Chemotherapy-induced differential cell cycle arrest in B-cell lymphomas affects their sensitivity to Wee1 inhibition

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Supplemental Figure 1. Ara-C treatment caused apoptosis. (A) Apoptosis of CH12 mouse lymphoma cells upon 1μM Ara-C treatment. Representative FACS plots of Annexin V and PI staining. (B) DNA fragmentation in Ara-C treated CH12 mouse lymphoma cells. Cytosolic DNA was isolated from untreated or treated CH12 cells with 1μM Ara-C for 24hrs, and resolved by electrophoresis with 1.5% agarose gel. (C) Cell death after Ara-C treatment. CH12 lymphomas cells were treated with 1μM Ara-C for 24 hours. Dead cells were detected by fluorescent microscopy with DAPI staining. (D) Western blotting analysis of caspase 3. Treatment of 1μM or 10μM Ara-C resulted in the increased expression of cleaved caspase 3 in CH12 cells in a dose-dependent manner. β-actin is the loading control. (E) Representative FACS plots of cleaved caspase 3. The level of cleaved caspase 3 was increased upon 1μM Ara-C treatment after 24 hours in CH12 cells. (F) Ara-C treatment had no effect on necroptosis. G1XP, A20, CH12 and 38C13 lymphoma cells were treated with 1μM or 10μM Ara-C for 24 hours. CaMK IIδ and RIP3 were detected by western blot and β-actin as the loading control.

Supplemental Figure 2. SOMAscan Data. (A) Top 10 upregulated (top panel) and downregulated (bottom panel) proteins are shown in Ara-C treated WT primary B cells (n=3) compared with untreated group (n=3). WT.nt: WT untreated; WT.trt: WT treated. (B) Top 10 upregulated (top panel) and downregulated (bottom panel) proteins are shown in Ara-C treated DKO primary B cells (n=3) compared with untreated group (n=3). DKO.nt: DKO untreated; DKO.trt: DKO treated. WT or DKO primary B cells were activated with anti-CD40/IL-4 for 4 days, then treated with 10μM Ara-C for 16 hours. Cell lysates were prepared and subjected to SOMAscan assay.

Supplemental Figure 3. Upregulation of Cyclin B1 and cyclin A2 in different types of B cells upon Ara-C treatment. (A) Increased expression of cyclin B1 and cyclin A2 in different types of B cells upon Ara-C treatment. DHL-16, Ramos, Ly1, Ly3 and Ly7 lymphomas cells were treated with 1μM Ara-C for 24 hours. Cyclin B1, cyclin A2, pCDK1 and total CDK1 were detected by western blot and β-actin as the loading control. (B) Upregulation of pChk1 and pChk2 in different types of B cells upon Ara-C treatment. DHL-16, Ramos, Ly1, Ly3 and Ly7 lymphoma cells were treated with 1μM Ara-C for 24 hours. pChk1, Chk1, pChk2, Chk2, CDC25A and pCDC25C were detected by western blot and β-actin as the loading control. Of note, Ly3 cells expressed a lower level of pCDC25C in both untreated and treated samples. In addition, Ly3 failed to upregulate pChk1 and pChk2 upon Ara-C treatment, probably due to defects in the signaling pathway of Chks. (C) Kinetics of cyclinB1/A2 upregulation induced by Ara-C treatment. CH12 cells were treated with 1μM Ara-C for 4, 20, or 24 hours. Cyclin B1, CDK1, pCDK1, cyclin A2 and CDK2 were detected by western blot and β-actin as the loading control.

Supplemental Figure 4. Ara-C results in more apoptosis regardless of the phase of cell cycle arrest. CH12 lymphoma cells were treated with 1μM Ara-C for 24 hours. Cells were collected and fixed by 70% ethanol. After PI and caspase 3 staining, cell cycle status was determined by flow cytometry (FL1-H/FL2-A). (A) Cell cycle analysis without Sub-G1 phase cells. Representative FACS plots for PI staining (top panel) or cleaved caspase 3 vs. PI staining (bottom panel) of untreated vs. Ara-C treated cells. (B) Cell cycle analysis with Sub-G1 phase cells included. Representative FACS plots for PI staining (top panel) or cleaved caspase 3 vs. PI staining (bottom panel) of untreated vs. Ara-C treated cells. Ara-C treatment increased the percentage of Sub-G1 phase cells (10.8% in untreated vs. 35.6% in Ara-C treated), and all sub-G1

phase cells were positive for cleaved caspase 3, indicating their apoptotic phenotypes. The ratio among G1/S/G2 remains the same with or without sub-G1 phase included.

Supplemental Figure 5. Mitotic entry is blocked upon Ara-C treatment. CH12 and G1XP lymphoma cells were treated with 1μM Ara-C for 24 hours. Cells were collected and fixed by 70% ethanol. After PI and pH3 staining, cell cycles were determined by FACS (FL1-H/FL2-A).

Supplemental Figure 6. Knockdown of cyclin B1 or/and cyclin A2 had no effects on cell cycle changes. CH12 lymphoma cells were transiently transfected with cyclin B1 or/and cyclin A2 shRNA constructs. After 24 hours incubation, cells were treated with 1μM Ara-C for 24 hours. (A) Cyclin B1, cyclin A2 and caspase 3 were detected by western blot and β-actin as the loading control. (B) Cells were collected and fixed by 70% ethanol. After PI staining, cell cycles were determined by flow cytometry (FL2-A).

Supplemental Figure 7. Wee-1 inhibitor (MK1775) did not enhance mitotic entry of Ara-C-induced G1 and S phase-arrested lymphoma cells. DHL-16, Ly1, Ramos and Ly7 were either untreated or treated with 1μM Ara-C, 100nM MK1775, or both for 24 hours. Cells were collected and fixed by 70% ethanol. After PI and pH3 staining, cell cycles were determined by flow cytometry (FL1-H/FL2-A).

Supplemental Figure 8. Wee-1 inhibitor (MK1775) did not increase activated caspase 3 of Ara-C-induced G1 and S phase-arrested lymphoma cells. DHL-16, Ly1 and Ly7 were either

untreated or treated with 1μM Ara-C, 100nM MK1775, or both for 24 hours. Cyclin B1, cyclin A2, caspase3 and pCDK1 were detected by western blot and β-actin as the loading control.

Supplemental Material and Methods

Cytosolic DNA isolation

Cytosolic DNA was isolated as previously described with minor modifications (1). Briefly, cells were lysed in 10mM HEPES (pH 7.9), 10mM KCl, 1.5mM $MgCl_2$, 0.34M sucrose, 10% (v/v) glycerol and 0.1% (v/v) Triton X-100 for 5 minutes on ice, and nuclei were removed by low-speed centrifugation (1,500×g, 10min). Cytoplasmic protein extracts were treated with 1mg/ml Proteinase K for 1 hour at 55°C. After phenol/chloroform extraction, the DNA-containing aqueous phase was precipitated. Precipitated DNA was resuspended in TE buffer and digested by 200μg/mL DNase-free RNase (Cat. EN0531, Thermo Scientific) for 30 minutes at 37°C. Cytosolic DNA was analysed by a 1.5% (w/v) agarose gel with ethidium bromide in 1×TBE buffer.

