Hypomethylating agent decitabine sensitizes diffuse large B-cell lymphoma to venetoclax

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Supplemental Information

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Inventory of Supplemental Information

Supplemental Methods

BH3 profiling. BH3 profiling was performed by flow cytometry, as described previously[1]. Briefly, DLBCL cells were exposed to 0.002% digitonin and BH3-only peptides for 60 min in MEB2 buffer (150 mM Mannitol, 10 mM HEPES-KOH pH 7.5, 150mM KCl, 1mM EGTA, 1mM EDTA, 0.1% BSA, 5mM Succinate, Polaxamer188 2.5g/L). After formaldehyde fixation and neutralization, fluorescent antibodies Alexa Fluor 488 anti-cytochrome c (Biolegend 612310, San Diego, CA, USA), and Hoechst 33364 (Invitrogen H3570, Waltham, MA, USA) were added. Cells were incubated with antibodies for at least 24 hours before being analyzed by flow cytometry.

Western blotting. Cells were lysed in CelLytic Cell Lysis Reagent (Sigma C2978, Burlington, MA, USA). The following antibodies were used: BCL-2 (Biolegend, 658702), MCL-1 (Cell Signaling Technology (CST), 94296, Danvers, MA, USA), BFL-1 (Abcam, ab45413, Waltham, MA, USA), BCL-xL (CST, 2764), BIM (CST, 2933), BAK (BD Biosciences, 556382, NJ, USA), BAX (Proteintech,50599-2-Ig, Rosemont, IL, USA), PUMA (CST, 12450), c-Myc (CST, 5605), phospho-GSK3β (CST, 9336), GSK3β (CST, 9832), total OXPHOS rodent WB antibody cocktail (Abcam, ab110413), DNMT1 (CST, 5032), γH2AX (CST, 2577), β-actin (CST, 5125), GAPDH (Proteintech, HRP-60004), SMAD1 (CST, 6944), phospho-SMAD1 (Abcam, ab214423). After blotting with the HRP-conjugated secondary antibody, the membrane was developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, 34580, Waltham, MA, USA). Position of protein marker (Bio-Rad, 1610375, Hercules, CA, USA) was indicated in the figure. Image J was used to quantify protein bands[2].

Drug treatments. Cells were treated with decitabine (Sigma, A3656), venetoclax (Selleckchem, S8048, Houston, TX, USA), S63845 (Selleckchem, S8383), A1331852 (Selleckchem, S7801),

LY2109761 (Selleckchem, S2704) and doxorubicin (Sigma, D1515) when needed. Combination indexes for drug treatment were calculated by CompuSyn (ComboSyn Inc, Paramus, NJ, USA) using the Chou-Talalay method[3].

Cell cycle analysis. Cell cycle was analyzed with BD Pharmingen BrdU Flow Kits (552598). Briefly, cells were pulsed with BrdU for 3 hours, followed by fixation and permeabilization procedures. Then, the cells were stained with APC-conjugated anti-BrdU antibody, followed by 7-AAD staining for the total amount of DNA. Cells were then analyzed by flow cytometry. DNA contents were analyzed using FlowJo software (FlowJo, LLC).

Apoptosis. Cell apoptosis was measured using Propidium Iodide (PI) (invitrogen, P1304MP), and Alexa Fluor 488 anti-Annexin V (Invitrogen, A13201) staining following manufacturer's protocol. Cells were then analyzed by flow cytometry.

Cell viability assay. Cell viability was measured using CellTiter-Glo 2.0 Assay (Promega, G9242, Madison, WI, USA) following manufacturer's instructions. Luminescence signal was read using SpectraMax M3.

SMAD1 knockdown. Short hairpin shRNA in the pLKO.1 lentiviral vector targeting SMAD1 was purchased from Horizon Discovery (RHS3979-201752806, Cambridge, UK).

Immunoprecipitation. 20 million DLBCL cells were lysed with 1ml of CHAPS lysis buffer (40mM Hepes, pH 7.5, 120mM NaCl, 1mM EDTA, 0.3% CHAPS). CHAPS-containing lysates were incubated with 5μg of BAX antibody (Proteintech, 50599-2) or IgG isotype control (CST, 2729) overnight before adding Dynabeads Protein G (Invitrogen, 10003D) for another 3 hours. After washing the beads with lysis buffer, bound proteins were analyzed by western blotting.

