Supplementary Methods

Reagents

Bortezomib, carfilzomib, lenalidomide, panobinostat, melphalan, and doxorubicin were from Selleck Chemical (Houston, TX). Drug stock solutions were prepared in dimethyl-sulfoxide (Fisher Scientific; Pittsburgh, PA) and stored at -20°C. All cell culture reagents and TRIzol® Reagent (Total RNA Isolation reagent) were from Invitrogen Corp. (Carlsbad, CA).

Myeloma cell lines and primary samples

MM1.S, NCI-H929, U266 and RPMI 8226 cells were from the American Type Culture Collection (Manassas, VA), while ANBL-6 and KAS-6/1 myeloma cells were a kind gift of Dr. Diane Jelinek (Mayo Clinic; Rochester, MN). Cells were grown in RPMI 1640 (Life Technologies; Carlsbad, CA) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich; St. Louis, MO) with supplementation using interleukin-6 for ANBL-6 and KAS-6/1 cells. Cell lines were validated by the MD Anderson Characterized Cell Line Core Facility using short tandem repeat DNA fingerprinting with the AmpFlSTR kit (Applied Biosystems; Foster City, CA). Primary plasma cells were collected under a protocol approved by the MD Anderson Institutional Review Board after informed consent was obtained in compliance with the Declaration of Helsinki. These were purified from bone marrow aspirates using anti-CD138 antibody magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Inc.; San Diego, CA).

Real Time RT-PCR

Total RNA was isolated using TRIzol® (Thermo Fisher Scientific; Grand Island, NY) and Direct-zol™ RNA MiniPrep Kit (Zymo Research; Irvine, CA). Then, cDNAs were synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). qPCR

was performed using the TaqMan Gene Expression Master Mix and probes purchased from Applied Biosystems on an Applied Biosystems StepOnePlus Real-Time PCR system. Syber Green PCR was performed only for XBP1 spliced and unspliced fragments, and BiP. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blotting

Protein concentrations were determined using the DC Assay Kit (Bio-Rad; Hercules, CA). A total of 40 μg boiled protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane. The primary antibodies used included: anti-BiP, anti-PERK, anti-MCL1, and anti-p21 from Cell Signaling; anti-phospho-IRE1α, anti-NRF2, anti-ATF6, anti-c-MYC from abcam; anti-HSP70 from Enzo Life Sciences, Inc. (Farmingdale, NY), anti-ubiquitin, anti-XBP1 and anti-TP53 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-MDM2 from Millipore Sigma (Burlington, MA), and anti-β-Actin (A1978) from Sigma-Aldrich as a loading control.

Tumor xenografts

For *in vivo* studies, 6-13 week old female CB-17 Severe combined immunodeficient (SCID) mice (Charles River Laboratories; Wilmington, MA) were inoculated subcutaneously in the flanks with MM1.S (5.0×10^6 cells/animal) or MOLP-8 cells (2.0×10^6 cells/animal) in RPMI 1640 media with Matrigel (BD Biosciences). Tumor growth was monitored with vernier calipers, and mean tumor volume was calculated using the formula $V=0.5\times(\text{length}\times\text{width}^2)$. When the mean tumor volume reached ~150-200 mm^3, the animals were randomized into groups (n=6 for MM1.S, n=7 for MOLP-8), and dosed intravenously twice weekly (BIW) with vehicle (5% or 10% 2-hydroxypropyl-β-cyclodextrin (HPbCD)) or TAK-243 formulated in 10% HPbCD. Tumor size

and body weights were measured twice weekly, and tumor growth inhibition ([mean tumor volume (MTV) of control group - MTV of treated group]/MTV of the control) was calculated on day 14, when the vehicle group was removed due to mean tumor volume reaching ~2000 mm^3.

For the pharmacodynamic study in MM1.S cells, when tumors reached 400-600 mm^3, a single IV dose of TAK-243 (25 mg/kg) or vehicle (10% HPbCD) was administered. Tumors were harvested at the indicated times and divided, with a portion placed in 10% neutral buffered formalin for immunohistochemistry, and another portion snap-frozen and stored at -80°. Protein lysates for Western blot were prepared from frozen tumors using a Covaris (Woburn, MA) sonicator with RIPA buffer, and 20 μg of protein was separated and blotted. Primary antibodies were used at 1:1000 dilution for Noxa (MilliporeSigma; Burlington, MA), cleaved caspase-3 and cleaved PARP (Cell Signaling Technology; Danvers, MA) and at 1:5000 for tubulin (AbCam).

