Supplementary Materials and Methods

Transfection

Plasmids (neomycin resistance) ectopic expressing human MCL-1, c-MYC, BCL-X_L or empty vector as negative control (pcDNA3.1) were purchased from Qiagen, Germantown, MD. pcDNA3-CDK7 HA (P#633) was a gift from Matija Peterlin (Addgene plasmid # 14647; http://n2t.net/addgene:14647; RRID:Addgene_14647) (1). Cells were transfected using the Amaxa Nucleofector (Amaxa GmbH, Cologne, Germany) device with Cell Line Specific Nucleofector Kit C (Amaxa GmbH, Cologne, Germany) as per the manufacturer's instructions. Clones were selected with 400 μg/ml G418. Knockdown CDK7 plasmids (TRCN0000000592 as shRNA-1, and TRCN0000218042 as shRNA-2) were purchased from Sigma, which targets the CDK7 UTR. pLKO was used as a negative control. Lentivirus production was generated using Lipofectamine 3000 (Invitrogen, ThermoFisher Scientific, NJ) following the manufacturer's protocol. Puromycin was used to treat cells for 5 days for selection, which eliminated all cells in the uninfected control group. Cells were harvested 7 days after the initial viral infection and subjected to either western blotting to assess the knockdown efficiency or for clonogenic cell growth assays.

For CDK7 rescue studies, OPM2 cells were infected with lentivirus encoding shRNA targeting the CDK7 UTR. After infection and selection with puromycin (0.2 μg/ml, 2 days), cells were transfected using the Amaxa Nucleofector (Amaxa GmbH, Cologne, Germany) device with Cell Line Specific Nucleofector Kit C, following by G418 selection for 5 days, and then cells were collected for monitoring the expression of HA tag, CDK7, c-MYC, MCL-1 and BCL-X_L by immunoblotting. α-Tubulin or β-actin was assayed to ensure equivalent loading and transfer.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analysis using TaqMan gene expression assays and a 7900HT real-time PCR system (Applied Biosystems, Foster City, CA) was performed to quantify mRNA levels. Briefly, total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was synthesized from 1 ug of total RNA by using a High Capacity cDNA reverse transcription kit (Applied Biosystems). One microliters of cDNA was employed for qPCR assays (TaqMan gene expression assays).

Assay identification numbers for MCL-1 were Hs03043899_m1;

Assay identification numbers for c-MYC were Hs00153408_m1;

Assay identification numbers for BCL-X_L were Hs00236329_m1;

Assay identification numbers for TP53BP1 were Hs00996818_m1;

Assay identification numbers for BRCA1 were Hs01556193_m1;

Assay identification numbers for RAD51 were Hs00947967_m1;

References for quantitation were human β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems). Data were analyzed by using SDS 2.3 software.

Immunoblot analysis

Samples were prepared from whole-cell pellets. Total protein was quantified using Coomassie Protein Assay Reagent (Pierce ThermoFisher Scientific, Rockford, IL). Equal amounts of protein (30 μg) were separated by SDS-PAGE and electro-transferred onto nitrocellulose membrane. Where indicated, the blots were probed or re-probed with antibodies against β-actin or α-tubulin (EMD/Millipore/Sigma, Billerica, MA) to ensure equal loading and transfer of proteins. The following primary antibodies were used: MCL-1 (BD Biosciences, San Jose, CA); CDK1/2, p-CDK2(T160); p-CDK1(T161); BCL-X_L, cleaved caspase 3 (Asp175), cleaved PARP (Asp214), c-MYC, and p-CDK9 (T186) (Cell Signaling, Beverly, MA); RNA Polymerase II (EMD/Millipore/Sigma, Billerica, MA), and RNA Polymerase II (RNA Polymerase II (H5) (the phosphoserine 5 form of pol II), and RNA Polymerase II (H14) (the phosphoserine 2 form of pol II) (BioLegend, San Diego, CA); human BCL-2 oncoprotein (DAKO, Carpinteria, CA); PARP (Enzo, Plymouth Meeting, PA); CDK7 and CDK9 (Santa Cruz Biotech, Dallas, TX).