BAX and BAK activity. To assess the activity of BAX and BAK, cells were fixed and permeabilized using BD Fixation/Permeabilization Kit (554714). Then cells were stained with conformation specific mouse-anti BAX (invitrogen, MA5-14003), mouse-anti BAK (BD Biosciences, 556382), or mouse IgG1 isotype control (Invitrogen, MA1-10407). Staining was visualized by adding Alexa Fluor 488 anti-mouse IgG (Invitrogen, A-11029) and flow cytometry.

Oxygen consumption rate. Oxygen consumption rate was measured by Seahorse bioscience extracellular flux XFe96 using Agilent (Santa Clara, CA, USA) Seahorse XF Cell Mito Stress Test Kit (103015-100). 1μM of oligomycin, 1μM of Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), 0.5μM of rotenone/antimycin A were used for OCR measurements.

RNA-seq analysis. Total RNA was extracted using GeneJET RNA Purification Kit (ThermoFisher Scientific, K0731) according to the manufacturer's protocol. DNBSEQ stranded mRNA library was prepared and sequenced using service from BGI Genomics. For RNA-seq analysis, mapping, alignment, and differential expression were analyzed on the Galaxy public server (usegalaxy.org)[4]. Briefly, raw reads were aligned to the human reference genome (UCSC hg38) with HISAT2. Transcripts/genes were assembled with StringTie. Differential expressions were analyzed with DESeq2. GSEA was performed locally by GSEA software (V4.0.3) (http://software.broadinstitute.org/gsea/index.jsp). For the GSEA analysis, molecular signatures databases h.all.v5.2 symbols.gmt was used. Additional 17 gene sets of mitochondrial functions, biogenesis, and electron transport chain were downloaded from the molecular signatures databases (available upon request). RNA-sequencing data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession number GSE223598.

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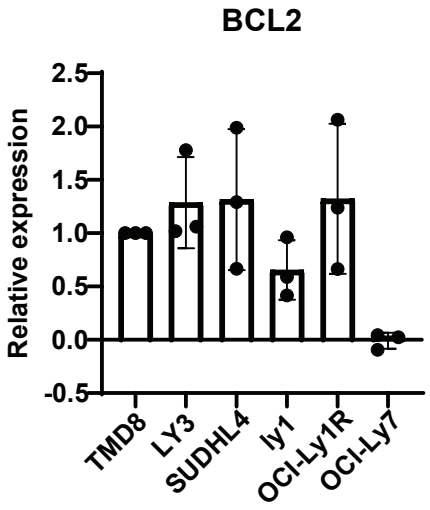


Figure S1. Quantification of BCL2 expression level in DLBCL cell lines. Experiments were performed as indicated in figure 1A. Image J was used to quantify protein band intensity. In each experiment, BCL2 level was normalized to GAPDH loading control first, then normalized to BCL2 expression in TMD8 cell line. Data were presented as mean ± SD, n=3.