Flow cytometry and cell cycle analysis

For caspase 3 staining, cells were fixed by 4% paraformaldehyde for 1 hour at room temperature. Then, cells were permeabilized by 0.1% Triton X-100 for 30 minutes. After 2% BSA blocking, cells were stained with Alexa Fluor® 488 conjugated rabbit anti-cleaved caspase 3 (clone 269518, R&D Systems, Minneapolis, MN). For Annexin V and PI staining, cells were collected and stained with Allophycocyanin conjugated Annexin V and PI according to the kit instruction (Cat. 88-8077, Thermo Fisher Scientific).

For cell cycle determination, cells were collected and fixed with 70% cold ethanol overnight at –20°C. The fixed cells were stained with 4μL propidium iodine (PI) (50μg/mL, MP Biomedicals, Solon, OH) and 0.4μg/mL Alexa Fluor® 488 conjugated rabbit polyclonal anti-phospho-histone 3 (Ser 10) (Cell Signaling Technology, Danvers, MA) in the presence of RNase A (200μg/mL, Cat.EN0531, Thermo Scientific) in PBS for one hour at 37°C in dark. All analyses

were performed with FACSCalibur (BD Bioscience), and results were analysed with FlowJo software.

SOMAscan assay and data analysis

Untreated or Ara-C treated cells were collected and cell lysates were prepared by M-PER™ Mammalian Protein Extraction Reagent (Cat.78503, Thermo Fisher Scientific). Samples were submitted to Genomic and Microarray Core Facility at University of Colorado. Based on SOMAmer™ (Slow Off-rate Modified Aptamer) technology, 1310 proteins were quantified simultaneously. Initial data analysis was performed by SomaLogic (Boulder, CO). Pre-normalized SOMAscan expression signal data were read in by R readat package (2). Using the treatment condition as independent variable and Log2 transformed expression data as dependent variable, SOMAscan proteomic data were analysed with linear regression model fit followed by Empirical Bayes statistical tests using limma package (3). Top differentially expressed protein targets in comparison of interest were reported with Aptamer ID, protein ID, Gene ID, together with the p-value and FDR of those targets in Supplemental Table 1 and 2.

CyclinA2 and B1 knockdown

For cyclinA2 and cyclinB1 knockdown, CH12 cells were plated at 5×10^5/mL and allowed to proliferate overnight. The plasmids containing shRNA of cyclinA2 (TRCN0000077767) and cyclinB1 (TRCN0000306520) were purchased from Sigma. Transient transfection was performed with 4μg shRNA constructs using Cell Line Nucleofector Kit-R and program K-005 (Lonza, Walkersville, MD). Ara-C was added into cell culture at 24hrs and cells were collected at 48hrs after transfection for analysis.

***In vivo* treatment of transplant G1XP lymphoma model**

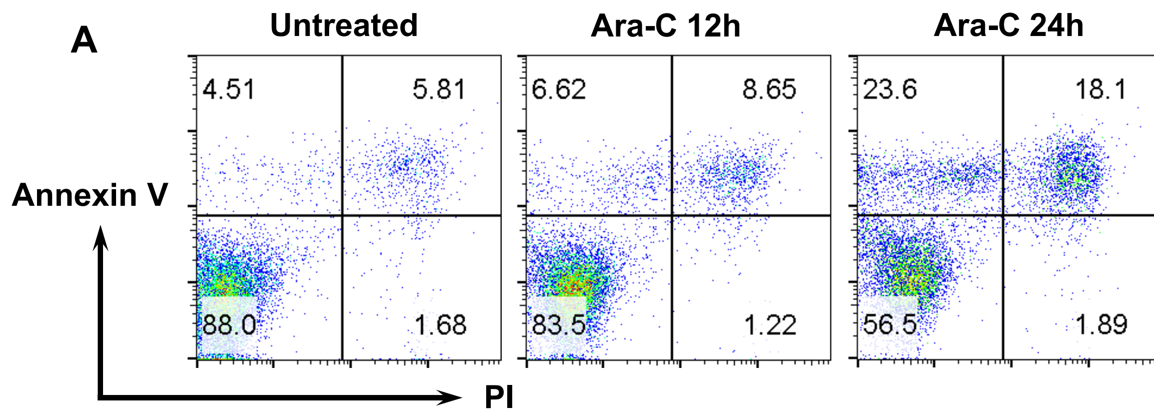
Littermate controls of G1XP mice (6-8 weeks) were injected subcutaneously at both flanks with 1×10^6 G1XP lymphoma cells. When tumor size reached ~1000 mm^3 (~20 days after inoculation), recipient mice were treated daily with vehicle control (intraperitoneal injection of PBS and oral gavage of methylcellulose vehicle of MK1775), Ara-C (100 mg/kg/dose) via intraperitoneal injection, MK1775 (40mg/kg/dose) via oral gavage, or both Ara-C and MK1775 as indicated in Figure 7. Mice were maintained under specific pathogen-free conditions in the vivarium facility of University of Colorado Anschutz Medical Campus.

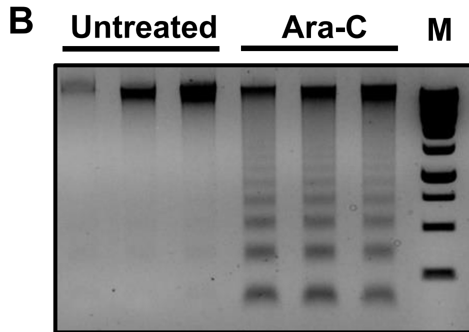
Reference:

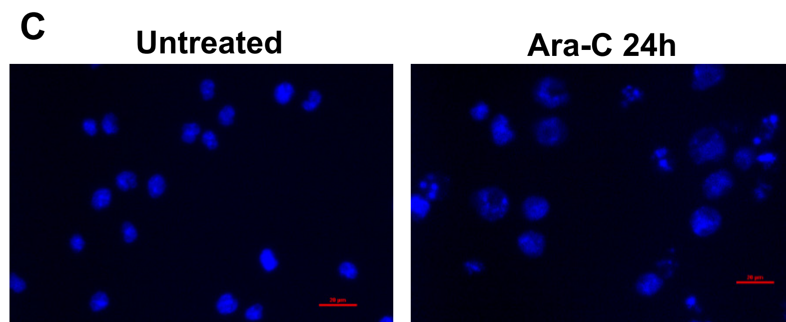
1. Hartlova A, Erttmann SF, Raffi FA, Schmalz AM, Resch U, Anugula S, et al. DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity. Immunity. 2015 Feb 17;42(2):332-43.

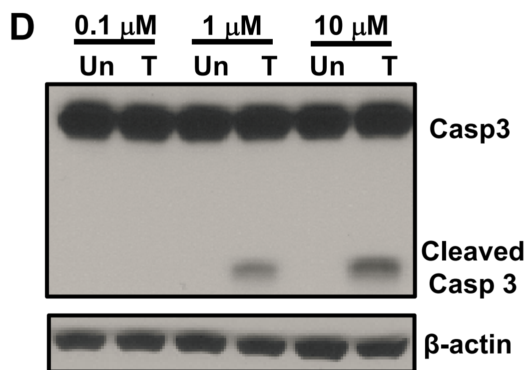
2. Cotton RJ, Graumann J. readat: An R package for reading and working with SomaLogic ADAT files. BMC Bioinformatics. 2016 May 04;17(1):201.