Immunohistochemistry was performed on 5 micron paraffin embedded tumor sections. The Discovery® XT automated staining system (Ventana Medical Systems; Tucson, AZ) was used to stain tumor sections for ATF4 (Cell Signaling Technology, 1:750) and XBP1s (Cell Signaling Technology, 1:7000) utilizing Amp HQ reagents (Ventana Medical Systems). Tumor sections were stained with BIP (Cell Signaling Technology, 1:100) using Bond RX and detected with Leica BOND™ Polymer Refine Detection kit (both from Leica Biosystems; Buffalo Grove, IL). After staining, whole slide images were captured using an Aperio Scanscope® XT (Aperio ePathology Solutions; Vista, CA). Quantitative analysis was done using Tissue Studio® image analysis software (Definiens; Cambridge, MA). Data are represented as the percentage of ATF4- or XBP1s-positive nuclei relative to the total number of nuclei in the viable tumor region, and the BIP data as a percentage of BIP positive area within the viable tumor area. All animal studies were conducted under the approval of the Millennium Pharmaceuticals, Inc. Institutional

Animal Care and Use Committee. Statistical analyses were performed with unpaired t tests in GraphPad, and p-values less than 0.05 were judged to be significant.

Supplementary Tables

Supplementary Table 1. Reverse phase protein array analysis of proteins enhanced in MM1.S cells exposed to TAK-243 at 25 nM for 24 hours

Enhanced	proteins
over 100%	Caspase-3, Caspase-7-cleaved, c-Myc, Mcl-1, TFRC, MDM2, PLK1
50%-100%	Connexin, Cyclin-B1, Cyclin-D3, DUSP4, H2AX, HSP70, NDRG1, P27, P38, Chk1
20%-50%	SLC1A5, PDCD4, JUN, BCL2L11, IGFBP2, KAT2A, HIF1A, PTPN11, TP53, CDKN1A, SOD2, BIRC3, EEF2, HIST3H3, IRF1, BRD4, GSK3A, GSK3B, RPS6K, TUFM, CCNE1, BAP1, ATRX, TYRO3, CDK1, ARID1A

Supplementary Table 2. Median inhibitory concentration values of TAK-243 in drug-naïve and –resistant cell lines*

	IC_{50} (nM)	Wild-type vs Resistant Cells (p value)
ANBL-6	86 ± 1.06	
ANBL-6/C10R	26.3 ± 0.11	<0.0001
ANBL-6/V10R	64 ± 0.38	<0.0001
KAS-6/1	74 ± 0.10	
KAS-6/1/C10R	45.1 ± 0.34	0.005
KAS-6/1/V10R	30.1 ± 0.32	0.001
KAS-6/1/R10R	23.3 ± 3.10	0.0001
U266	44 ± 0.14	
U266/C10R	16.5 ± 0.49	0.04
U266/R10R	10.8 ± 0.97	0.001
RPMI 8226	1750 ± 1.5	
RPMI 8226/DOX40	1500 ± 1.21	0.04
RPMI 8226/LR5	1050 ± 3.11	0.002
MM.1S	24 ± 1.1	
MM.1R	20 ± 1.3	0.5
MM1.S/R10R	35 ± 0.57	0.034

*Abbreviations: C10R, carfilzomib resistant; DOX40, doxorubicin resistant; LR5, melphalan resistant; MM1.R, MM1.S cells resistant to dexamethasone; R10R, lenalidomide resistant; V10R, bortezomib resistant

Supplementary Table 3. Combination index values (CI) for TAK-243 with either bortezomib or carfilzomib in wild-type (WT) myeloma cell lines, or in bortezomib-resistant (V10R) or carfilzomib-resistant (C10R) cells

