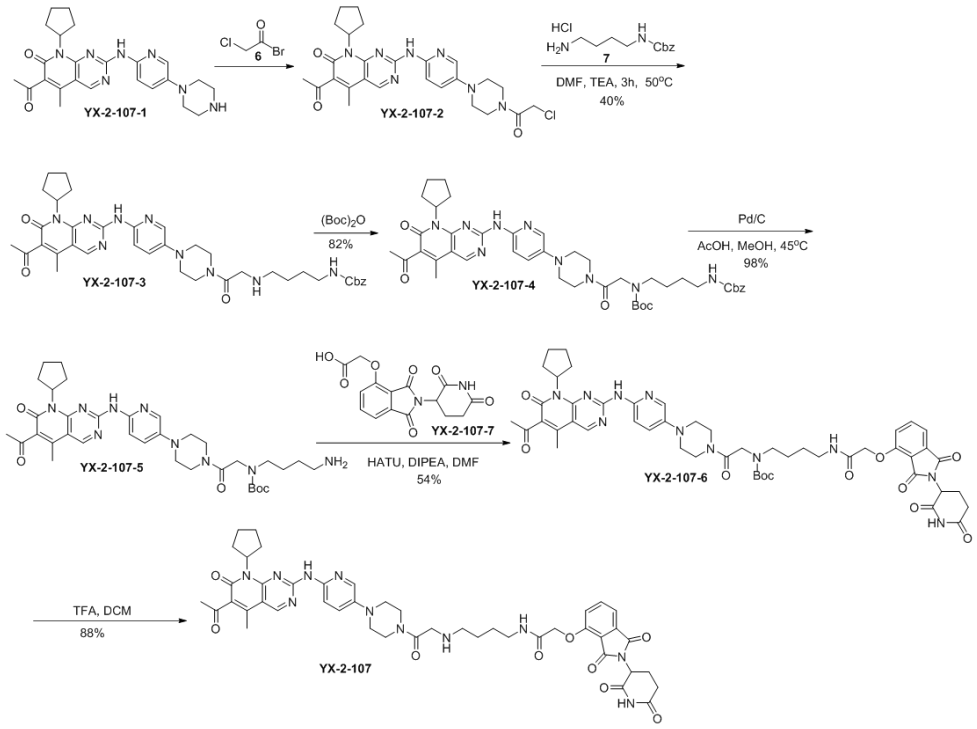

Figure S2. **Schematic steps in the synthesis of PROTAC YX-2-107.**

Figure S3

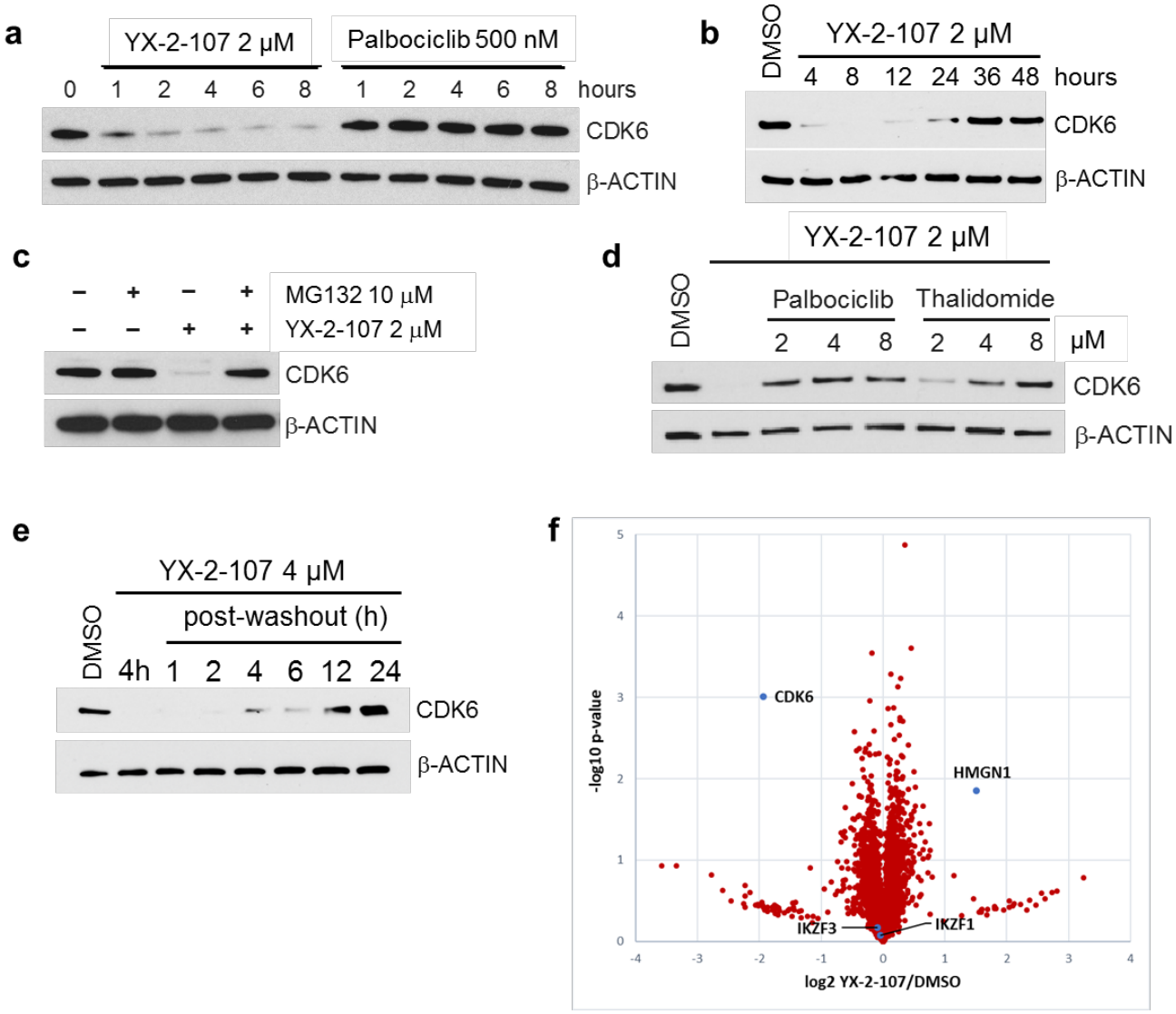

Figure S3. **Proteasome-dependent degradation and CDK6 stability in PROTAC YX-2-107-treated cells.** Immunoblot shows CDK6 expression in BV173 cells treated with**:** a, b) YX-2-107 or Palbociclib; c) YX-2-107 with or without the proteasomal inhibitor MG132 for 4 hours; d) YX-2-107 (2μM) with Palbociclib or Thalidomide at the indicated concentrations for 4 hours; and e) YX-2-107 for 4 hours, washed and cultured without YX-2-107 for 