Romidepsin enhances the efficacy of cytarabine *in vivo*, revealing histone deacetylase inhibition as a promising therapeutic strategy for *KMT2A*-rearranged infant acute lymphoblastic leukemia

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SUPPLEMENTARY MATERIALS AND METHODS

Flow cytometry

All flow cytometry studies were performed using single cell suspensions, and cells were stained using standard protocols. Flow cytometry was performed on a BD LSRII (BD Biosciences, Franklin Lakes, NJ, USA). Cell suspensions were counted using a Vi-CELL® Cell Viability Analyzer (Beckman Coulter, Indianapolis, Indiana, USA). To monitor the development of leukemia, mononucleated cells were treated with eBioscience red blood cell lysis buffer (Life Technologies Australia, Scoresby, VIC, Australia) and stained with anti-human CD19-APC (BD Biosciences) for the PER-785 xenograft model and anti-human CD45-PE (BD Biosciences) for the MLL-5 and MLL-14 xenograft models. For phospho-H2A.X staining, cells were fixed and permeabilized with 1% formaldehyde and 0.1% triton-X. Cells were then incubated with 1μL of Alexa Fluor 647-conjugated phospho-Histone H2A.X (Ser139) rabbit mAb (Cell Signaling Technology, Danvers, MA, USA) for 20 minutes before flow cytometric analyses.

Western blotting

Cells were harvested and lysed in Evan's protein lysis buffer (1mM EDTA, 1mM EGTA, 1%NP-40, 50mM Tris-HCl, 120nM NaCl and protease inhibitors – cOmplete EDTA-free Protease Inhibitor Cocktail (Roche, North Ryde, NSW, Australia). 20μg of protein extracts from each cell line were separated electrophoretically (SDS-PAGE). The membrane was blocked with either 5% milk or 5% bovine serum albumin dissolved in TBS containing 0.1% Tween 20 and stained separately with the following antibodies: phospho-ATM (Ser1981) Mouse mAb, ATM (D2E2) Rabbit mAb, phospho-ATR (Ser428) Rabbit pAb, ATR Rabbit pAb, phospho-CHK1 (Ser345) Rabbit pAb, CHK1 (2G1D5) Mouse mAb, phospho-CHK2 (The68)(C13C1) Rabbit mAb, CHK2 (1C12) Mouse mAb, phospho-P53 (Ser15) Rabbit pAb, P53 (1C12) Mouse mAb, phospho-DNA PKcs Rabbit pAb, DNA PKcs Rabbit pAb, acetyl-KU70 Rabbit pAb, KU70 (D10A7) Rabbit mAb, histone H2A.X (D17A3) Rabbit mAb and phospho-histone H2A.X (Ser139) Rabbit mAb. All antibodies were obtained from Cell Signaling Technology with the exception of acetyl-KU70 and

phospho-DNA PKcs obtained from Abcam (Melbourne, VIC, Australia). Secondary staining was conducted with either anti-rabbit horseradish peroxidase or anti-mouse horseradish peroxidase (GE Healthcare, Pittsburg, PA, USA) based on primary antibody host species. Horseradish peroxidase activity was detected with a chemiluminescence detection kit. After exposure, the membrane was probed for housekeeping protein mouse anti-β-actin (Sigma-Aldrich, Castle Hill, NSW, Australia). The band density on representative gels was measured using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analyses and graphics were performed using GraphPad Prism 7 and Microsoft Excel for Mac 2011. Data were analyzed using the two-tailed unpaired Student's t-test. Survival studies were analyzed using log-rank test. The results are presented as means ± standard deviation (SD). A *P* value < 0.05 was considered as statistically significant.

SUPPLEMENTARY TABLES

Supplementary Table 1. P values for Figure 1C calculated by log-rank test

	PER-785	MLL-5	MLL-14
Romidepsin vs Vehicle	0.41	0.25	0.29
Cytarabine vs Vehicle	0.001	0.0005	0.41
Cytarabine vs Romidespin/Cytarabine	0.0051	0.0025	0.0009