Immunofluorescence

Cytospin slides were fixed in 4% paraformaldehyde for 1 h, permeabilized in 0.25% Triton X-100 in PBS, and blocked with PBS containing 1% BSA and 2% FBS. For immunofluorescent staining of cleaved-caspase-3 (Cell Signaling), secondary Alexa Fluor 488 was employed. Slides were mounted using DAPI (4', 6-diamidino-2-phenylindole) Fluoromount-G (Southern Biotech, Birmingham, AL). Images were captured using an Olympus IX71 Inverted System Microscope.

Chromatin Immunoprecipitation Assay

ChIP was performed using a SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) (Cell signaling, Tokyo, Japan, #9002). U266 were cross-linked in 1% formaldehyde, and blocked with glycine. Cells were further washed and digested by micrococcal nuclease. The nuclear pellet was suspended in the chromatin immunoprecipitation (ChIP) buffer and sheared by sonication. The sheared, cross-linked, chromatin was incubated at 4°C overnight with H3K27Ac (1:100 Cell Signaling, 8173S) and control normal Rabbit IgG (1:100 Cell Signaling 2729S). This was later suspended in ChIP-Grade Protein G Agarose beads (Cell Signaling, 9007) and incubated at 4°C for 2 hours. The immunoprecipitated chromatins were then eluted with ChIP elution buffer and the DNA fragments were released by treating with ribonuclease A and then proteinase K at 65°C for 2 hours. The DNA fragments were then purified with columns and amplified by site-specific primers, by quantitative reverse transcriptase polymerase chain reaction (qRTPCR) using SYBR green reagent (Cell Signaling, 88989S).

Isolation of primary MM cells and cord blood

Bone marrow (BM) samples were obtained with informed consent according to the Declaration of Helsinki and Virginia Commonwealth University IRB approval from four patients with MM undergoing routine diagnostic aspirations. $CD138^+$ cells were separated using a MACS magnetic separation technique (Miltenyi Biotech, San Diego, CA) as per the manufacturer's instructions. The purity (> 90%) of CD138+ and viability (> 95%) were determined by flow cytometry and trypan blue exclusion, respectively. Normal $CD34^+$ hematopoietic progenitor cells were isolated from cord blood (CB) samples similarly. Isolated cells were cultured in RPMI1640 medium containing 10% FBS.

Co-culture of MM cells with stromal cells

The human stromal cell line HS-5 was obtained from ATCC. HS-5 cells were maintained in RPMI1640 medium containing 10% FBS and sub-cultured twice weekly by trypsinization at a subcultivation ratio of 1:5-1:6. For co-culture experiments, HS-5 cells were cultured for 6-8 h prior to seeding MM cells.

Analysis of cell death

Apoptosis was evaluated by flow cytometry utilizing Annexin V-FITC/PI or 7-AAD staining as previously reported (2).

For CD138-/CD19+/CD20+/CD27+ analysis, CD138- mononuclear cells isolated from primary MM bone marrows were blocked by TruStain FcX on ice for 10min, stained with C19-PE/Cy7, CD20-APC/Cy7 and/or CD27-APC (Biolegend, San Diego, CA) on ice for 30min followed by staining with Annexin V- FITC at room temperature for 15min. The percentage of apoptotic (Annexin V+) cells in the $CD138^{-}/CD19^{+}/CD20^{+}/27^{+}$ population was then determined using a FACSCanto flow cytometer (BD Biosciences).

Supplementary Figure legends

Supplementary Table 1. Comparison of THZ1 IC50 values between MM cell lines. H929, OPM2, 8226/s, 8266/V10R, 8226/R10R, U266, and KMS28-BM/PE were exposed to the indicated concentrations of THZ1 for 24 hr. Followed by flow cytometric analysis of cell death after staining with 7-AAD.

Supplementary Fig S1. THZ1 induces G2/M arrest and inhibits CDK7-mediated phosphorylation of CDK1/2 Cell cycle distribution of U266 (A and B) and OPM2 (C) following THZ1 treatment for 24 hr was determined by flow cytometry. (D) The expression of CDK1, p-CDK1 (T161), CDK2 and p-CDK2 (T160) were detected by Western blotting both in U266 and PS-R cells after THZ1 treatment for 16 hr. β-actin was assayed to ensure equivalent loading and transfer.