			WT Cells	V10R Cells
Combination Dosage	TAK-243 (nM)	Bortezomib (nM)	CI with Bortezomib	CI with Bortezomib
ANBL-6				
1	25	2.5	2.19	1.45
2	50	5.0	1.84	1.14
3	100	10.0	3.19	1.22
KAS-6/1				
1	25	2.5	1.04	1.19
2	50	5.0	1.80	1.14
3	100	10.0	1.85	1.66
RPMI 8226				
1	25	2.5	1.70	2.40
2	50	5.0	2.20	3.15
3	100	10.0	2.53	2.97
			WT Cells	C10R Cells
Combination Dosage	TAK-243 (nM)	Carfilzomib (nM)	CI with Carfilzomib	CI with Carfilzomib
ANBL-6				
1	25	2.5	0.79	0.75
2	50	5.0	2.14	1.14
3	100	10.0	1.19	1.13
KAS-6/1				
1	25	2.5	1.80	2.30
2	50	5.0	1.53	2.22
3	100	10.0	1.90	1.06
U266				
1	25	2.5	1.50	2.35
2	50	5.0	1.20	3.05
3	100	10.0	2.53	2.87

Supplementary Table 4. Combination index values (CI) for TAK-243 with lenalidomide in wild-type (WT) myeloma cell lines, or in lenalidomide-resistant (R10R) cells

			WT Cells	R10R Cells
Combination Dosage	TAK-243 (nM)	Lenalidomide (nM)	CI with Lenalidomide	CI with Lenalidomide
KAS-6/1				
1	25	625	0.20	0.058
2	50	1,250	0.24	0.023
3	100	2,500	0.31	0.019
U266				
1	25	625	2.71	3.44
2	50	1,250	4.34	5.50
3	100	2,500	5.74	1.50

Supplementary Figure Legends

Supplementary Figure 1. TAK-243 and other inhibitors targeting the UPP. Ubiquitin-dependent proteolysis through the ubiquitin-proteasome pathway (UPP) is illustrated, as are some of the agents that target the UPP. The E1 ubiquitin-activating enzyme forms a high-energy ubiquitin-E1 bond through the hydrolysis of ATP, and this is then transferred to an E2 conjugating enzyme. An E3 ubiquitin (Ub) ligase typically binds to the E2, and target proteins destined for degradation are then recognized. Energy stored in the Ub-E2 bond is used to transfer ubiquitin to a lysine in the target protein, and ubiquitin chains are formed on the target after several rounds of this reaction. These chains are recognized by the cap complex of the proteasome, ubiquitin is cleaved off and recycled, and the target protein is unwound and fed into the proteasome for degradation, producing oligopeptides. Please note that the various protein components are not drawn to scale, and only representative inhibitors and inducers of E1, E2, E3, and proteasome function are indicated.

Supplementary Figure 2. TAK-243 and apoptosis. (A) B-cell lymphoma (U2932 and HBL-1), pancreatic carcinoma (MIA PaCa-2 and PANC-1), breast carcinoma (MCF-7 and MDA-MB-231), melanoma (A375), ovarian carcinoma (ID8 and HEY A8), HS-5 stromal, and MCF 10A non-transformed breast epithelial cell lines were exposed to either vehicle (DMSO) or TAK-243 at the indicated concentrations. Viability was then determined after 72 hours using the tetrazolium reagent WST-1, and data representative of one of three independent experiments, each performed in triplicate, are presented, along with the calculated median inhibitory concentrations. (B) A representative profile obtained by fluorescence-activated cell sorting is shown of U266 cells exposed to vehicle for 24-hour, TAK-243, or bortezomib after staining with Annexin-V and TO-PRO-3. (C) U266 cells were exposed to either TAK-243 or bortezomib, and

extracts were then probed by Western blotting to determine the abundance of full-length and cleaved caspase 7 after a 24-hour drug exposure.

Supplementary Figure 3. E1/ShRNA cells are resistant to TAK-243. (A) MM1.S and U266 cell lines were prepared in which E1 knockdown was achieved using two distinct shRNAs compared to an shRNA control (shRNA/C), and reduced protein expression was confirmed by Western blotting with β-actin used as a loading control. (B) These cells were then exposed to either vehicle (DMSO) or TAK-243 at the indicated nanomolar concentrations, and viability was determined after 72 hours. Data are presented as mean ± SE, with asterisks indicating $p \leq 0.05$ compared to the controls. (C) The impact of TAK-243 on selected components of the ER stress pathway is shown by Western blotting of extracts from MM1.S and U266 myeloma cells, with bortezomib serving as a positive control.

Supplementary Figure 4. TAK-243 and protein abundance changes. Heat map of the reverse phase protein analysis data in MM1.S cells treated with TAK-243 at 25 nM for 24 hours. A red color indicates an increase in the abundance of the indicated protein, while green indicates a decrease. CTRL indicates cells treated with vehicle only.