1,2,4,6, and 24 hours; f) Volcano plot illustrates significantly differentially abundant proteins identified by at least two unique peptides found in all three replicates of the YX-2-107-treated or control (DMSO-treated) samples. The –log10 p-value is plotted against the log2-fold change (YX-2-107/DMSO). CDK6 and HMGN1 represent proteins with $p < 0.05$ and an absolute fold-change >2.

Figure S4

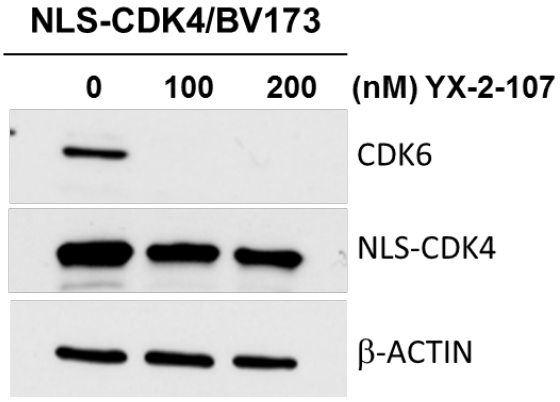

Figure S4**. NLS-CDK4 is resistant to degradation by PROTAC YX-2-107.** Immunoblot shows expression of NLS-CDK4 and CDK6 in YX-2-107-treated NLS-CDK4-BV173 cells.