Supplementary Fig S2. THZ1 downregulates c-MYC, MCL-1 and BCL-X_L expression at transcriptional level. (A) H929 cells were exposed to the indicated concentrations of THZ1 for 24 hr, after which c-MYC, MCL-1 and BCL-X_L were monitored by immunoblotting analysis. β-actin was assayed to ensure equivalent loading. (B) H929 cells were collected at 3, 6, and 24 hr after THZ1 treatment. Expression of cleavage PARP, BCL-XL, c-MYC, and MCL-1 were monitored by immunoblotting analysis. α-Tubulin was assayed to ensure equivalent loading and transfer. (C) H929 cell were treated with or without Z-VAD for 6 hr, after which c-MYC, MCL-1, BCL-X_L and cleaved PARP and Caspase-3 were monitored by immunoblotting analysis. α-Tubulin was assayed to ensure equivalent loading. (D) Expression of c-MYC, MCL-1 and BCL-

X_L were analyzed by real-time RT-PCR, and results are presented relative to GAPDH expression in the un-treatment control group.

Supplementary Fig S3. THZ1 downregulates DDR genes expression. (A) U266 cells were treated with indicated THZ1 for 24 hr, after which CtIP, FANCD2, RAD51, BRAC1, ERCC1 and CDT1 proteins were monitored by immunoblotting analysis. β-actin was assayed to ensure equivalent loading and transfer. (B) U266 and H929 were exposed for 1, 3, or 5 hours to THZ1 (200 nM), after which RNA was extracted from the cells. Expression of RAD51, BRAC1, and 53BP1 were analyzed by real-time RT-PCR, and results are presented relative to GAPDH expression in the un-treatment control group.

Supplementary Fig S4. Co-culture with HS-5 cells fails to protect MM cells from THZ1 (A and B) U266 or PS-R cells stably expression luciferase were co-cultured with or without HS-5 cells (pre-cultured for 24 hr), and then exposed to indicated concentrations of THZ1 for 24 hr. Relative luciferase activity was determined to reflect cell viability. (C) GFP-expressing U266 or PS-R cells were co-cultured with or without HS-5 cells, after which cells were treated with THZ1 for 24 hr. Cells were then stained with 7AAD and images were captured by an fluorescence microscope (Olympus 1X71, 20× objective).

Supplementary Fig S5. (A) H929 cells were infected with lentivirus encoding Cas9 and sgRNA targeting GFP or CDK7. After infection and selection with puromycin (0.2 μg/ml, 5 days), cells were collected for monitoring the expression of CDK7 by immunoblotting. Cell viability was monitored by Cell titer-Glo assay at day 2, 4, 6 and 8. , **P<0.01. (B) PS-R cells were incubated with THZ1 ± Btz, Cfz, or ABT-199 for 24 hr, after which cell death was analyzed by flow cytometry after staining with 7-AAD. *P<0.05, **P<0.01. (C) H929 cells were incubated with THZ1 ± Btz at a fixed ratio (1: 10) for 24 hr, after which the percentage of Annexin V cells was determined. Combination Index (CI) values less than 1.0 denote a synergistic interaction. The results are representative of three separate experiments.

Supplementary Fig S6. OPM2 cells (A) were infected with lentivirus encoding Cas9 and sgRNA targeting GFP or CDK7. After infection and selection with puromycin (0.2 μg/ml, 5 days), cells were collected for monitoring the expression of CDK7, c-MYC, MCL-1, BCL-X_L by immunoblotting. The results are representative of three separate experiments. (B) For CDK7 rescue studies, OPM2 were infected with lentivirus encoding shRNA targeting the CDK7 UTR. After infection and selection with puromycin (0.2 μg/ml, 2 days), cells were transfected using the Amaxa Nucleofector (Amaxa GmbH, Cologne, Germany) device with Cell Line Specific Nucleofector Kit C, following by G418 selection for 5 days, and cells were collected for monitoring the expression of HA tag, CDK7, c-MYC, MCL-1 and BCL-X_L by immunoblotting. (C) Cells were transfected using the Amaxa Nucleofector (Amaxa GmbH, Cologne, Germany) device with Cell Line Specific Nucleofector Kit C, following by G418 selection for 5 days, and then treated with THZ1 (200, 500nM) for 6 hr and collected for monitoring the expression of HA tag, CDK7, c-MYC, MCL-1 and BCL-X_L by immunoblotting. α-Tubulin or β-actin was assayed to ensure equivalent loading and transfer.