Supplementary Figure 5. TAK-243 and U266 cells. Heat map of the reverse phase protein analysis data in U266 cells treated with TAK-243 at 200 nM for 24 hours. A red color indicates an increase in the abundance of the indicated protein, while green indicates a decrease. CTRL indicates cells treated with vehicle only.

Supplementary Figure 6. TAK-243 and U266 cells. Reverse phase protein array analysis was performed on U266 cells exposed to vehicle (Control) or TAK-243 (Treated). Expression

levels are shown for 45 selected proteins whose abundance was possibly enhanced at the left, while the right side shows 8 whose abundance was possibly decreased.

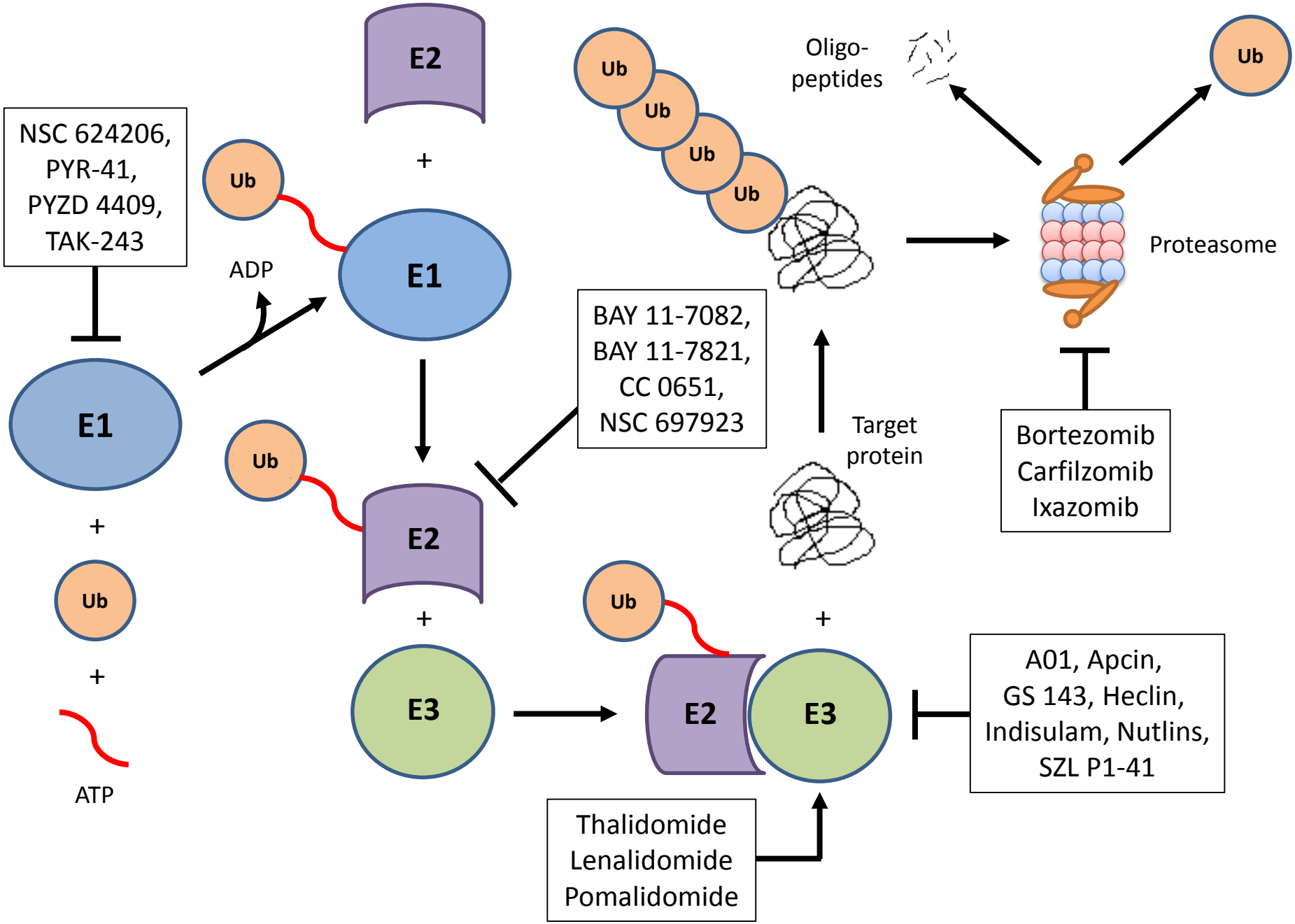
Supplementary Figure 7. TAK-243 and proteasome inhibitors. Wild-type, drug-naïve ANBL-6, KAS-6/1, and RPMI 8226 cells, and their bortezomib-resistant (V10R) or carfilzomib-resistant (C10R) counterparts were exposed to TAK-243, bortezomib (A) or carfilzomib (B), or to the combinations, and viability was evaluated after a 72-hour exposure. Statistical analyses showed no enhanced effect of the combinations compared with the single-agent treatments.