Supplementary Table 2. Differential *in vitro* sensitivity to cytarabine

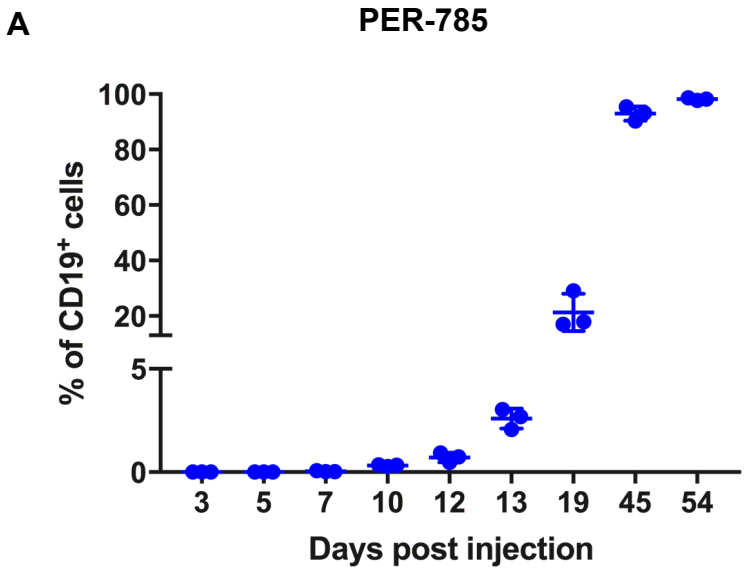
	PER-785	PER-826
Average IC50 (μM): Telethon Kids Institute, Perth	0.138	3.366
Average IC50 (μM): Children's Cancer Institute, Sydney	0.059	2.298

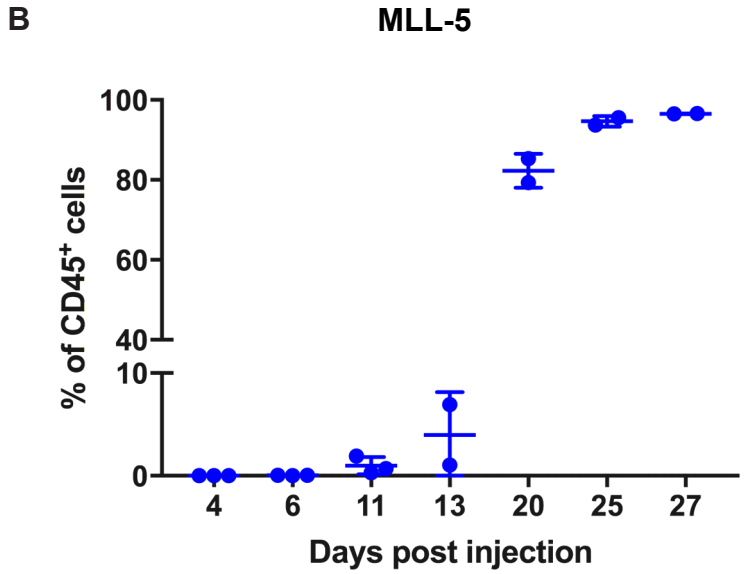
Average IC50 - half maximal inhibitory concentration averaged from two independent experiments, performed as described in Cruickshank MN *et al.* Leukemia 2017;31(1):40-50.

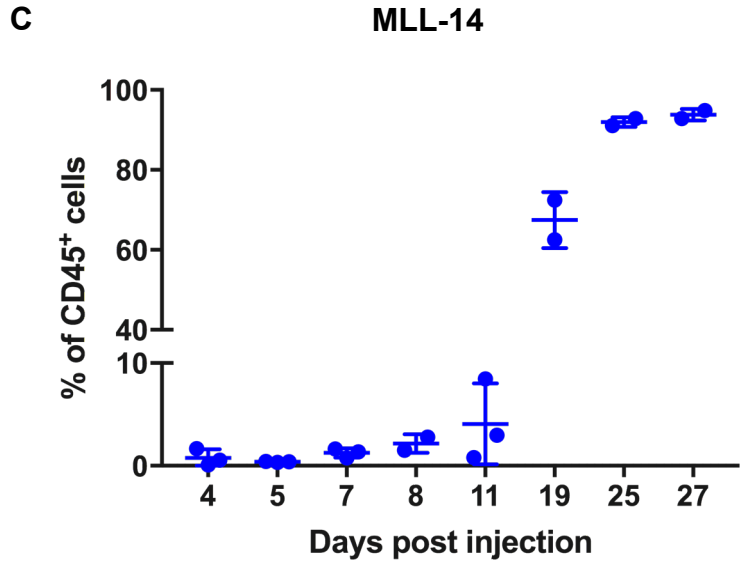
In vitro determination of cytarabine cytotoxicity, conducted in two independent centers, identified that PER-826, which is a cell line derived from the same patient as MLL-14, was less sensitive to cytarabine than PER-785, consistent with findings using single agent cytarabine *in vivo*.