Figure S5

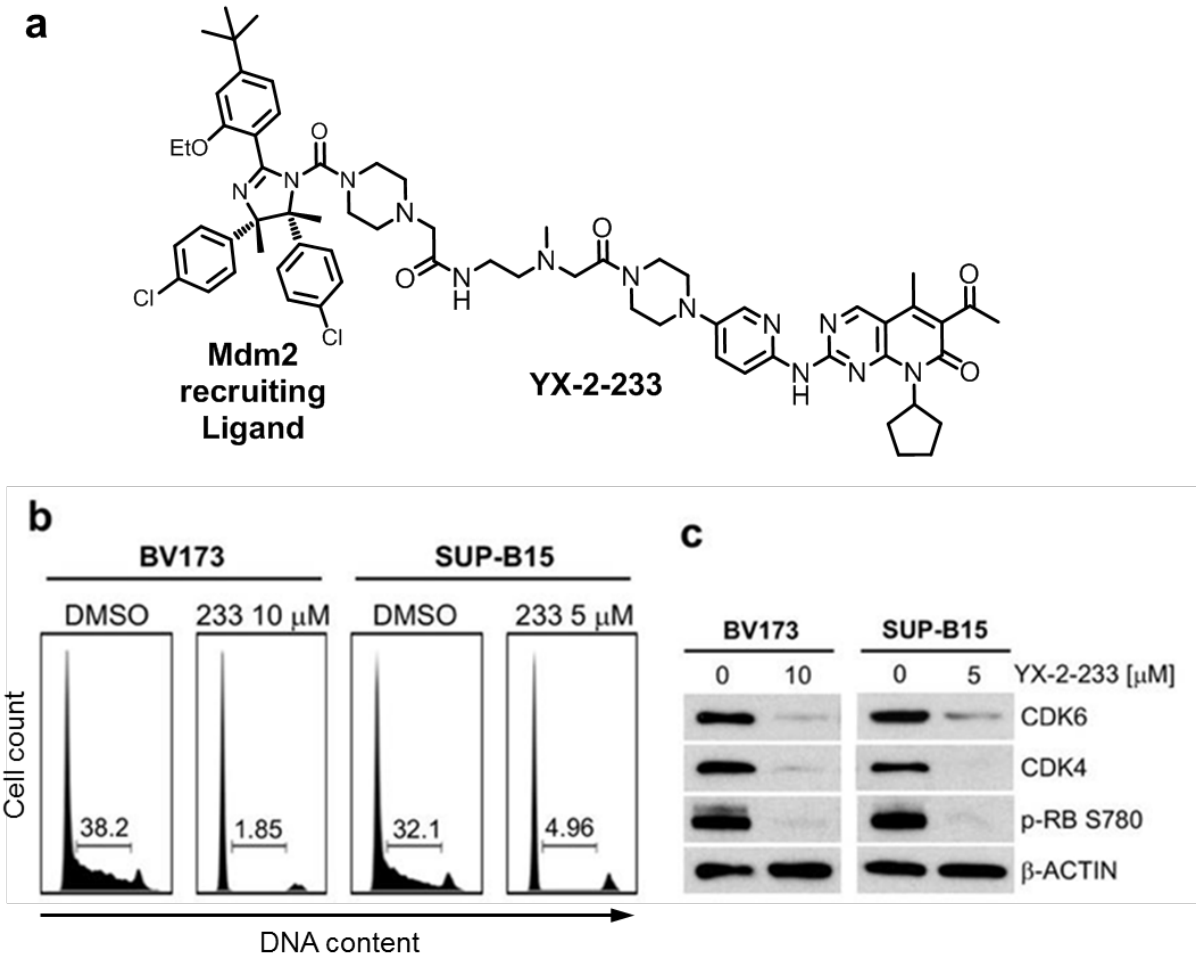

Figure S5**. Effects of PROTAC YX-2-233 in Ph+ ALL cell lines.** a) Structure of PROTAC YX-2-233; b) cell cycle analysis at 24 h; and c) immunoblot of PROTAC YX-2-233-treated (24 h) BV173 or SUP-B15 cells.