Supplementary Figure 8. TAK-243 and sensitivity to conventional drugs. TAK-243 was combined with either melphalan (MEL; left panel) or doxorubicin (DOX; right panel) at the indicated concentrations to determine if additive or synergistic interactions were seen during a 72-hour drug exposure.

Supplementary Figure 9. UAE inhibition and XBP1s abundance. A murine xenograft model based on MM1.S cells was generated and exposed to vehicle or a single dose of TAK-243 at 25 mg/kg. Immunohistochemistry was performed on paraffin embedded tumor sections obtained at the indicated time points as described in the Materials and Methods to detect expression of XBP1s.

Supplementary Figure 10. UAE inhibition and ATF4 abundance. A murine xenograft model based on MM1.S cells was generated and exposed to vehicle or a single dose of TAK-243 at 25 mg/kg. Immunohistochemistry was performed on paraffin embedded tumor sections obtained at the indicated time points as described in the Materials and Methods to detect expression of ATF4.

Supplementary Figure 1



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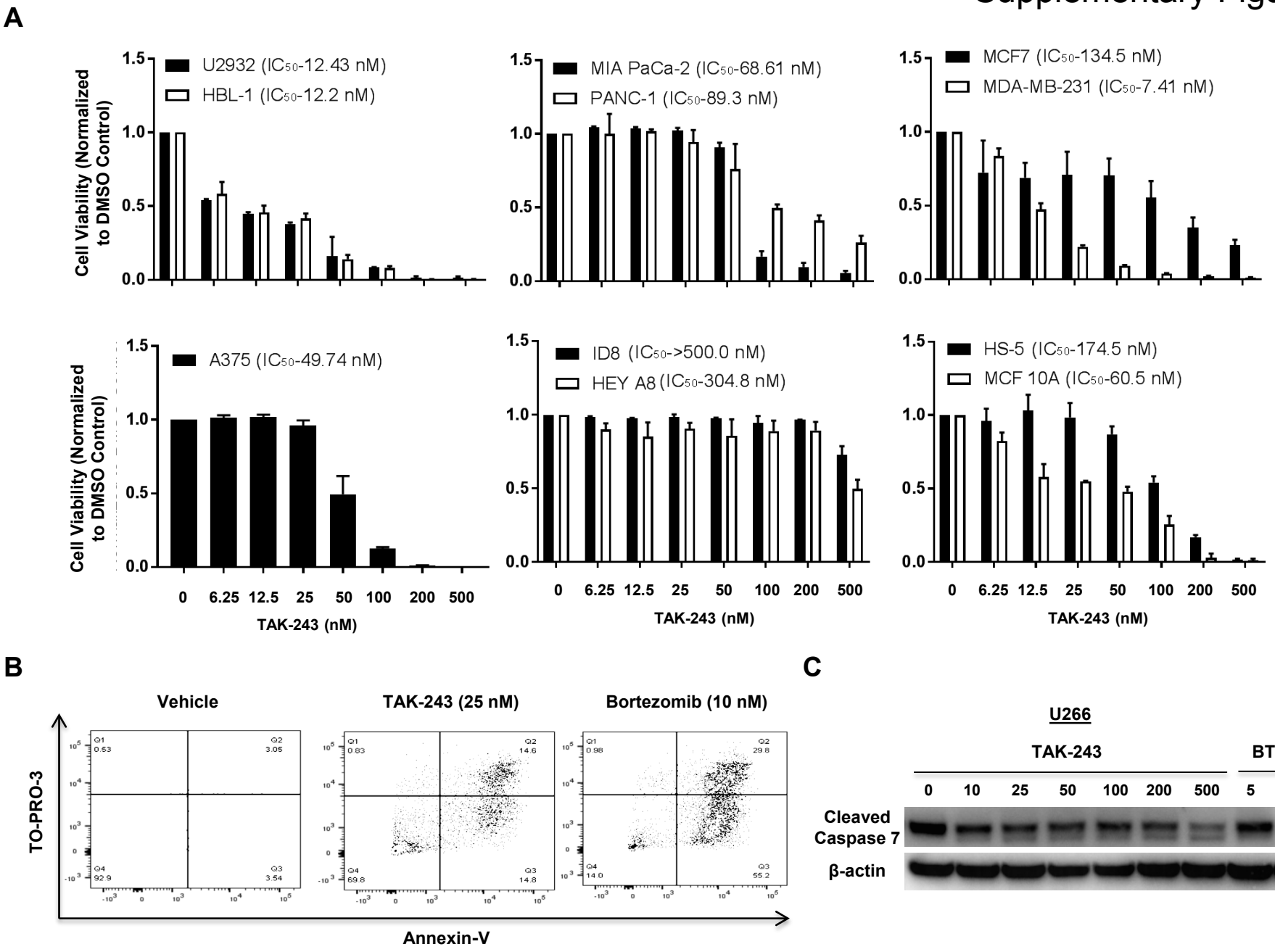