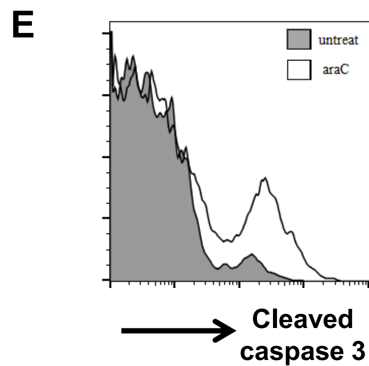
3. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Apr 20;43(7):e47.

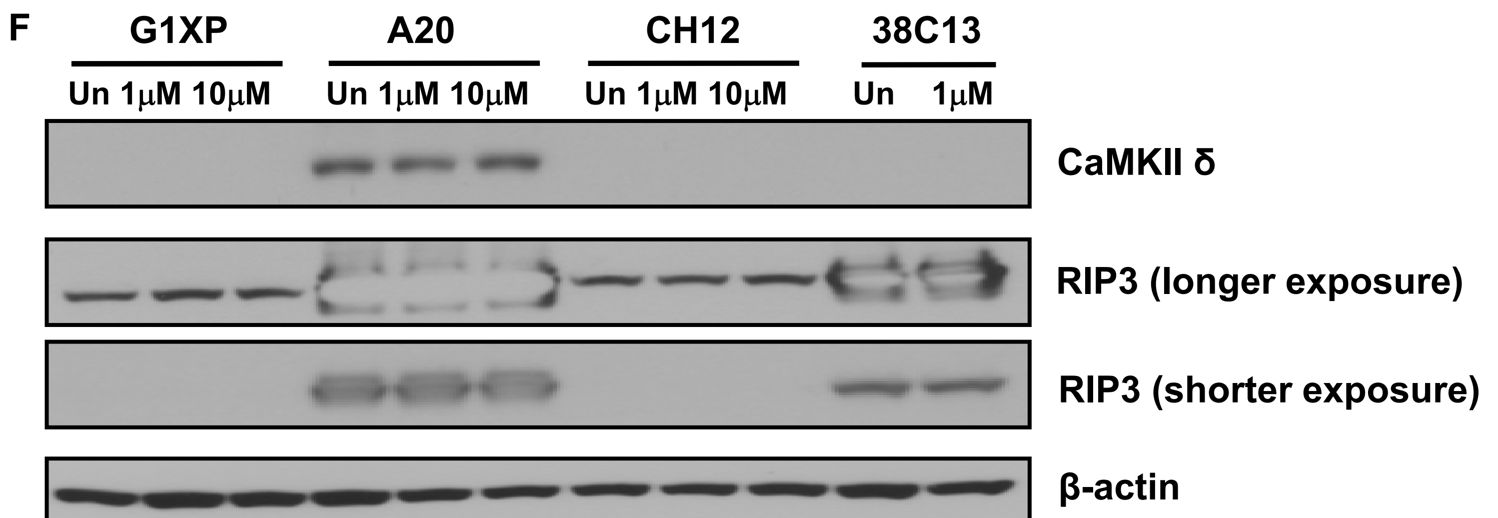