Figure S6

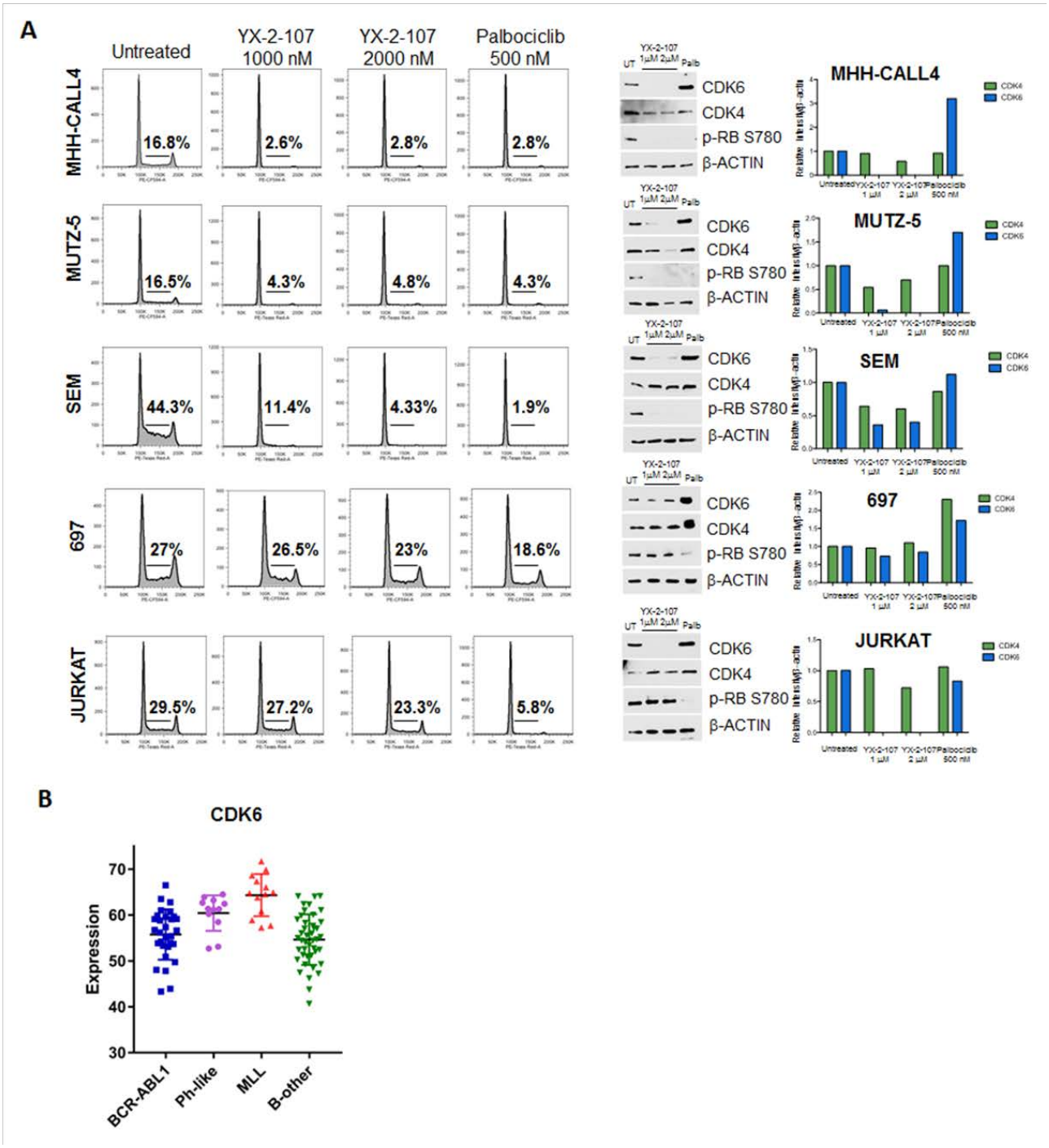

Figure S6. **Effect of PROTAC YX-2-107 on the cell cycle of non-Ph+ B-ALL cell lines**.

a) Percentage of S phase (left), phospho-RB and CDK4/6 western blots (middle), and CDK4/6 densitometric normalization to β-actin (right) in YX-2-107-treated B-ALL (CALL-4, MUTZ-5, SEM, 697) and T-ALL (Jurkat) cell lines; b) CDK6 expression in BCR-ABL1 (n = 30), Ph-like (n = 12), MLL-r (n = 13) or other B-ALL subtypes (n = 43).