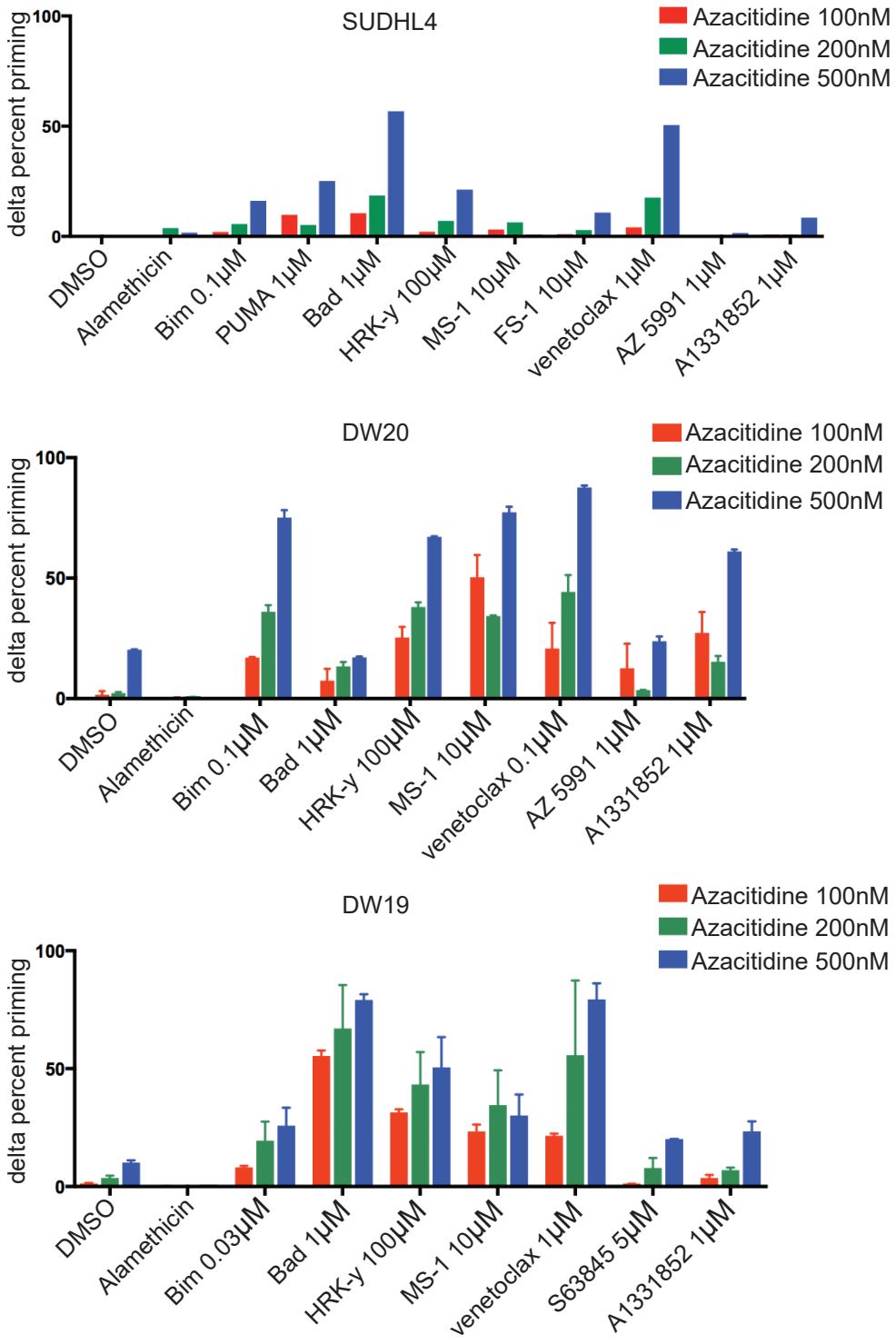
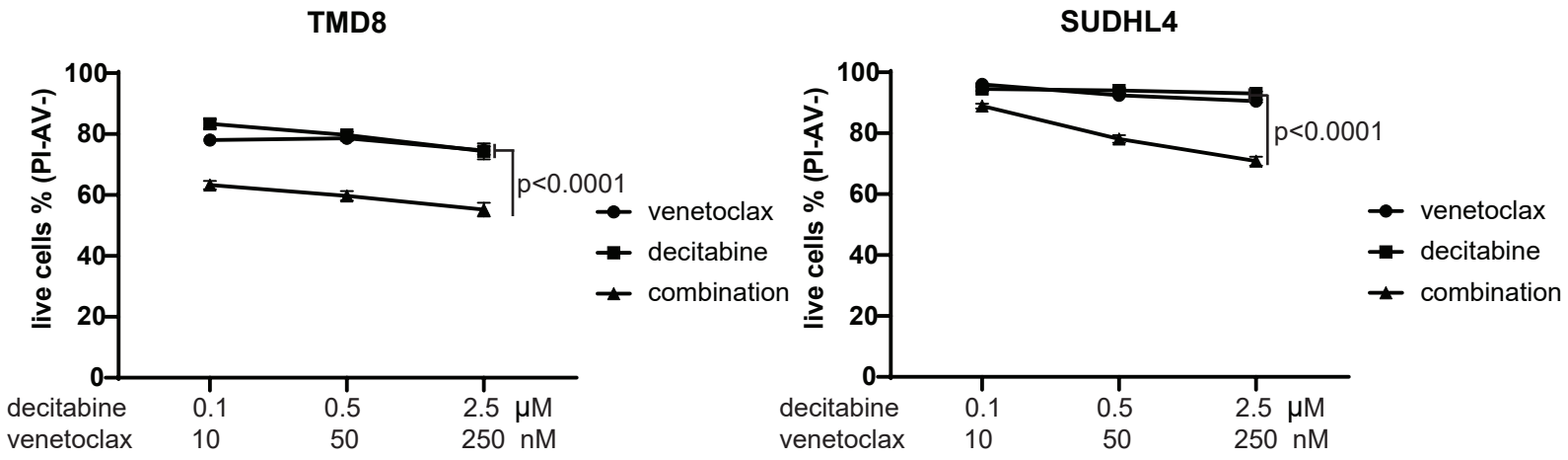