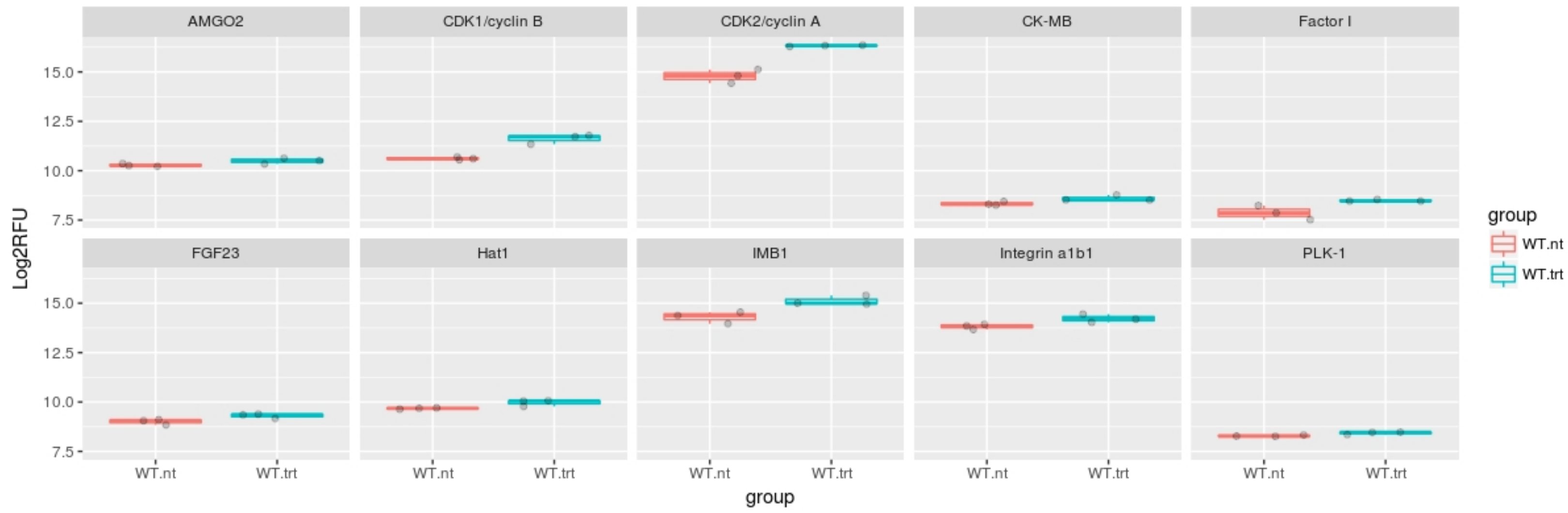


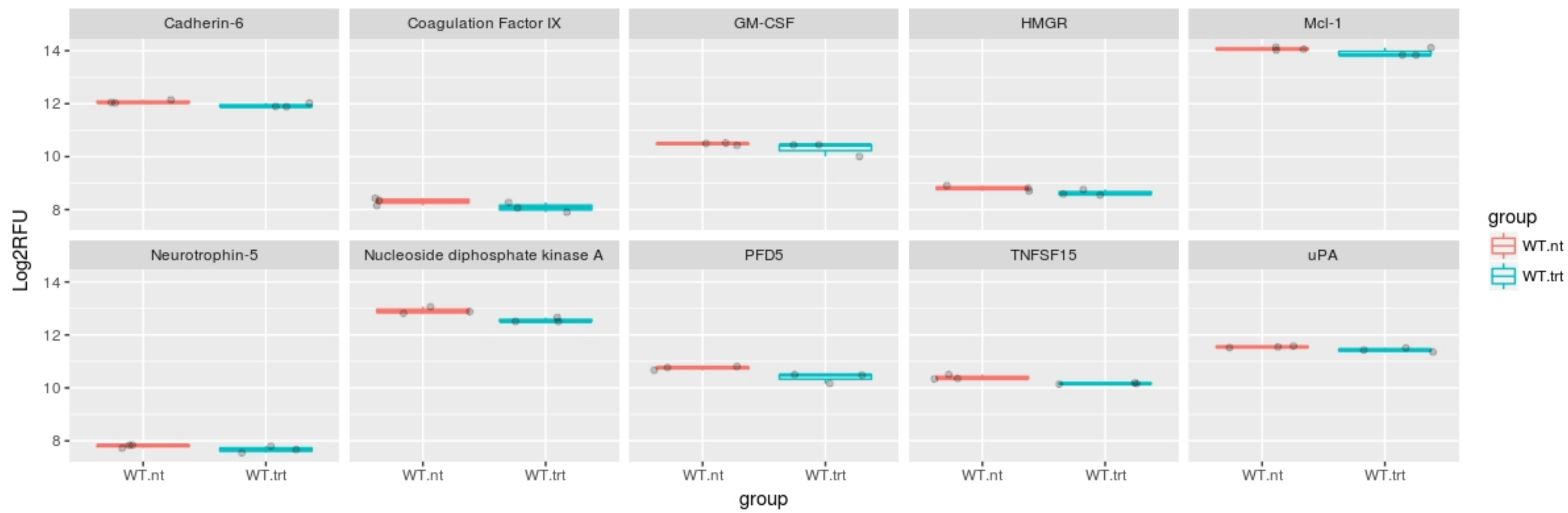




Supplemental Figure 1A-F

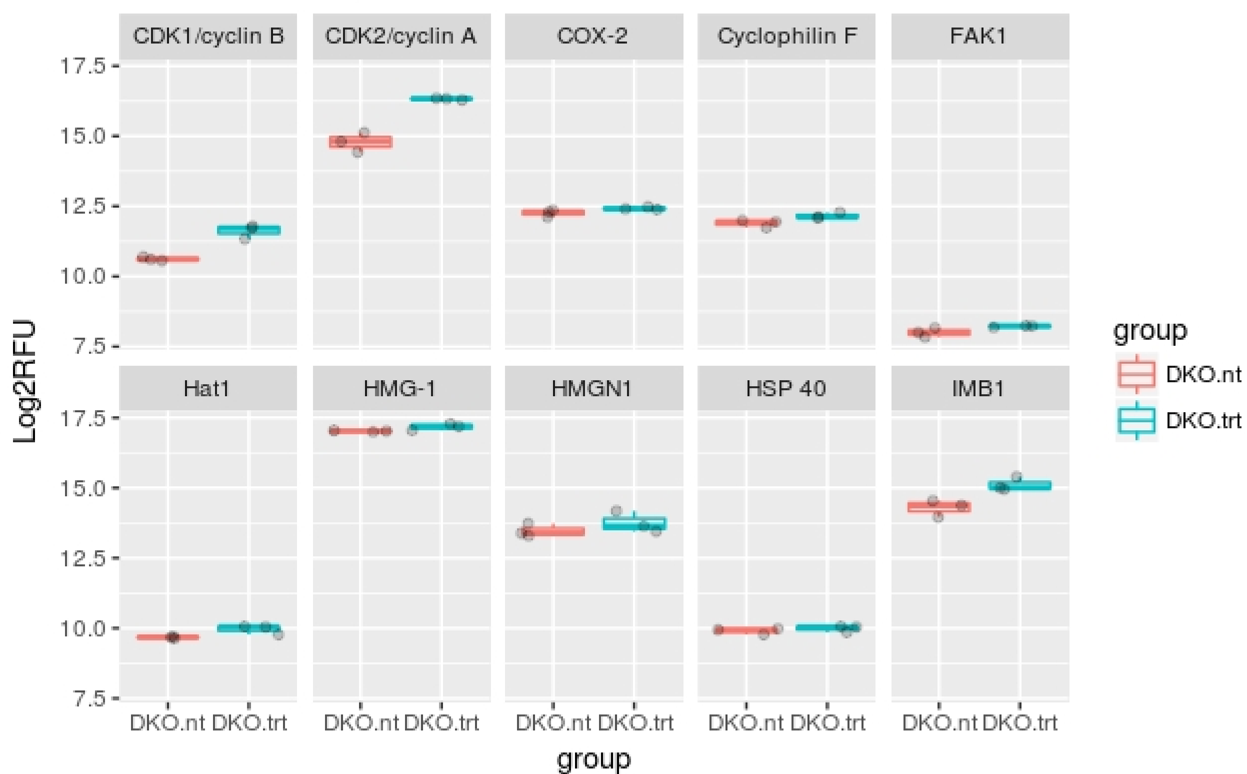
A