Supplementary Fig S7. H3K27ac q-ChIP (IgG was used as control) on MCL-1, c-MYC, BCL-X_L genes in U266 cells at baseline (vehicle, blue columns) and after 6 h exposure to THZ1 100 nM and 200 nM treatment (orange and grey columns). Data are presented as mean with standard error of the mean (SEM) for two independent replicates performed in triplicate. P-values were obtained using a T-test.

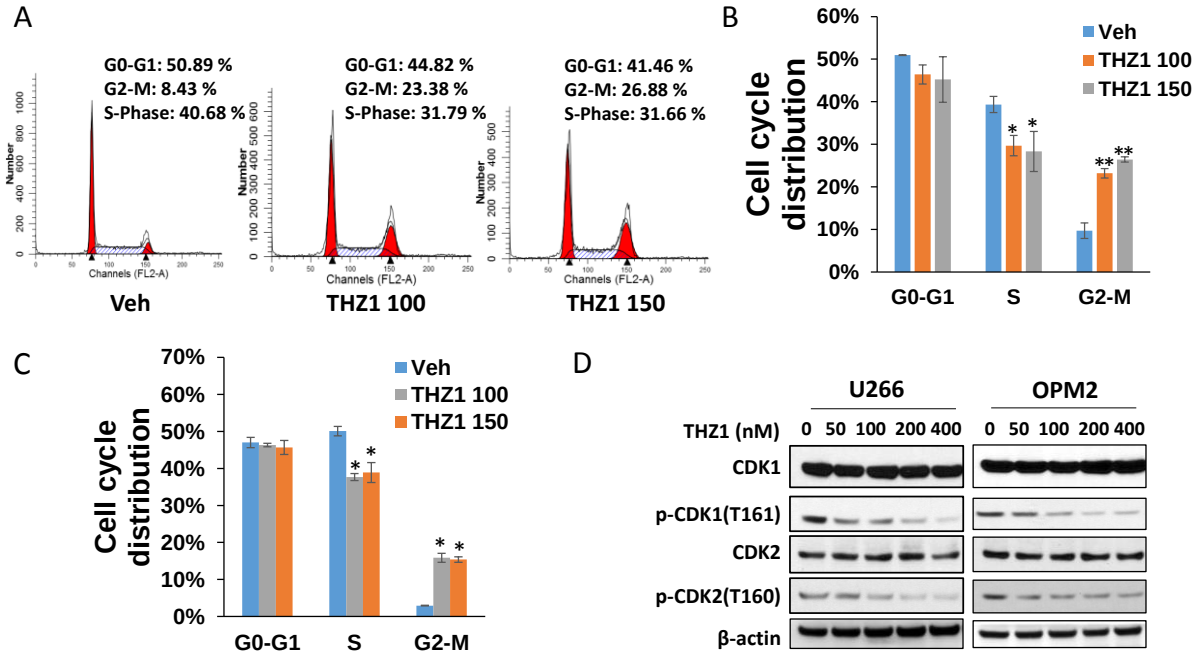
1. Kanazawa S, Soucek L, Evan G, Okamoto T, Peterlin BM. c-Myc recruits P-TEFb for transcription, cellular proliferation and apoptosis. *Oncogene* **2003**;22:5707-11.
2. Dai Y, Grant S. Methods to study cancer therapeutic drugs that target cell cycle checkpoints. *Methods Mol Biol* **2011**;782:257-304.