Figure S2. DBP of DLBCL cells treated with DMSO control or azacitidine at indicated dosages for 18 hours. Data were presented as delta percent priming.

A



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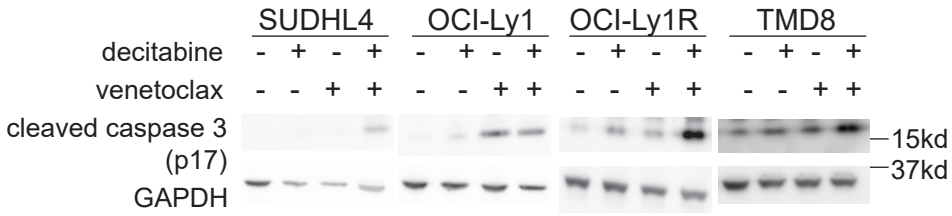


Figure S3. Combination of decitabine and venetoclax enhances apoptosis of DLBCL cells. (A) TMD8 and SUDHL4 cells were treated with indicated dosages of decitabine for 3 days, and venetoclax was added on the last day. Cell viability was determined by PI/Annexin V assay. Data were presented as mean ± SD, n=4. P value was calculated by two-way ANOVA. (B) DLBCL cell lines were treated with 0.5μM of decitabine for 3 days and/or 50nM of venetoclax for 1 day (10nM of venetoclax for OCI-Ly1 cells), and cells were lysed for western blotting analysis of cleaved caspase 3. GAPDH was used as loading control.