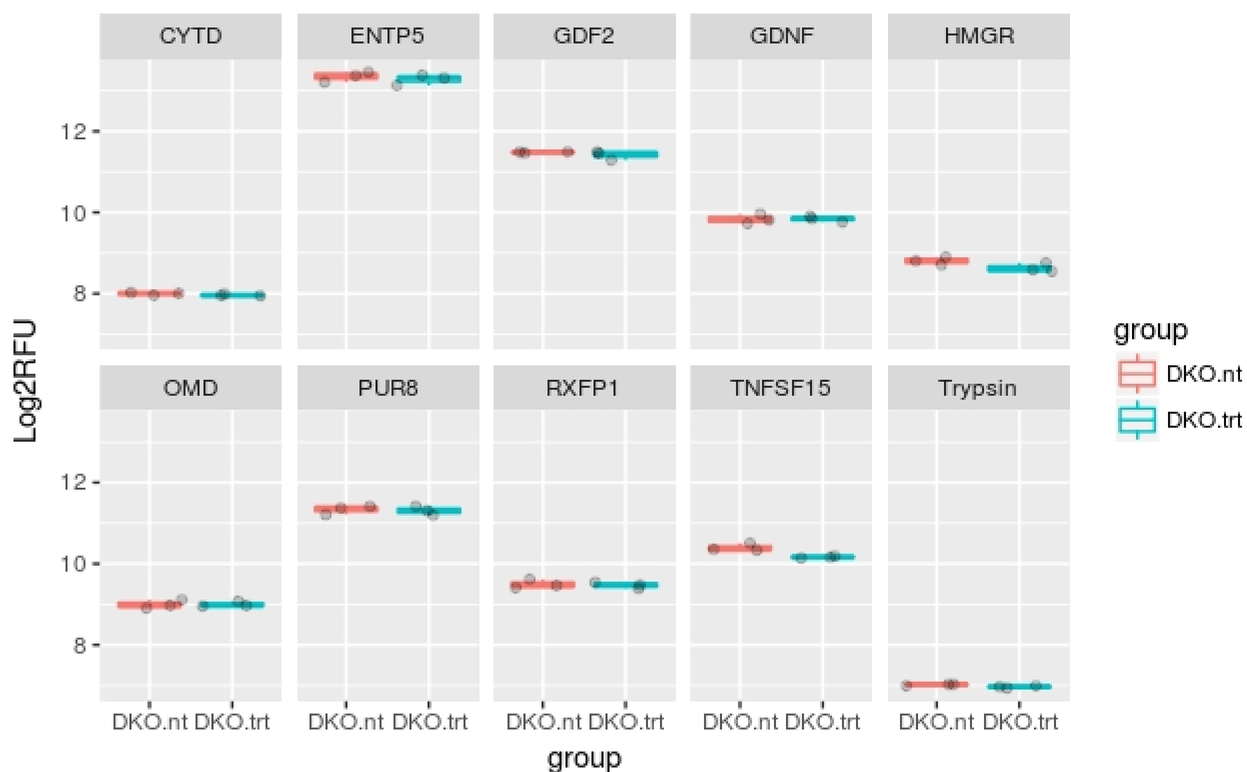




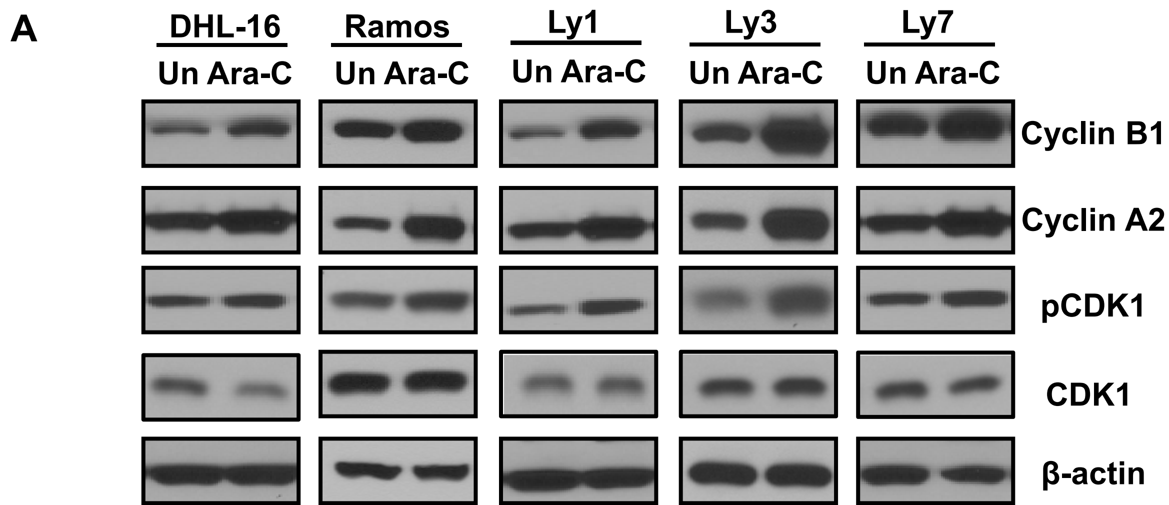
Supplemental Figure 2A

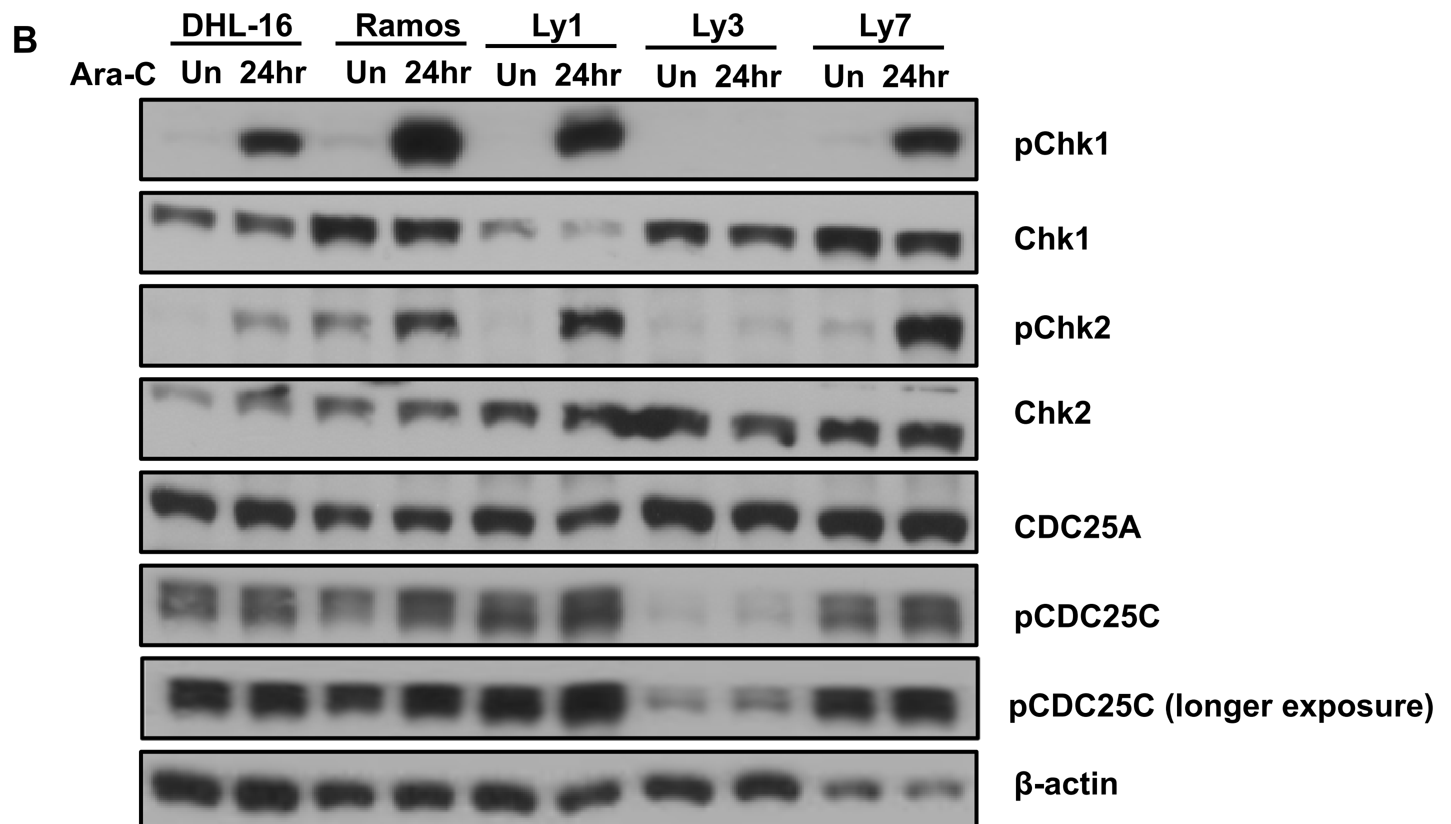
B

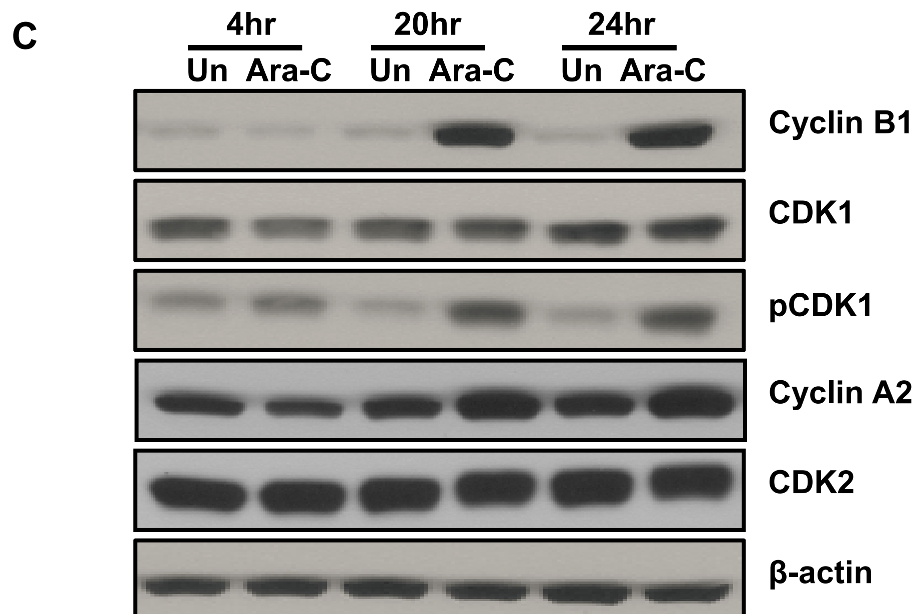