Supplemental Table 1

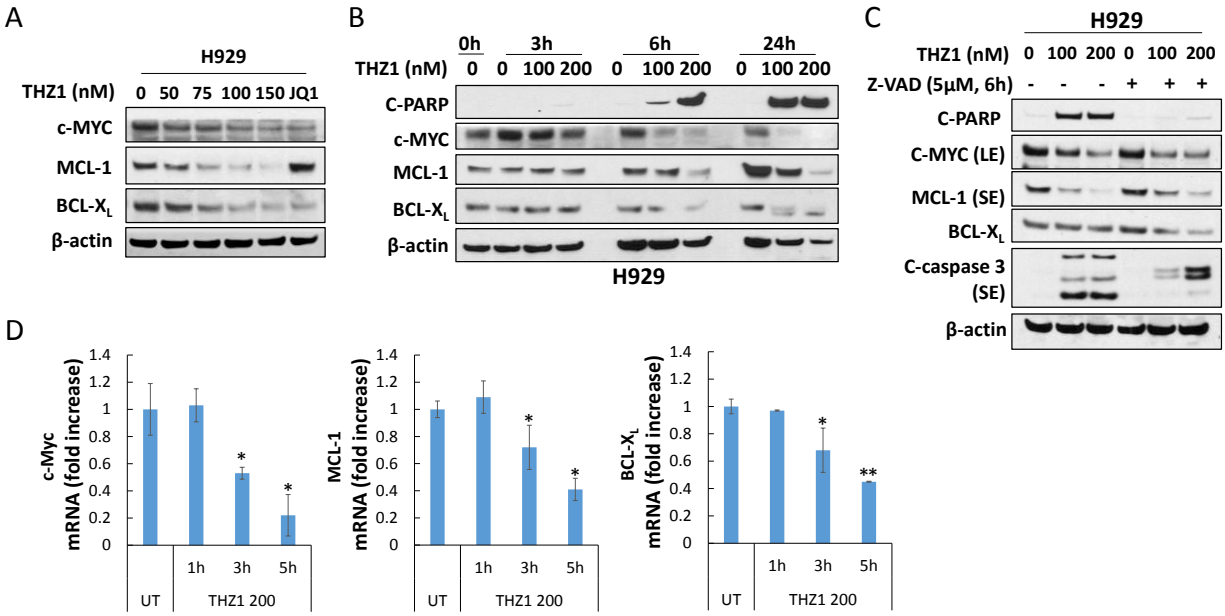
Cells treated with the indicated concentrations of THZ1 were assayed for apoptosis (7-AAD) at 24 hr. IC50 values were calculated using GraphPad Prism 5 software. IC50 values for THZ1 for each cell line represent the means for three independent experiments (n = 3, Mean ± S.D.)

Cell lines	**IC50 for THZ1 (nM) 24 hr**
H929	57.95 ± 1.05
OPM2	111.84 ± 4.43
8226/s	97.61 ± 2.62
U266	262.80 ± 20.19
KAS 6/1	12.41 ± 0.87
KMS28-PE	37.72 ± 1.15
KMS28-BM	43.76 ± 1.82