Figure S7

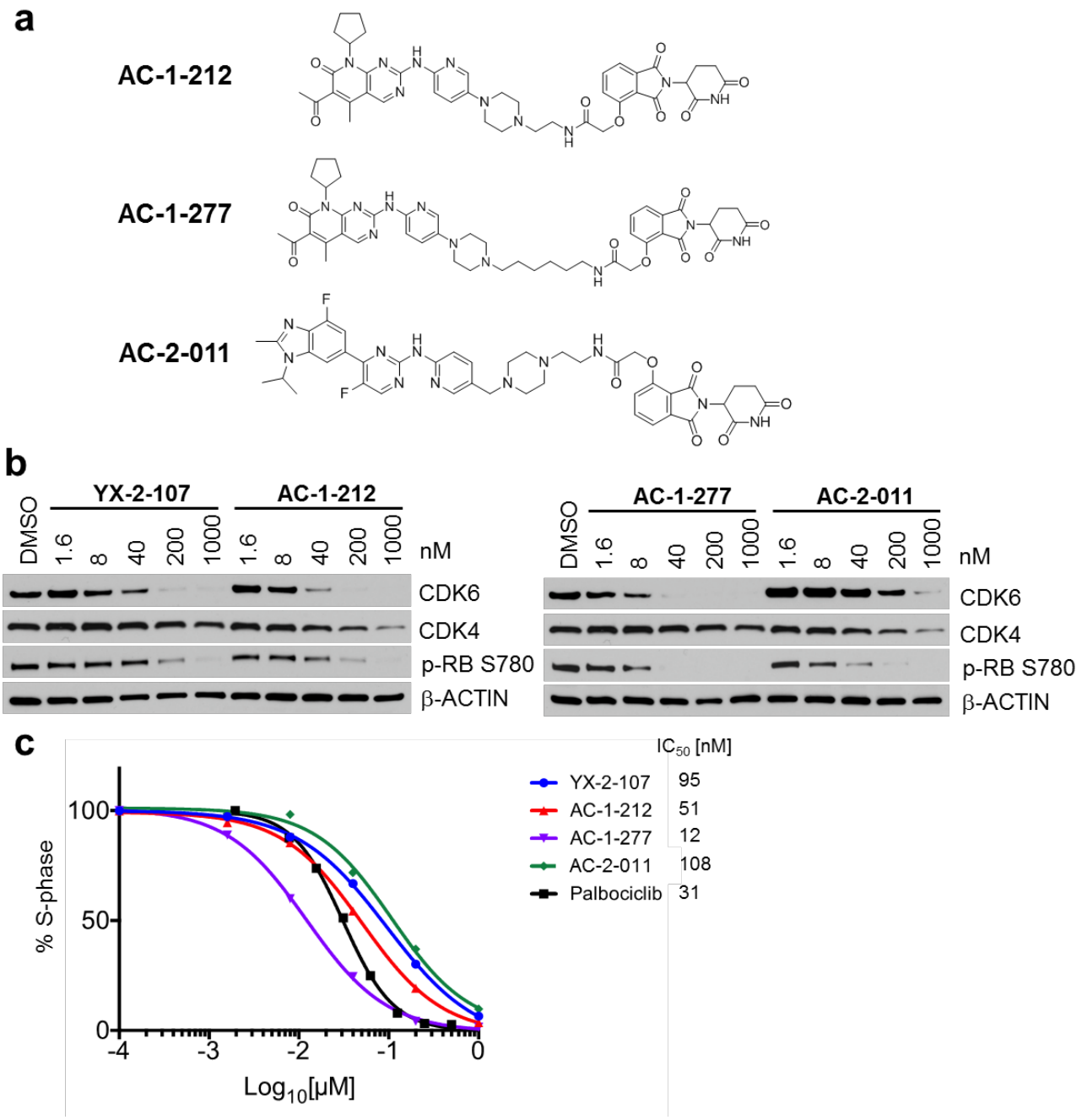

Figure S7. **Effects of CDK6-degrading PROTACs or Palbociclib on the proliferation of BV173 cells.** a) structure of YX-2-107-related PROTACs; b) immunoblots of BV173 cells treated with PROTACs at the indicated concentrations for 24 h; c) percentage of S phase cells by propidium iodide staining of BV173 cells treated with PROTACs as in (b) or with Palbociclib for 24 hours. IC_{50s} were calculated based on the percent reduction of S-phase cells using graphpad PRISM software.

Figure S8

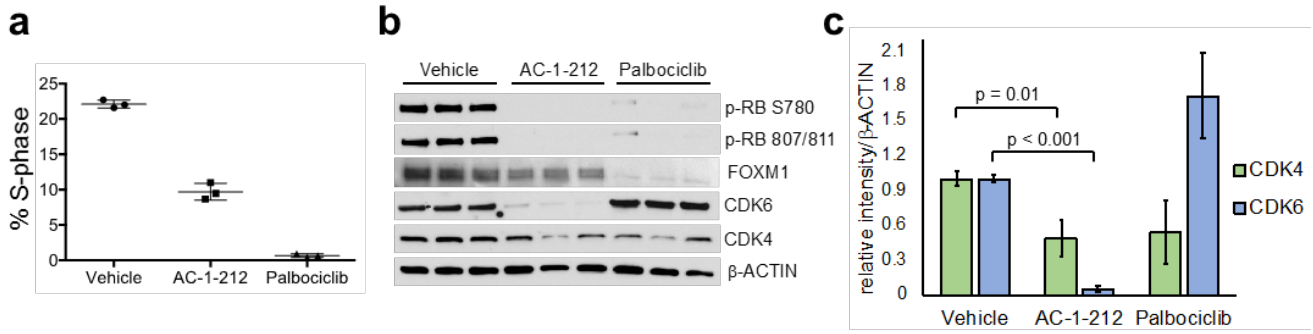

Figure S8**. In vivo effects of PROTAC AC-1-212 or Palbociclib on the proliferation of Ph+ ALL cells.** Mice were injected with human Ph+ ALL cells (sample #004) and, when peripheral blood leukemic cells (CD19+CD10+) were > 50%, treated (3 mice/group) with vehicle, PROTAC AC-1-212 20 mg/kg IP BID or Palbociclib 150 mg/kg by gavage for 3 consecutive days. Twelve hours after the last treatment, bone marrow cells (>90% CD19+CD10+) were purified and assessed for the percentage of S phase cells (a) or expression of CDK4/6, phospho-RB and FOXM1 (b). Quantitation of CDK4 and CDK6 levels (based on panel b immunoblot) is shown in panel c.