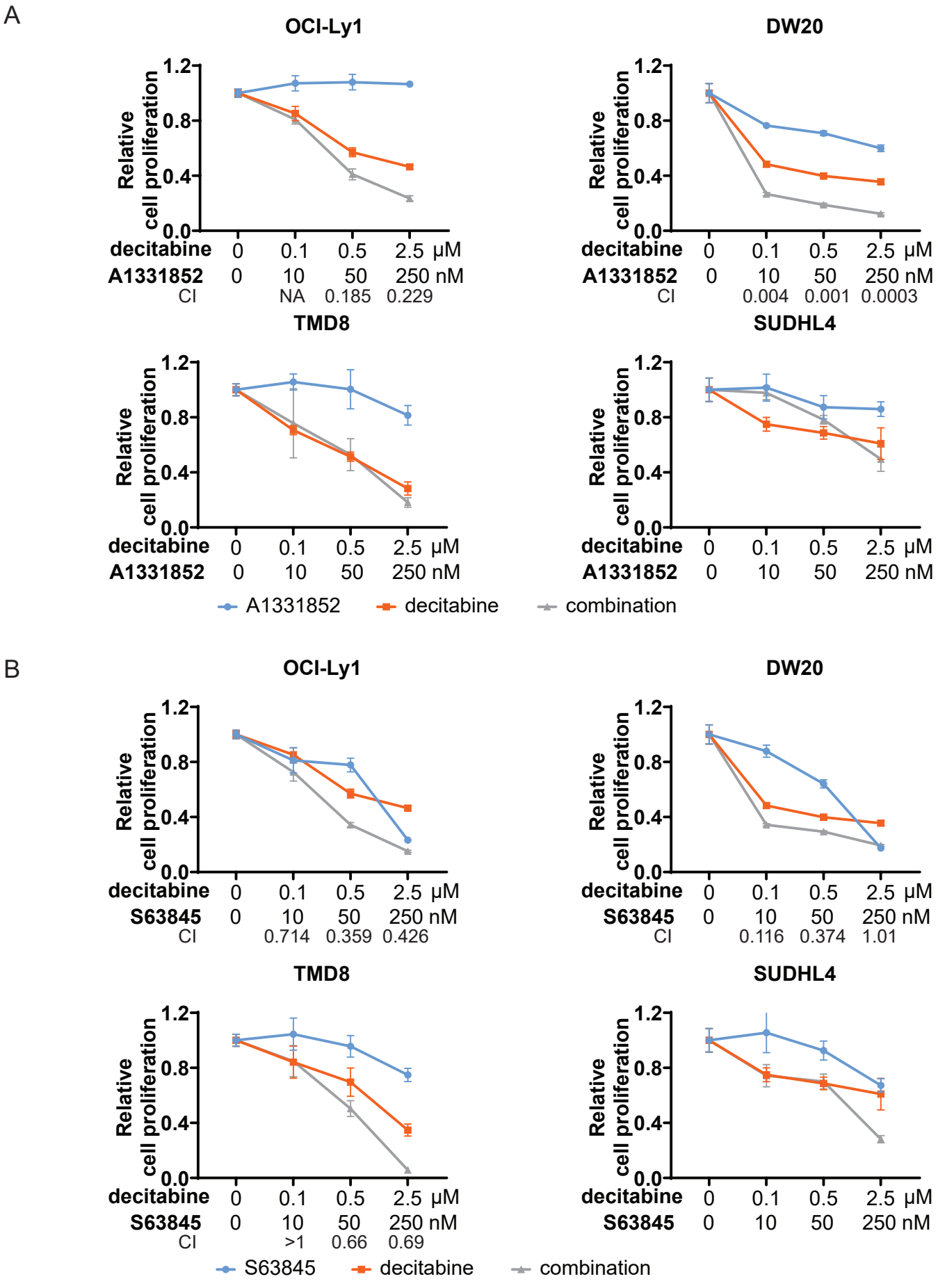


Figure S4. Combination of decitabine and A1331852 (A) or S63845 (B) in DLBCLcells. DLBCL cell lines were treated with indicated dosages of decitabine for 3 days, and A1331852 or S63845 was added on the last day. Cell proliferation was determined by CellTiter-Glo assay. Data were normalized to DMSO treated cells. Data were presented as mean ± SD, n=3. Combination index (CI) was calculated by Compusyn.