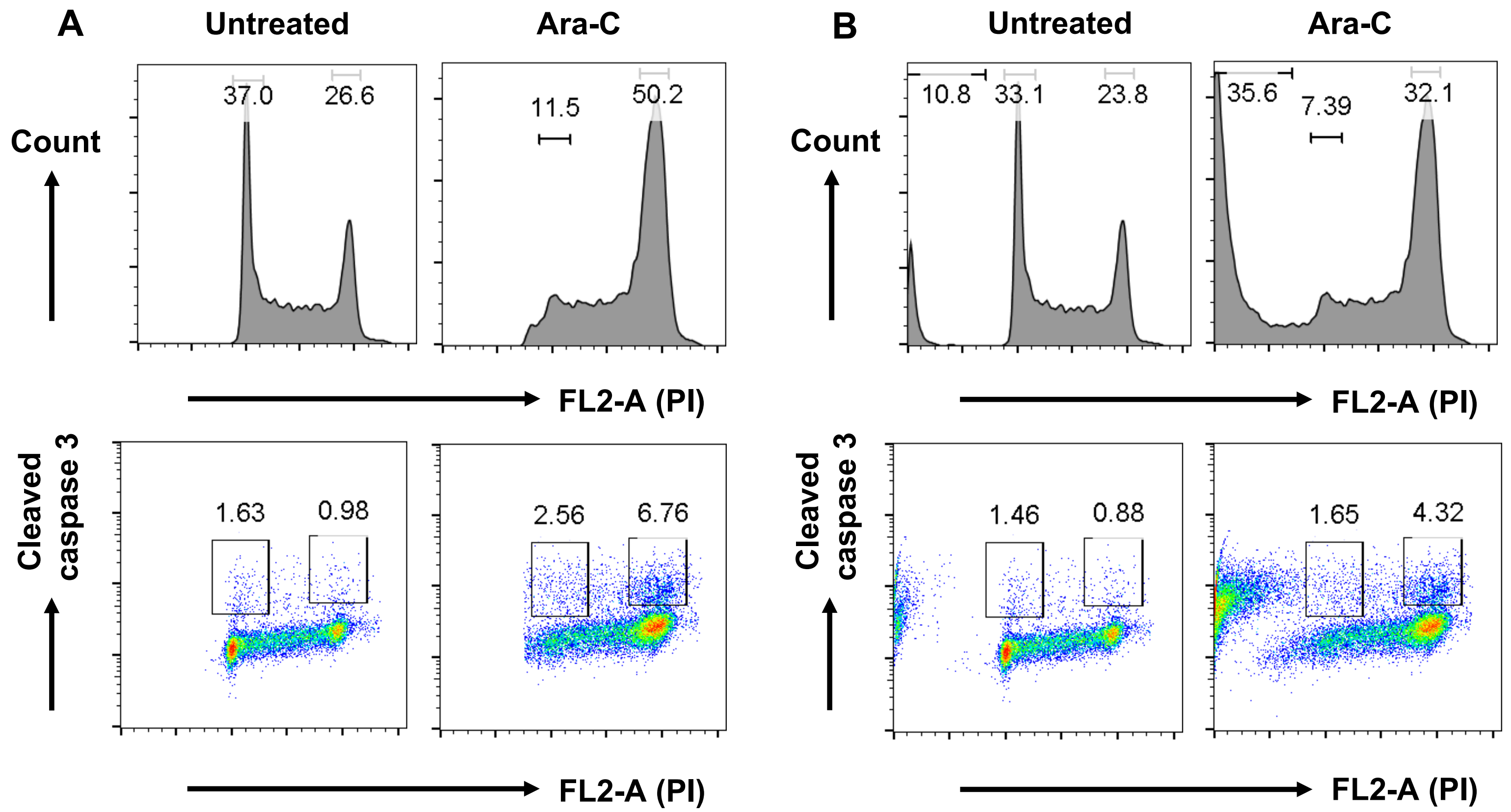
Supplemental Figure 2B







Supplemental Figure 3A-C



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