Figure S9

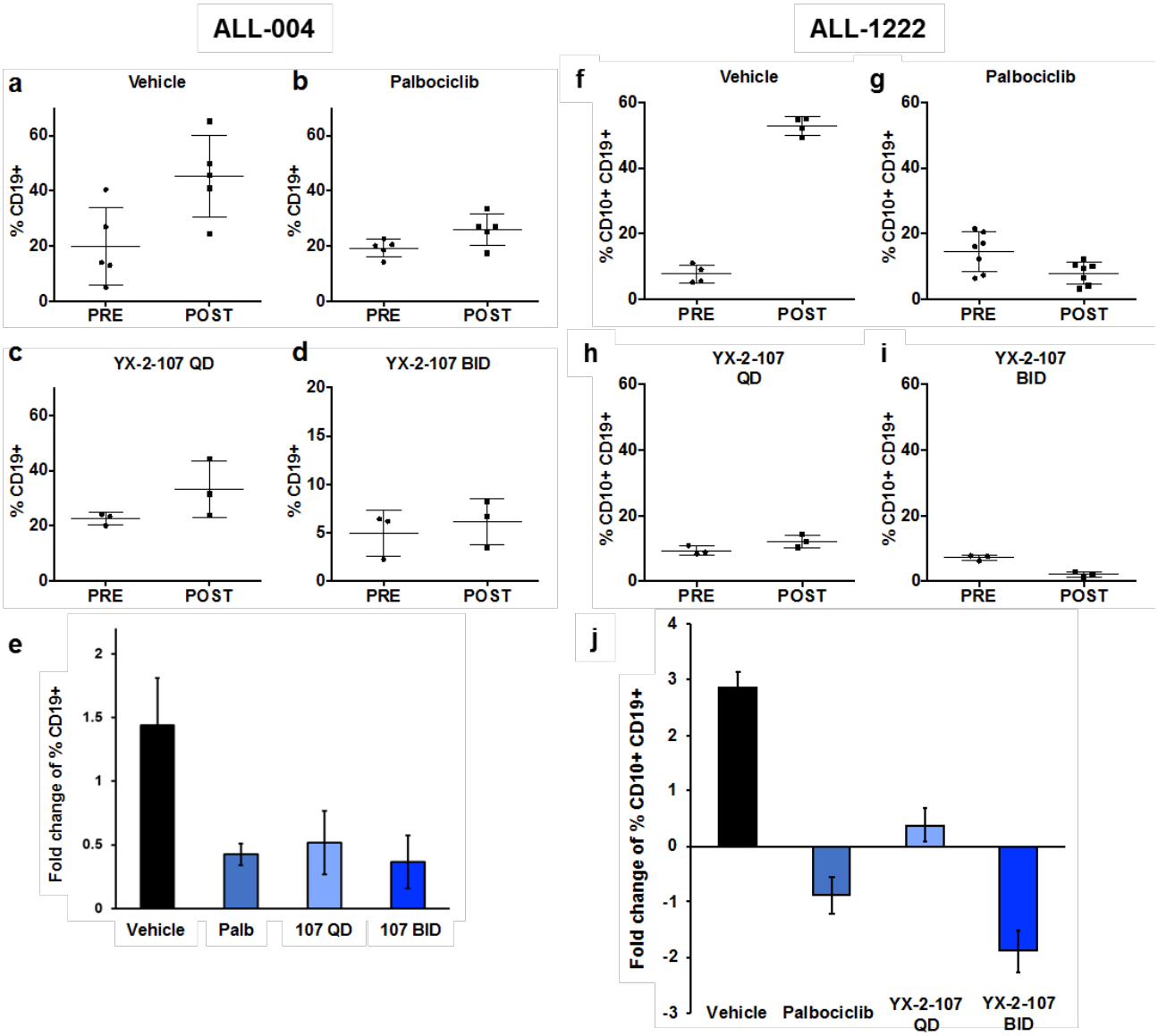

Figure S9. **Leukemia load in mice injected with Ph+ ALL primary samples and treated with PROTAC YX-2-107.** NSG mice injected with primary Ph+ ALL-004 (a-e) or ALL-1222 (f-j) were tested five weeks later (PRE) by anti-CD19 flow cytometry to assess the frequency of leukemic cells in the peripheral blood. Subsequently, mice were treated with: vehicle (a, n = 5; f, n = 4), Palbociclib 150 mg/kg once per day (b, n = 5; g, n = 7), YX-2-107 125 mg/kg (ALL-004) or 150 mg/kg (ALL-1222) once per day (c, n = 3; h, n = 3) or YX-2-107 twice per day at half dose per injection (d, n = 3; i, n = 3) for 10 consecutive days. Then, the percentage of

leukemia cells (CD19+CD10+) in the peripheral blood was determined at week 7 (POST). e, j) fold changes of the percentages shown above.