Supplemental Figure 1



Supplemental Figure 2



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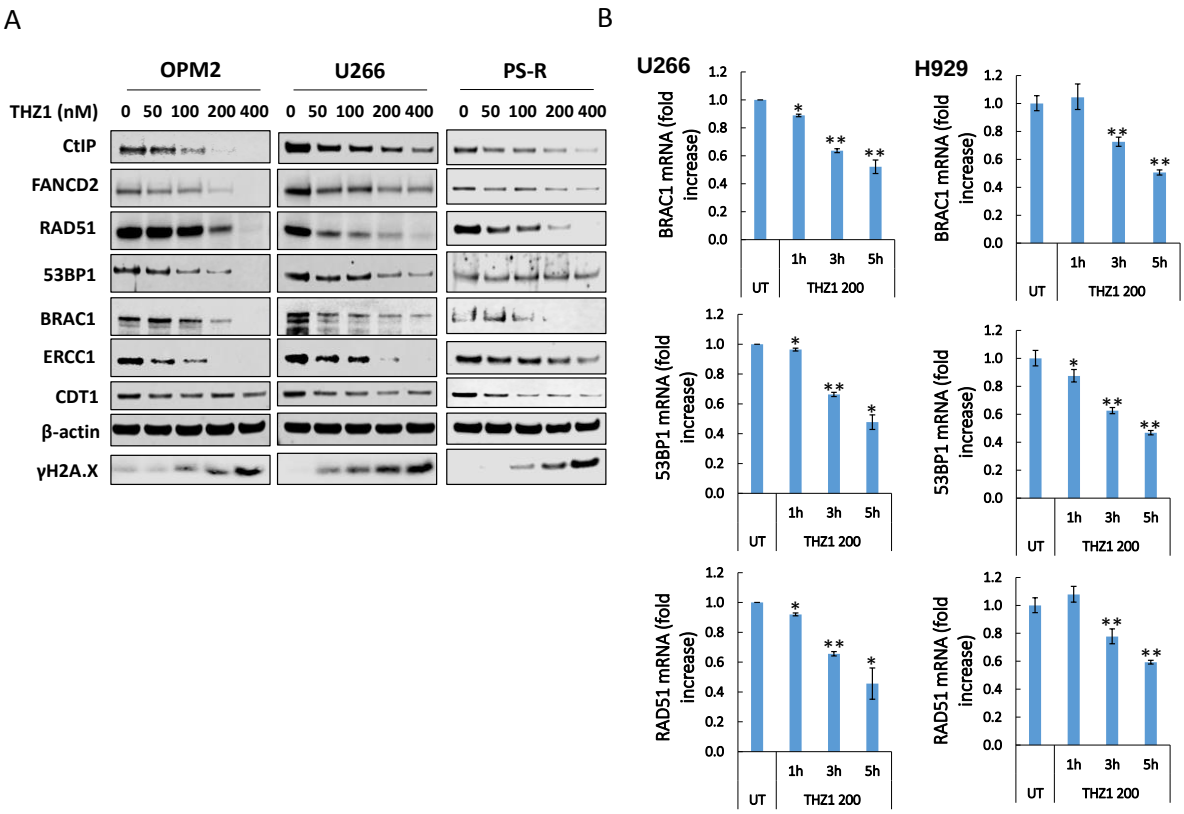

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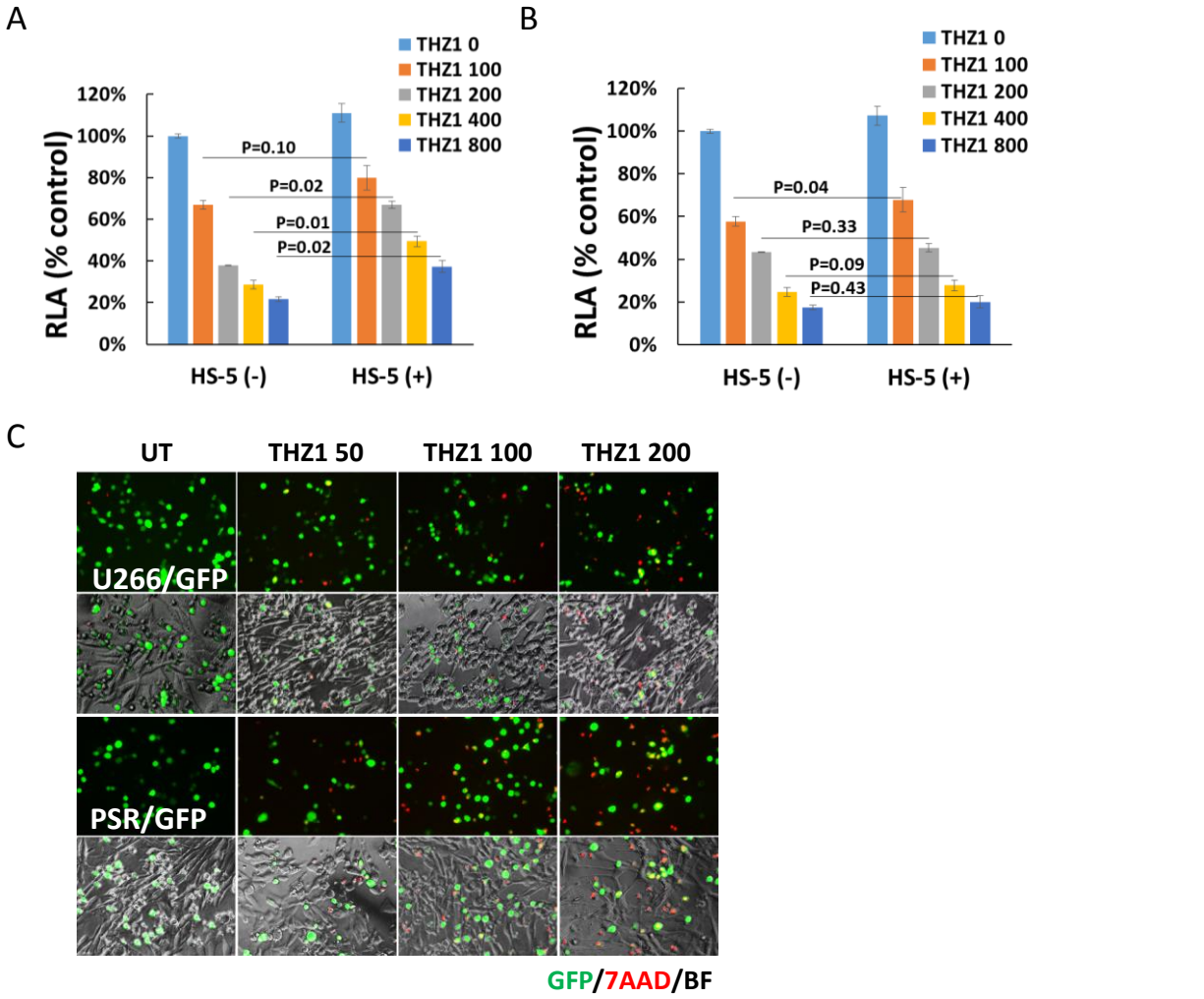