SUPPLEMENTARY FIGURES

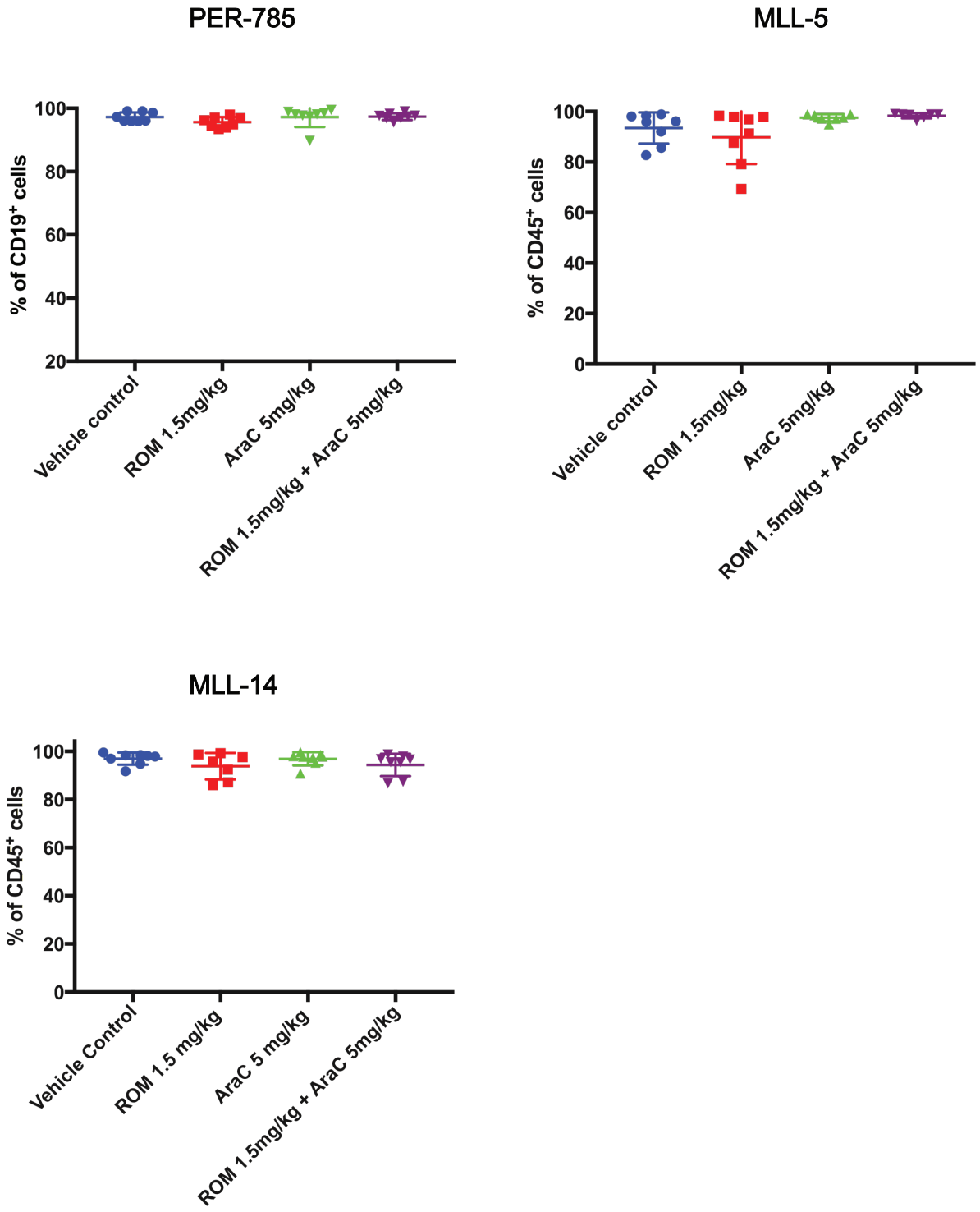
Supplementary Figure 1. Leukemia cell kinetics in the bone marrow. Mice were sacrificed at specified days post leukemia cell injection (n = 2-3 mice per time point) and leukemia burden in the bone marrow was measured by flow cytometry. (A) PER-785. (B) MLL-5. (C) MLL-14. Error bars represented mean ± SD.