Supplementary Methods

Protein analysis

Cells were counted and lysed at a density of 10,000/μL in Laemmli Buffer supplemented with 5% β-Mercaptoethanol. Lysates were resolved on 4-20% gradient polyacrylamide gels (Biorad, #4561095) and transferred onto a nitrocellulose membrane (Santa Cruz Biotechnology, #sc-3718) using a semi-dry trans-blot transfer cell (Bio-Rad). Membranes were then blocked in 5% non-fat dry milk/TBS-T and incubated with the following primary antibodies: CDK6 (rabbit, CST #13331), CDK6 (mouse, CST #3136), CDK4 (rabbit, CST #12790), CDK4 (rabbit, Bethyl Laboratories #A304-224), FOXM1 (rabbit, Santa Cruz Biotechnology #sc-502), phospho-RB Ser-780 (rabbit, CST #9307), phospho-RB Ser-807/811 (rabbit, CST #9308), β-ACTIN, (mouse, CST #3700).

Membranes were incubated with 1:10,000 HRP-conjugated secondary antibodies (Thermo Fisher Scientific, anti-mouse-HRP #31430, or anti rabbit-HRP #31460) and signals were visualized by chemiluminescent reaction using SuperSignal West Pico (Thermo Fisher Scientific #34580) or Dura (Thermo Fisher Scientific #34075) Chemiluminescent Substrates. When different antibodies were used to probe the same nitrocellulose membrane, previous signals were removed by incubation with 0.5% sodium azide for 10 minutes at RT or by stripping in 62 mM Tris-HCL pH 6.8, 2% SDS, 0.7% β-mercaptoethanol for 20 min at 50 °C. The degradation constant (DC_{50}) of the PROTAC-degraded proteins was calculated in GraphPad Prism 6.0 software by plotting the densitometric values of CDK4/6 intensity (obtained by ImageJ software) normalized by the intensity of the loading control (β-ACTIN).

RNA-sequencing

BV173 cells were plated at 5 x 10^5 cells/ml and treated with Palbociclib (1 μM) or DOX (1 μg/ml) for 48 hours. RNA was isolated with the RNeasy Plus Mini Kit (#74134, Qiagen) and 100 ng of

total RNA used to prepare libraries using TruSeq Stranded Total RNA kit (Illumina, CA, USA), following the manufacturer's protocol. Libraries were sequenced on a NextSeq 500 instrument using 75-bp paired-end chemistry. Raw FASTQ sequencing reads were mapped against the reference human genome Ensembl Version GRCh38 utilizing additional information from the gene transfer format (.gtf) annotation from GENCODE version GRCH28 using RSEM. Total read counts and normalized Transcripts Per Million (TPM) were obtained using RSEM's calculate-expression function. Before determining differential expression, batch effects and sample heterogeneity were tested using iSeqQC[1]. Differential gene expression was tested using the DESeq2 package in R/Bioconductor. Genes were considered differentially expressed (DE) if they had adjusted $p \leq 0.05$ and absolute fold change ≥ 2. Plots were generated using R/Bioconductor.MA, USA).

Proteomic Analysis

$15x10^6$ BV173 cells were treated for 4 hours with 1 µM of PROTAC YX-2-107 or DMSO in triplicate. Cells were centrifuged, washed in ice-cold PBS and lysed in 300 µL of lysis buffer (50 mM Tris, pH 7.5, 1% SDS, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor cocktail (VWR #M221). Lysates were sonicated with Bioruptor (Diagenode) and cleared by centrifugation (10,000 g, 30 min, 4 °C). Proteins (25 µg/sample) were loaded on 10% acrylamide gels (Bio Rad, #4561035), electrophoresed into the gel for 0.5 cm and stained with Coomassie brilliant blue R-250 (Bio Rad # 1610400) 0.1% in 40% ethanol, 10% Acetic Acid glacial, 50% water. The entire protein-containing gel regions were excised, digested with trypsin and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a 240-min gradient as described[2].