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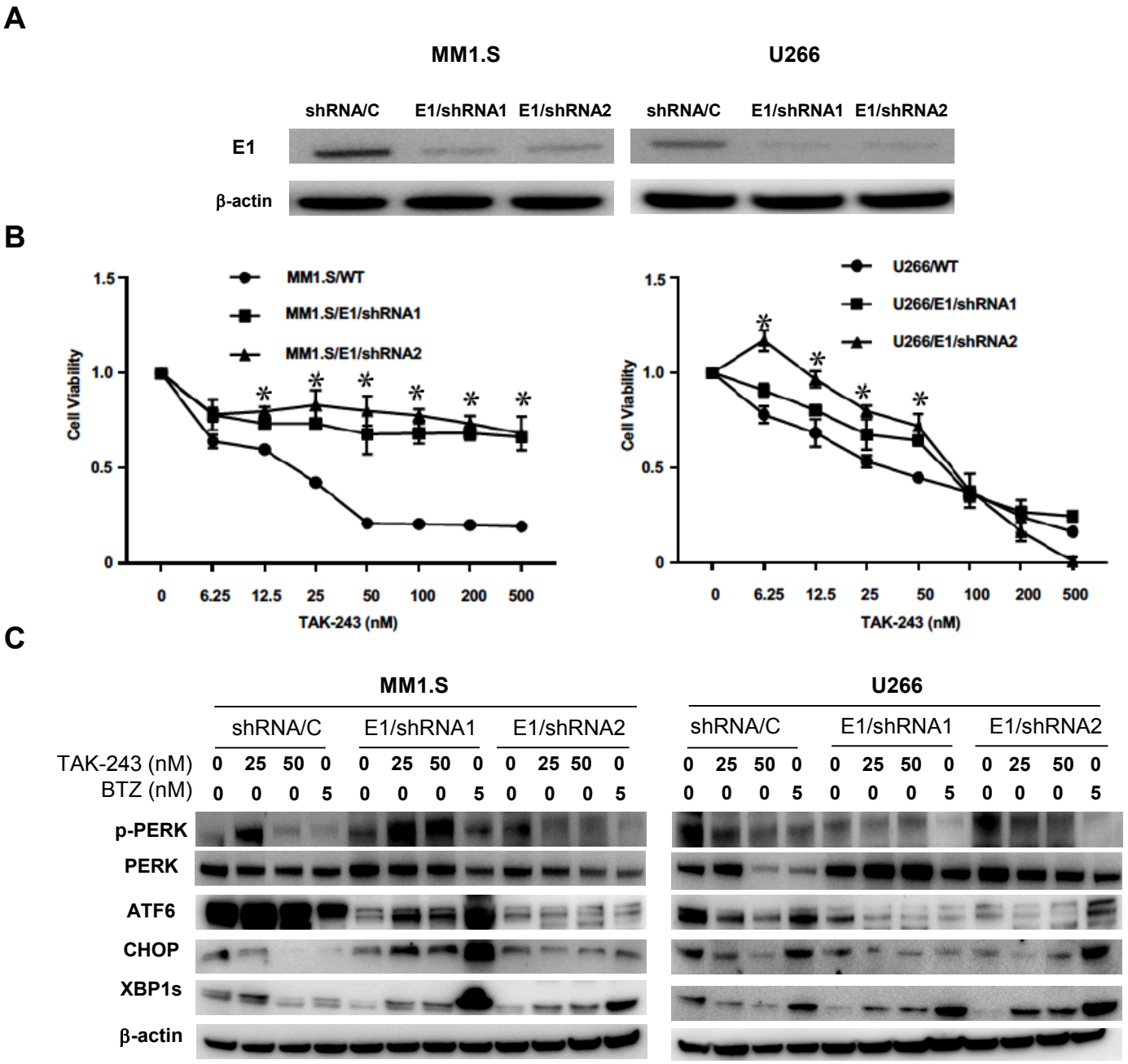