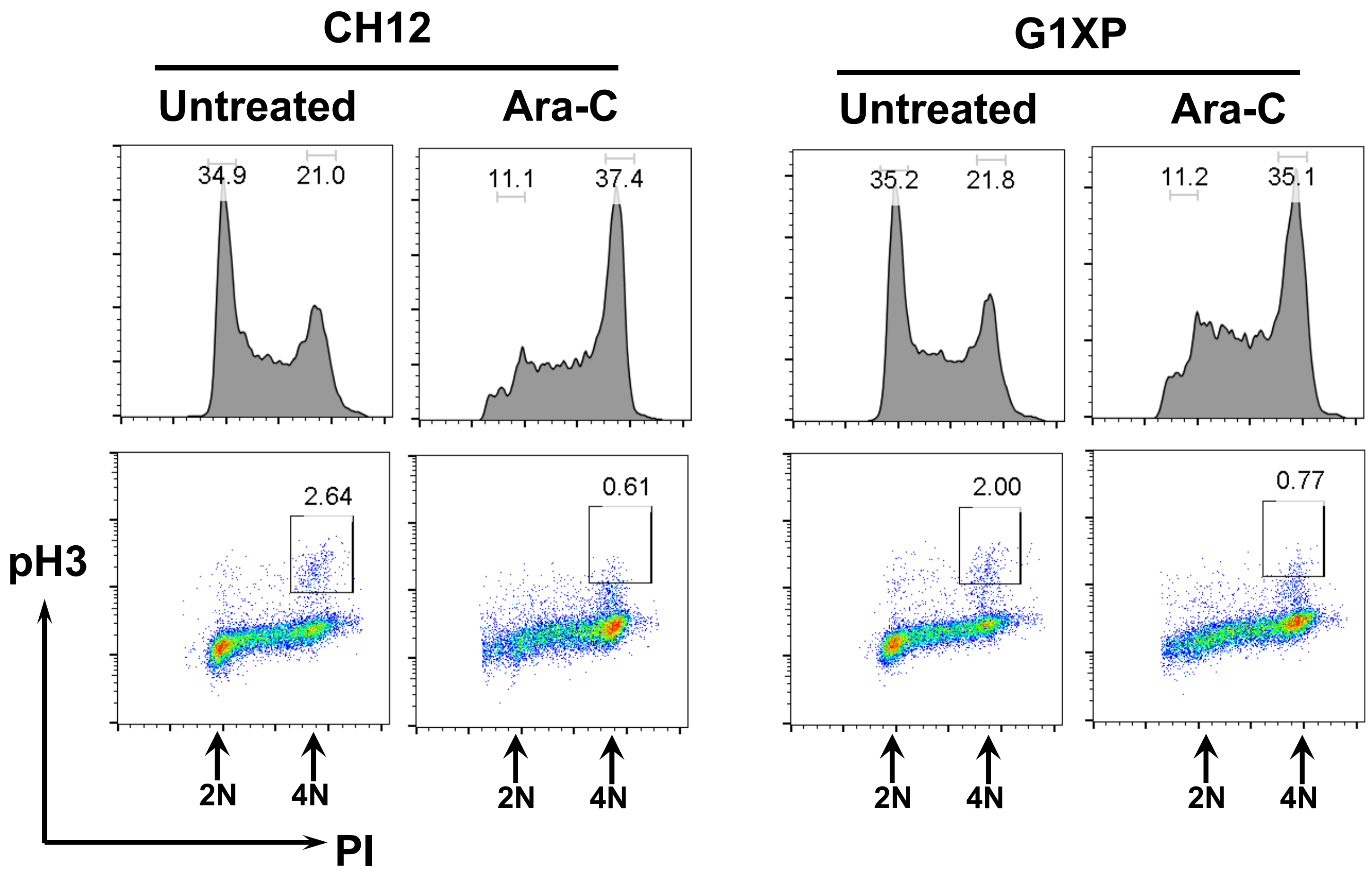
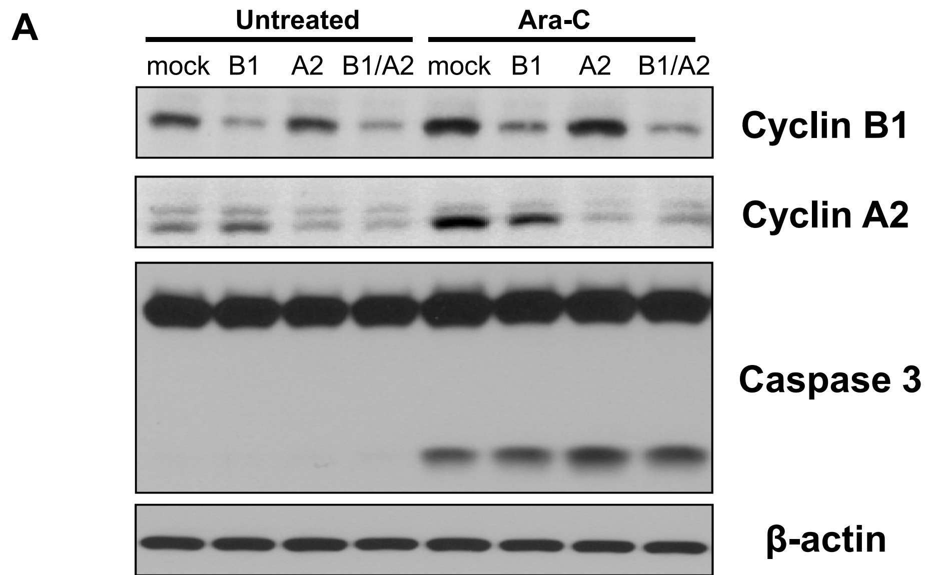
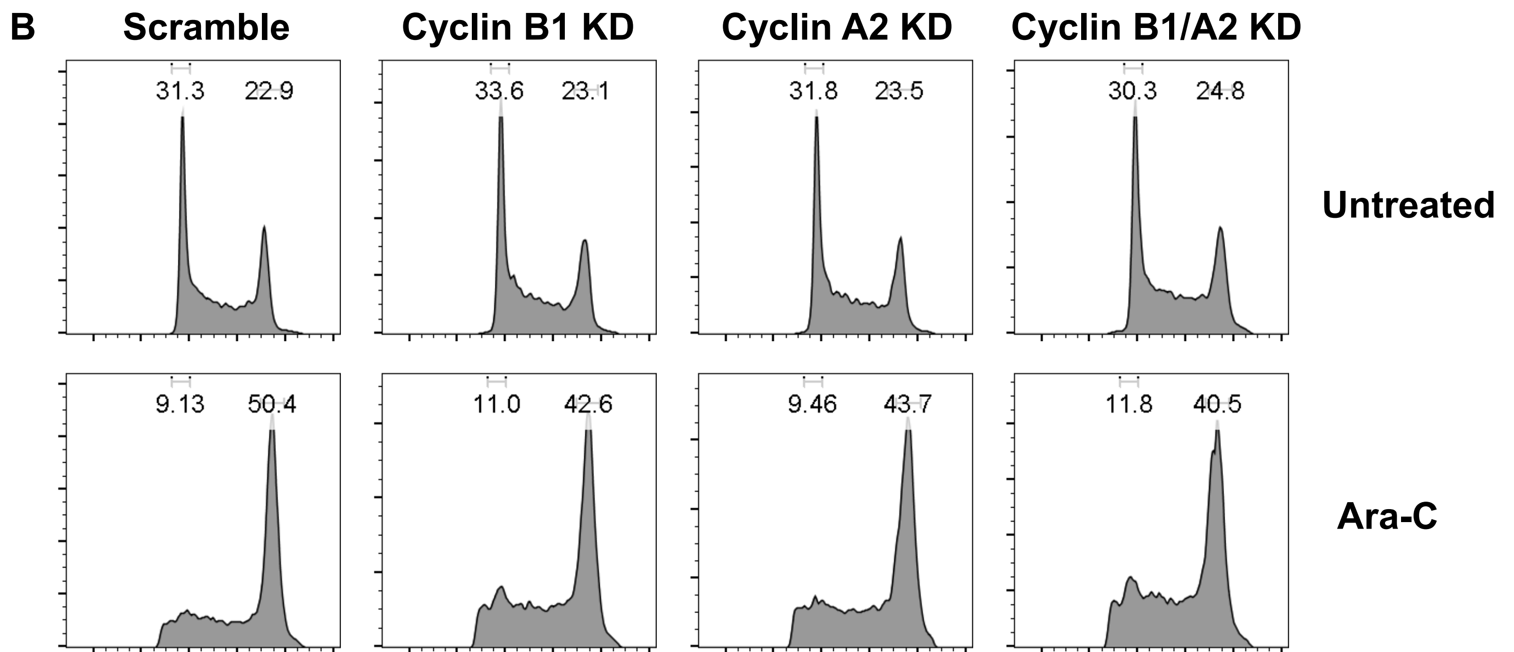
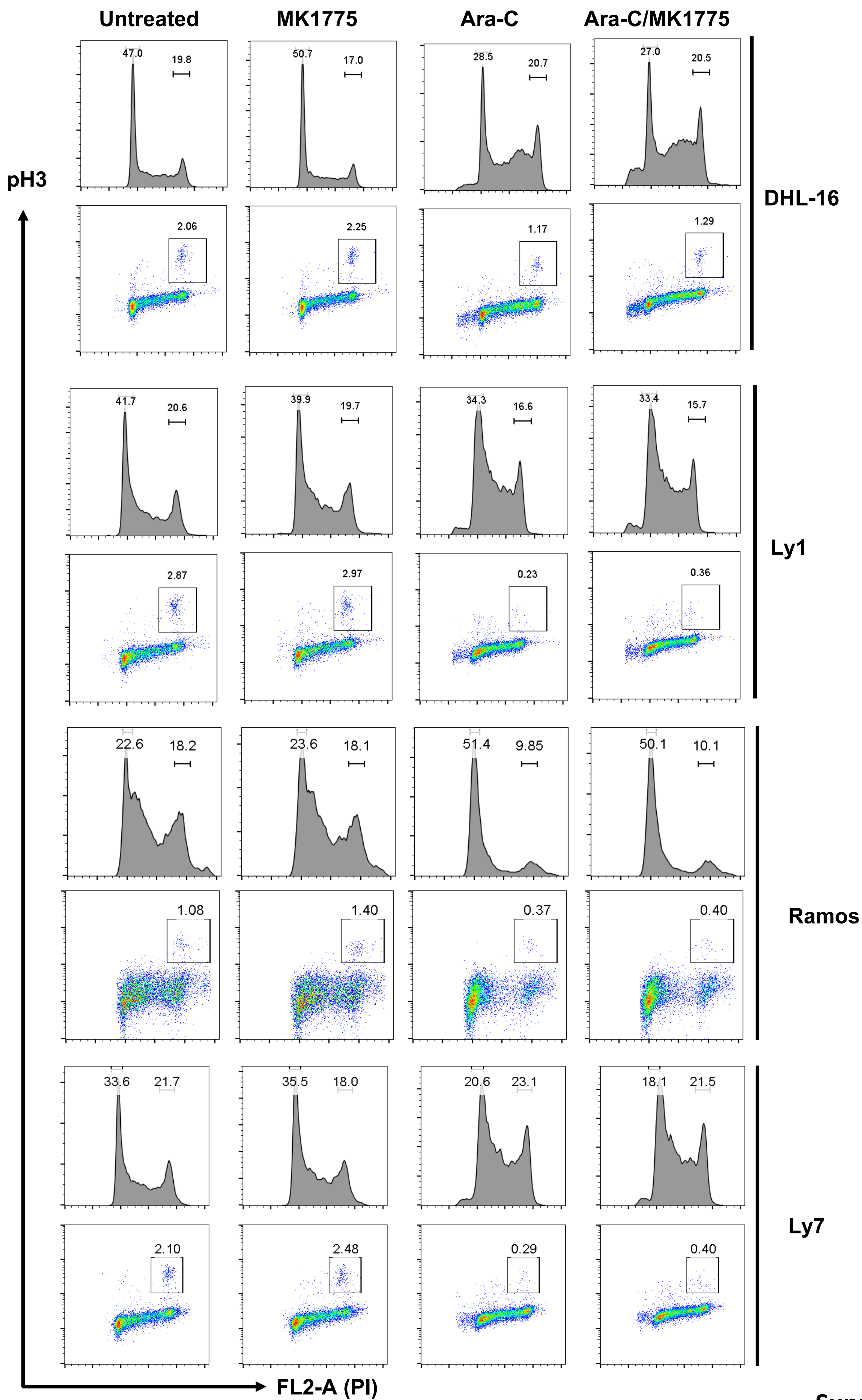