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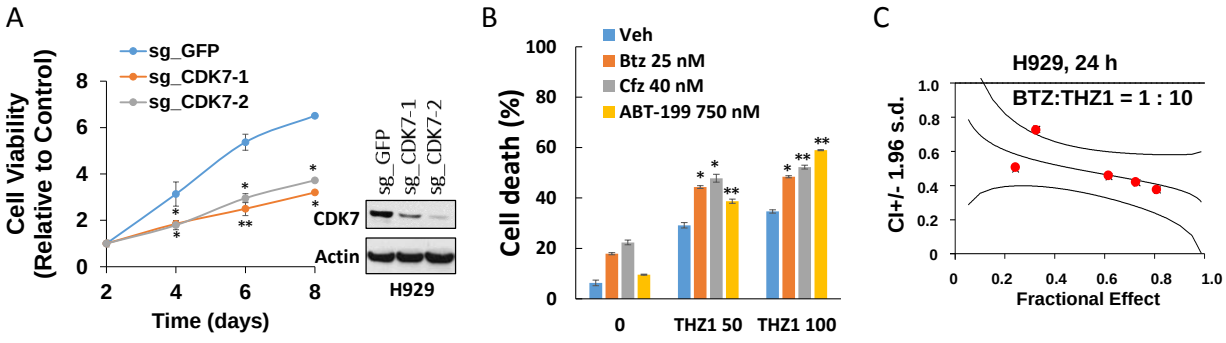
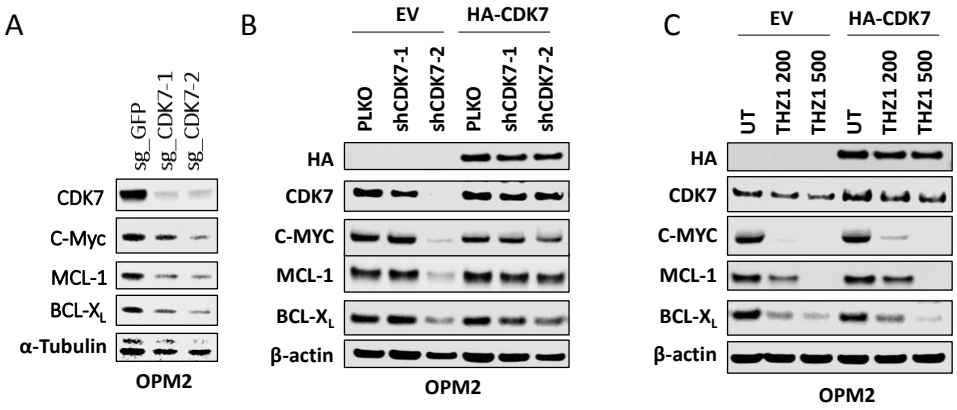


Supplemental Figure 6



Supplemental Figure 7