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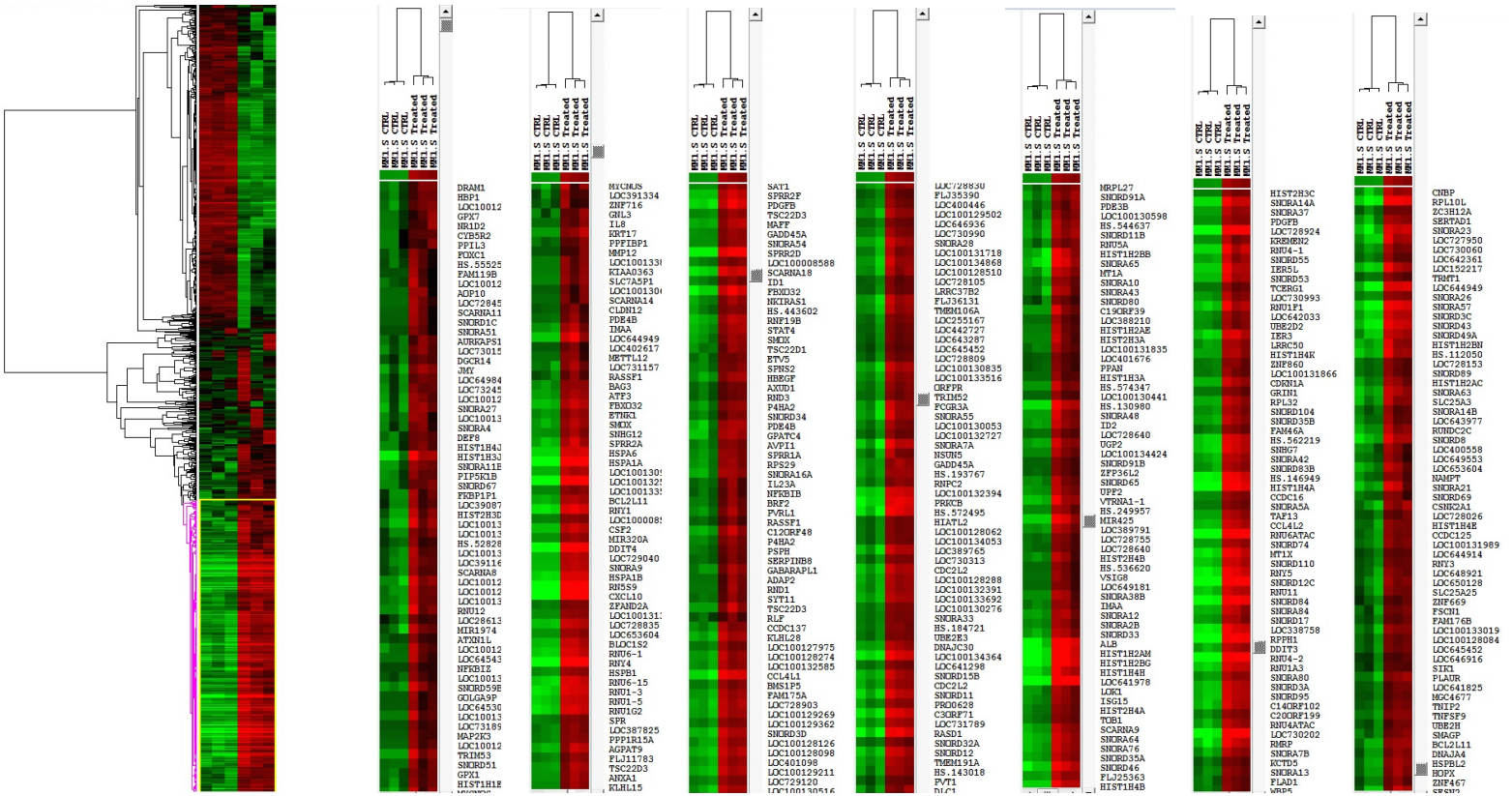