Peptide sequences were identified using MaxQuant 1.6.3.3[3]. MS/MS spectra were searched against a UniProt human protein database (10/01/2018) using full tryptic specificity with up to

two missed cleavages, static carboxamidomethylation of Cys, and variable oxidation of Met and protein N-terminal acetylation. Proteins were quantified by label-free quantitation (LFQ). The “match between runs” feature was used to help transfer identifications across experiments to minimize missing values. Peptide and protein identifications were filtered at <1% false discovery rate (FDR) against a reversed-sequence database. Missing LFQ protein values were imputed with the dataset minimum value divided by two. The protein list was filtered to remove low confidence identifications by requiring proteins to be identified by at least 2 unique peptides in all triplicate of either sample. A total of 3,682 protein groups were quantified using these criteria. Protein levels were considered significantly different between the two samples if the absolute fold-change is > 2 and the Student’s t-test p-value is < 0.05.

Metabolic stability of PROTACs in mouse liver microsomes

Test and control compounds were incubated at 0.5 µM with 0.5 mg/mL of liver microsomes and an NADPH-regenerating system (cofactor solution) in potassium-phosphate buffer (pH 7.4). At 0, 5, 15, 30, and 45 minutes, aliquots were taken, and reactions quenched with a solution of acetonitrile containing an internal standard. As controls, samples lacking the cofactor solution were also examined. At the end of the experiment, samples were analyzed by liquid chromatography with mass spectrometry (LC-MS/MS). The intrinsic clearance (CLint) was determined from the first-order elimination constant by nonlinear regression. This analysis was performed by Alliance Pharma (Malvern, PA).

Pharmacokinetic analysis of PROTAC YX-2-107 in CD-1 mice

A 1-arm PK study was performed in 18 CD-1 mice (n=3 mice per time point). Animals were injected intraperitoneally (IP) with a single dose (10 mg/kg) of PROTAC YX-2-107 dissolved in a solution of 10% DMSO, 10% Solutol, 80% PBS. Plasma samples were collected at 0.25, 0.5, 1, 2, 4, and 6 hours post-IP injection and analyzed by LC-MS/MS. Concentrations of PROTAC

YX-2-107 were calculated by linear regression analysis. This analysis was performed by Alliance Pharma (Malvern, PA).

CDK6 DC_{50} determination in PROTAC YX-2-107-treated BV173 cells

PROTAC YX-2-107 concentration for half-maximal degradation (DC_{50}) of CDK6 was assessed by immunoblot analysis of CDK6 in cells treated with PROTAC at various doses. Levels of CDK6 were measured by densitometric analysis using ImageJ software and normalized by the levels of β-ACTIN. The DC_{50} was determined by analyzing the dose-effect curve in Graphpad PRISM.

Quantitative PCR analysis

RNA was isolated from untreated, Palbociclib-treated (500 ng/ml; 48 hrs), or doxycycline-treated (2.5 µg/ml; 48 hrs) shCDK6-88 BV173 cells, using the RNeasy Plus Mini Kit (Qiagen, Limburg, The Netherlands) and then reverse-transcribed (2 µg) using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA, USA). Quantitative PCR was performed with the QuantStudio 12k Flex (Life Technologies) instrument and QuantStudio 12K Flex software, using the following primers: HDCA1 FW, 5'-CATGCTGTGAATTGGGCTG-3'; RV, 5' CCCTCTGGTGATACTTTAGCAGT-3'; SMARCD2 FW, 5'-GCATGCTGCCCGGACC-3'; RV, 5'-ACATGCCAGGTCGCTGGT-3'; JAK1 FW, 5'-TCCGCGACGTGGAGAATATC-3', RV, 5'-TGGTGTGGTAAGGACATCGC-3'; HADHA FW, 5'-TCAACATGTTAGCCGCTTGC-3'; RV, 5'-ATGGCAACCTCAAGTCCTCC-3'; SLC2A3 FW, 5'-ACTTGCTGCTGAGAAGGACAT-3'; RV, 5'-GGGTGACCTTCTGTGTCCCC-3'; LCK FW, 5'-GGAGGACCATGTGAATGGGG-3'; RV, 5'-CATTTCGGATGAGCAGCGTG-3'; ULK2 FW, 5'-GTGGGAACGTGAGGAAGAGG-3'; RV, 5'-GCCTTCCACTAGGCAGATGG-3'; OTUD1 FW, 5'-AGCAGACGGTGCACTACATC-3'; RV, 5'-TTGGGCAGCAGCGATGATAA-3'.

References

1. Kumar G, Ertel A, Feldman G, kupper J, and Fortina P. iSeqQC: A tool for expression-based quality control in RNA sequencing. BioRxiv preprint; https://doi.org/10.1101/768101

2. Chae YC et al. Mitochondrial Akt Regulation of Hypoxic Tumor Reprogramming. Cancer Cell. 2016; 30:257-272.

3. Cox J, and Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008.26:1367-1372.