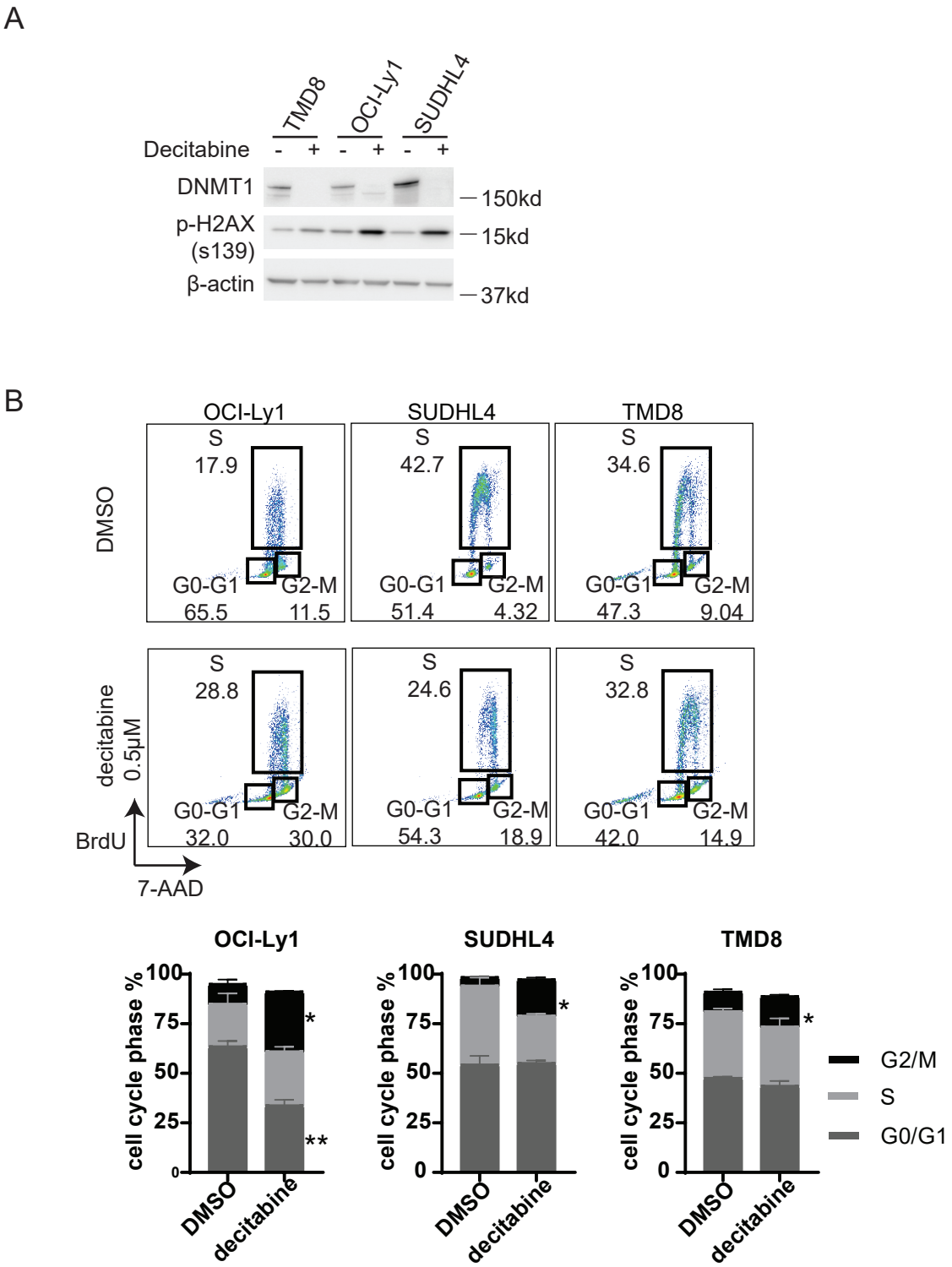
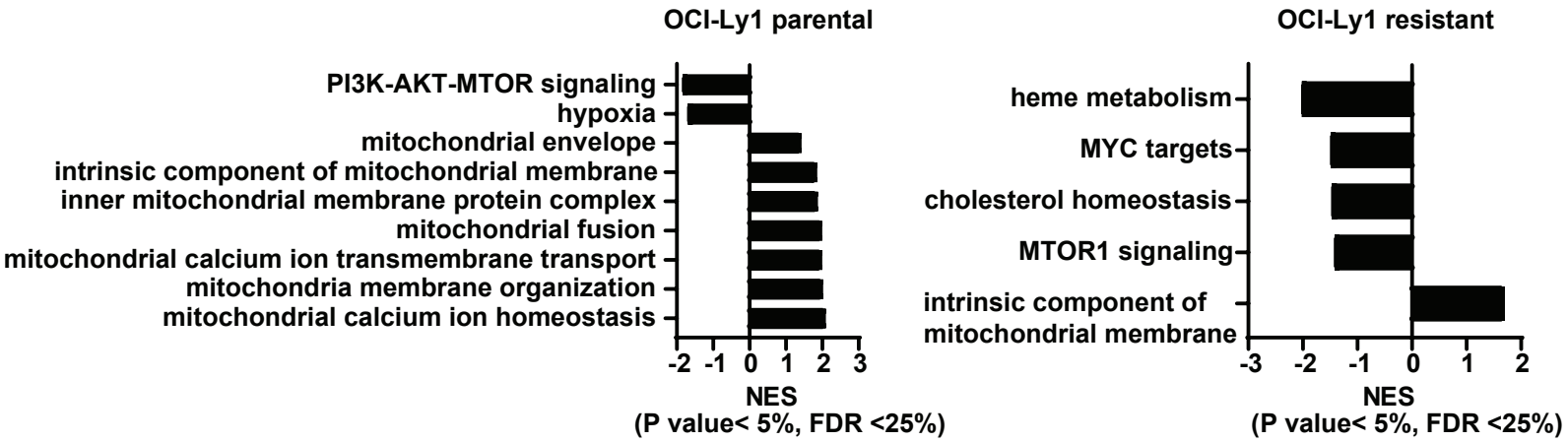