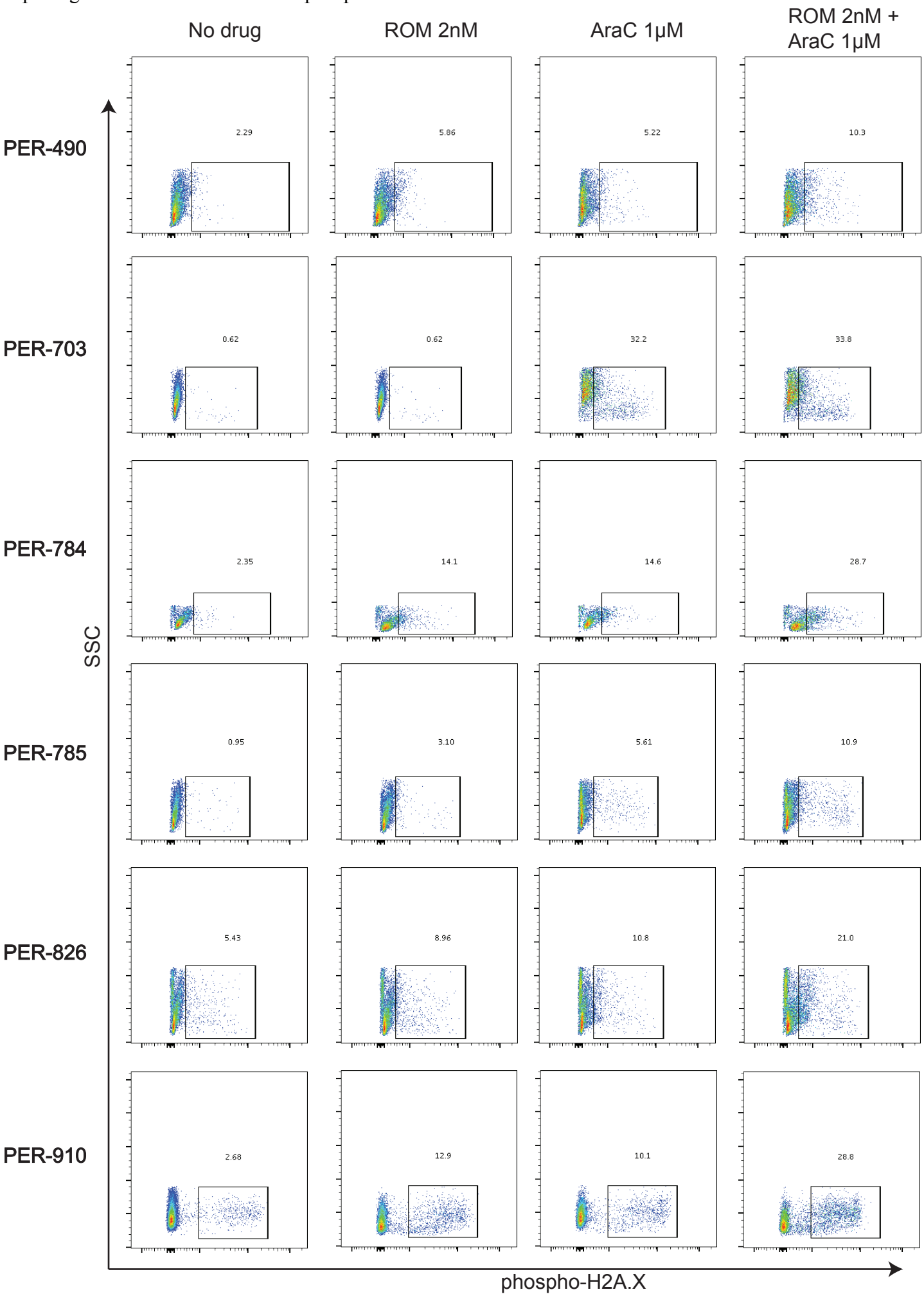




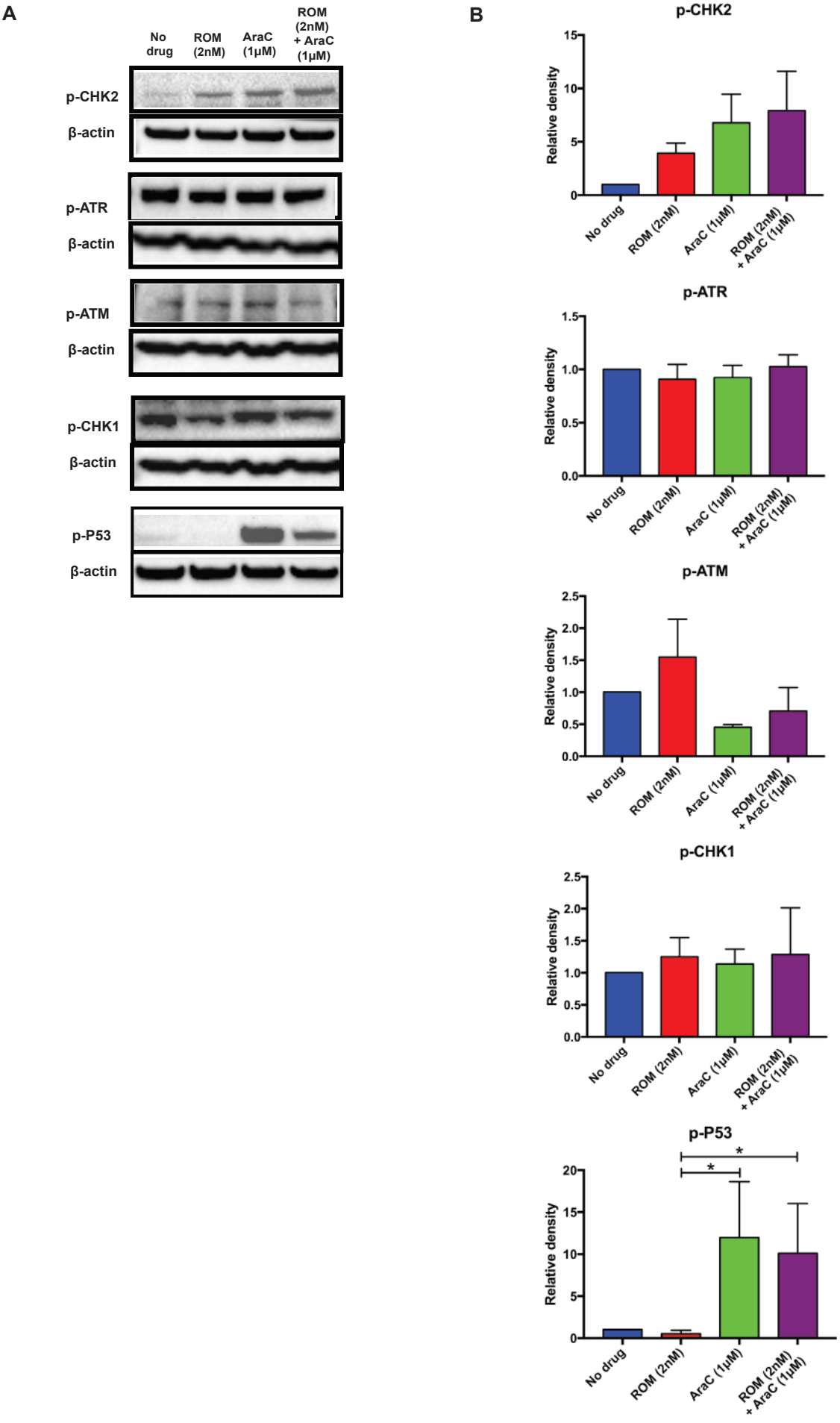
Supplementary Figure 2. Leukemia burden in the bone marrow when mice succumb to disease. Percentages of leukemia cells in the bone marrow of mice (n = 7-8 mice/group) treated with vehicle control, 1.5mg/kg romidepsin (ROM), 5mg/kg cytarabine (AraC), or combination of 1.5mg/kg ROM and 5mg/kg AraC for three weeks. Mice were sacrificed once they reached a humane end-point. Error bars represented mean ± SD.



Supplementary Figure 3. Romidepsin increases the intracellular levels of phospho-H2A.X when combined with cytarabine. Leukemia cell lines were treated with 2nM romidepsin (ROM), 1μM cytarabine (AraC), or combination of 2nM ROM and 1μM AraC for 24 hours. Representative flow cytometric data plots depicting the levels of intracellular phospho-H2A.X of the leukemia cell lines.



Supplementary Figure 4. Expression of DNA-damage signaling pathway modulators following treatment of leukemia cells with romidepsin and/or cytarabine. Leukemia cell line PER-490 was treated with 2nM romidepsin (ROM), 1μM cytarabine (AraC), or combination of 2nM ROM and 1μM AraC for 24 hours. (A) Western blot analysis of p-CHK2, p-ATR, p-ATM, p-CHK1, and p-P53 protein levels in the treated cell line. (B) Bar graphs depict densitometry ratios that were normalized to ratios calculated for non-treated controls. Data were collected from three independent experiments and analyzed using the two-tailed unpaired Student's t-test. Error bars represented mean ± SD. * $P < 0.05$.



Supplementary Figure 5. Expression of DNA repair proteins following treatment of leukemia cells with romidepsin and/or cytarabine. Leukemia cell line PER-490 was treated with 2nM romidepsin (ROM), 1μM cytarabine (AraC), or combination of 2nM ROM and 1μM AraC for 24 hours. (A) Western blot analysis of acetyl-Ku70 and p-DNA-PKcs protein levels in the treated cell lines. (B) Bar graphs depict densitometry ratios that were normalized to ratios calculated for non-treated controls. Data were collected from three independent experiments. Error bars represented mean ± SD.