Supplemental Figure 5

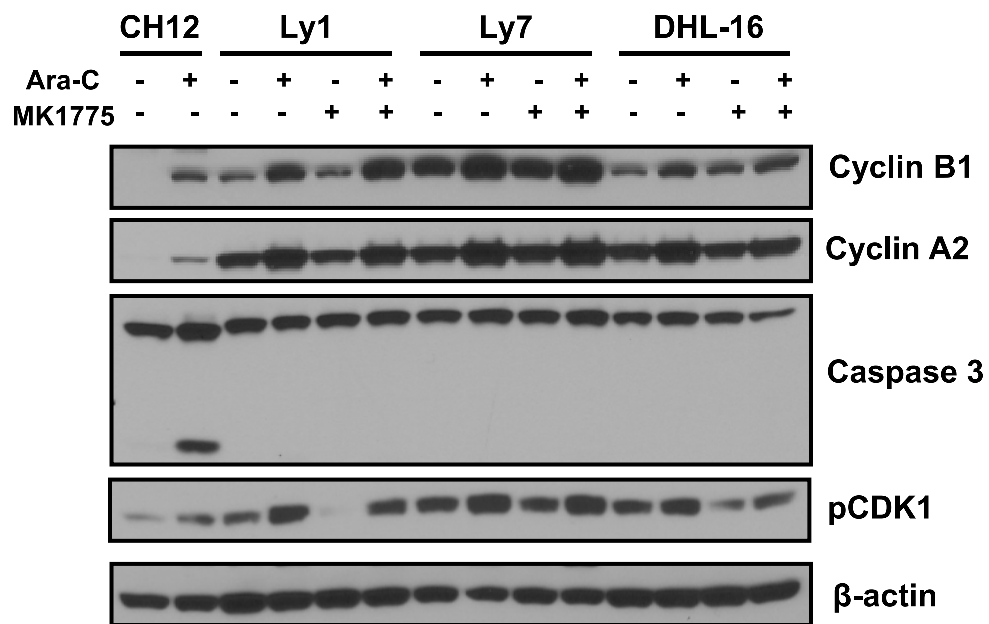




Supplemental Figure 6A-B



Supplemental Figure 7



Supplemental Figure 8

Supplemental Table 1. SOMAscan Data of WT primary B cells. All proteins tested are shown in Ara-C treated WT primary B cells (n=3) compared with untreated group (n=3). Data are reported with SomaID, protein ID, Gene ID, together with log2FC, the p-value, and adjusted p-value (FDR) of those targets.

Supplemental Table 2. SOMAscan Data of DKO primary B cells. All proteins tested are shown in Ara-C treated DKO primary B cells (n=3) compared with untreated group (n=3). Data are reported with SomaID, protein ID, Gene ID, together with log2FC, the p-value, and adjusted p-value (FDR) of those targets.

Supplemental Table 3. Antibodies used in the study.

Antibody	Catalogue	Clone	Company	Concentration
mouse anti-cyclin B1	Sc-245	GNS1	Santa Cruz Biotechnology	0.4 μg/ml
mouse anti-Cdk1	Sc-54	17	Santa Cruz Biotechnology	0.2 μg/ml
rabbit anti-phospho-CDK1	AF888	Poly	R&D Systems	0.2 μg/ml
goat anti-cyclin A2	AF5999	Poly	R&D Systems	0.4 μg/ml
goat anti-Cdk2	AF4654	Poly	R&D Systems	0.2 μg/ml
mouse anti-β-actin	Sc-47778	C4	Santa Cruz Biotechnology	0.4 μg/ml
rabbit anti-caspase 3	9662	Poly	Cell Signaling Technology	0.2 μg/ml
rabbit anti-RIP3	2283	Poly	Prosci	1 μg/ml
mouse anti-CaMKIIδ	Sc-100362	L-04	Santa Cruz Biotechnology	0.2 μg/ml
Rabbit anti-pChk1	2348	133D3	Cell Signaling Technology	0.2 μg/ml
Mouse anti-Chk1	2360	2G1D5	Cell Signaling Technology	0.2 μg/ml
Rabbit anti-pChk2	NB100-92502	Poly	Novus Biologicals	1 μg/ml
Rabbit anti-Chk2	2662	Poly	Cell Signaling Technology	0.2 μg/ml
Mouse anti-Cdc25A	MAB1648	336445	R&D Systems	0.2 μg/ml
Rabbit anti-pCdc25C	4901	63F9	Cell Signaling Technology	0.2 μg/ml