Figure S5. Decitabine induces DNA damage and leads to cell cycle arrest in DLBCL cells. (A) DLBCL cells were treated with DMSO or 0.5μM of decitabine for 3 days, and lysed for western blotting analysis of DNMT1 and phosphorylation of H2AX. β-actin was used as loading control. (B) Cell cycle analysis of DLBCL cells treated with DMSO or 0.5μM of decitabine for 3 days. Data were presented as mean ± SD, n=2. P value was calculated by student's T-test, * $p<0.05$, ** $p<0.01$.

A



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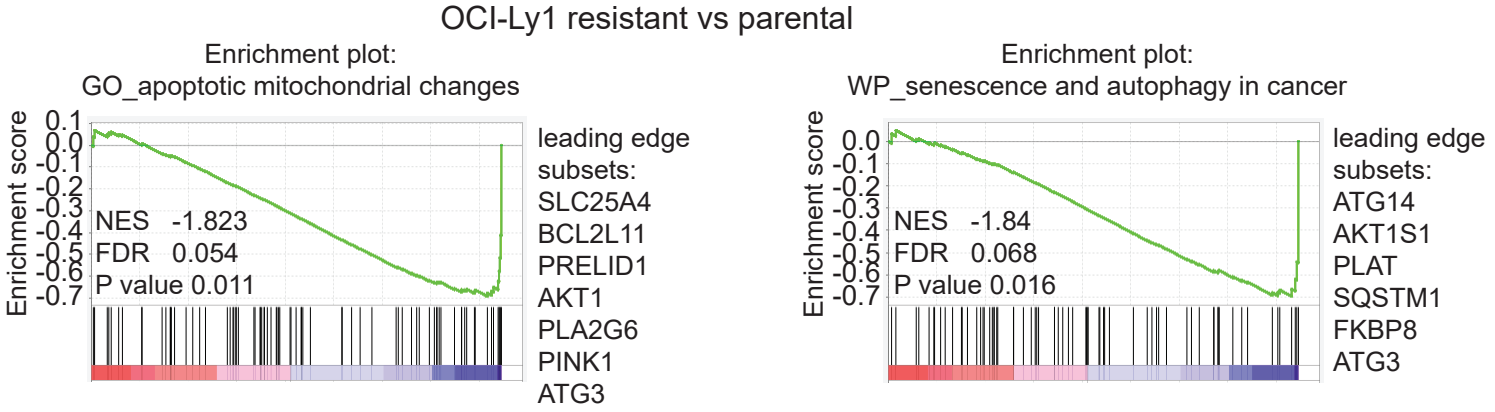

Figure S6. Decitabine regulates gene expression in DLBCL cells. (A) Significantly upregulated and down-regulated gene signatures revealed by GSEA in venetoclax sensitive (OCI-Ly1 parental) and resistant (OCI-Ly1R) cells treated with 1μM of decitabine for 3 days. Normalized enrichment score (NES) was calculated for each gene set and shown in the figure. (B) Significantly downregulated gene signatures in OCI-Ly1R cells compared to OCI-Ly1 parental cells.

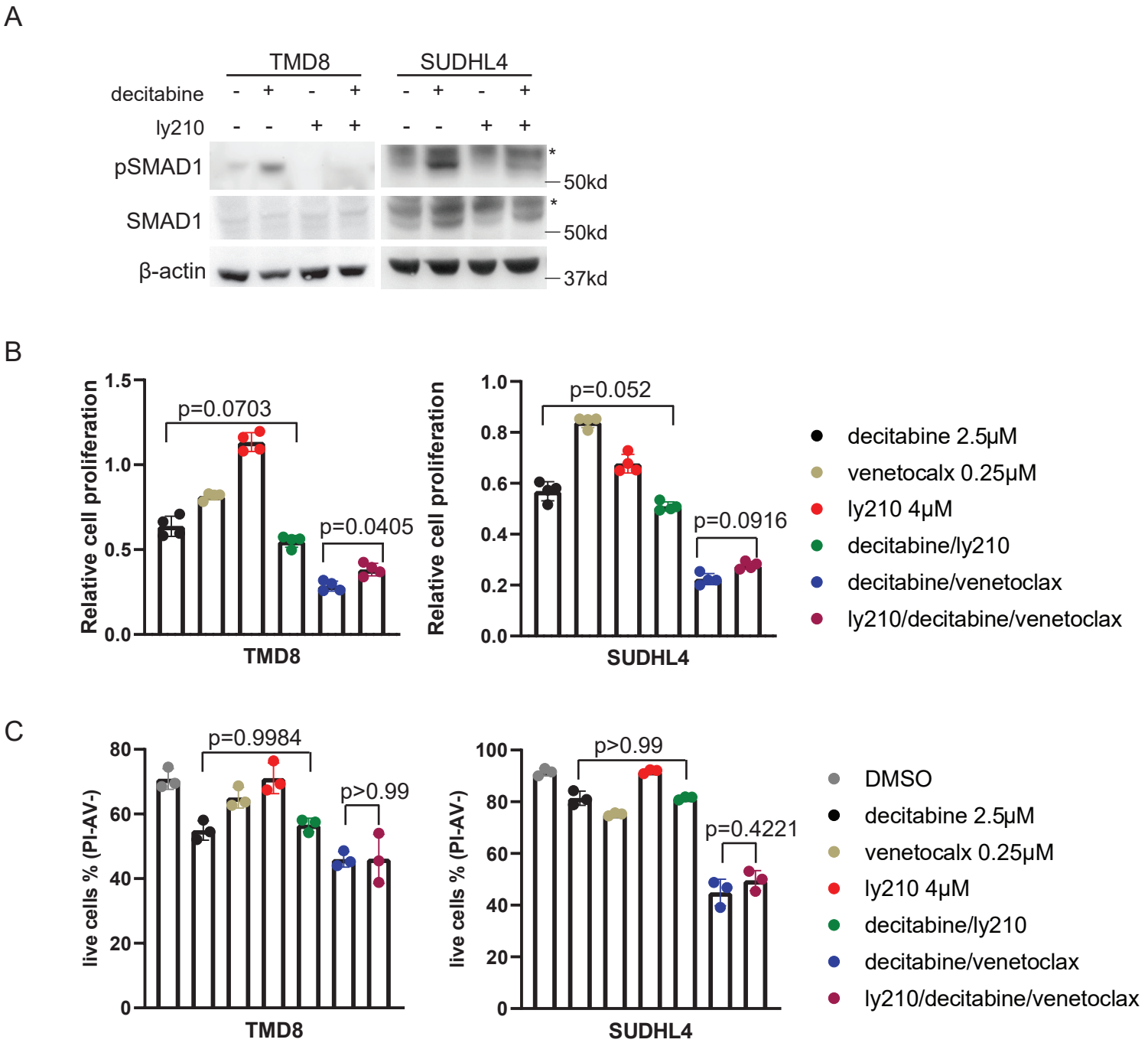

Figure S7. Inhibition of TGF-β signaling partially blocks the synergism between decitabine and venetoclax. (A) DLBCL cell lines TMD8 and SUDHL4 were treated with 2.5μM of decitabine, 4μM of TGF-β receptor type I/II (TβRI/II) dual inhibitor, LY2109761 (ly210), alone or in combination for 3 days, phospho-SMDA1 (s463/s465) and total SMAD1 were measured by western blotting. * non-specific band. β-actin was used as loading control. (B-C) DLBCL cell lines were treated with indicated drugs alone or in combination. Decitabine and ly210 were added to cells for 3 days, venetoclax was added on the last day. Cell proliferation was determined by CellTiter-Glo assay. Data were normalized to DMSO treated cells. Data were presented as mean ± SD, n=4 (B). Cell viability was determined by PI/Annexin V assay. Data were presented as mean ± SD, n=3 (C). P value was calculated by one-way ANOVA with multiple comparisons test.