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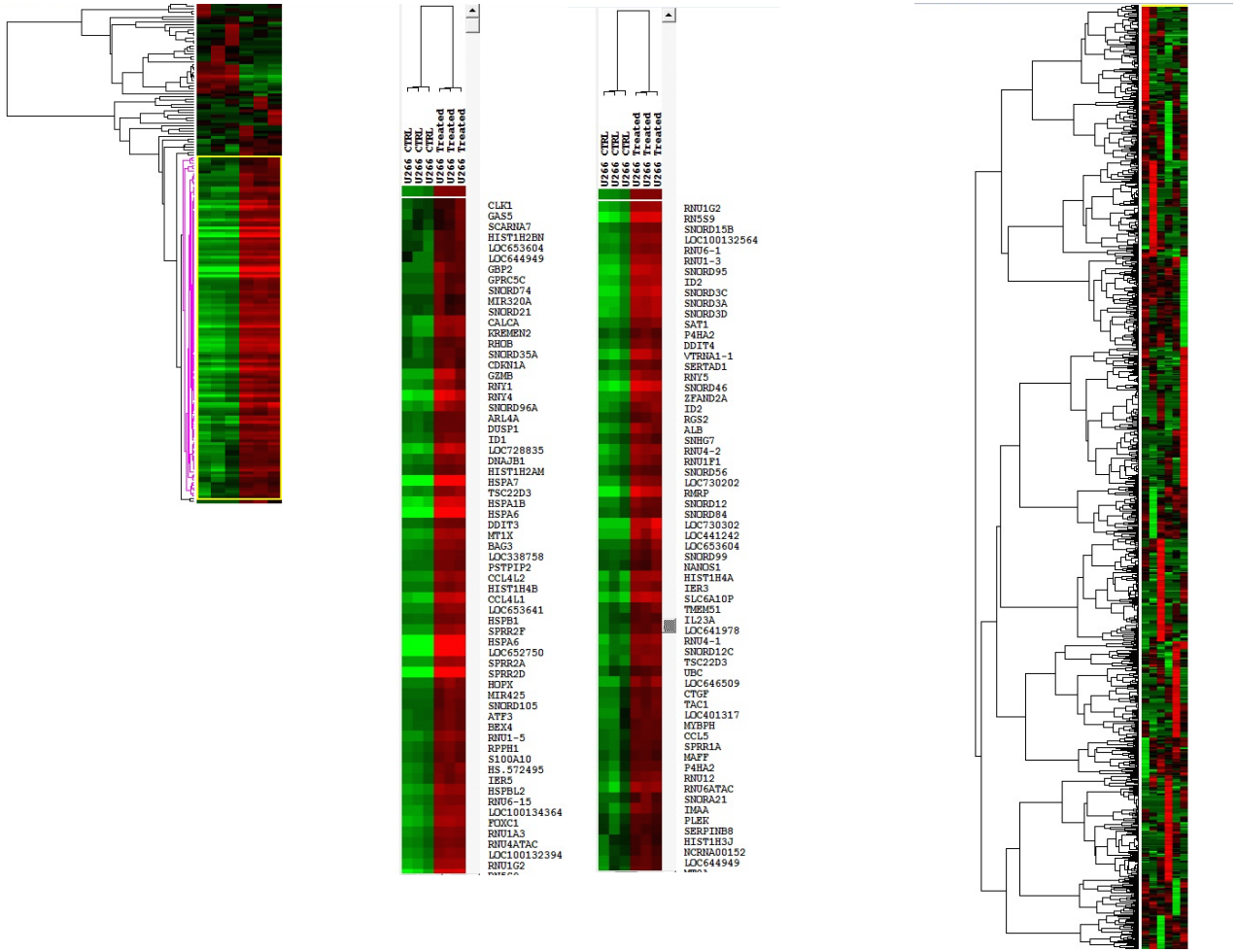

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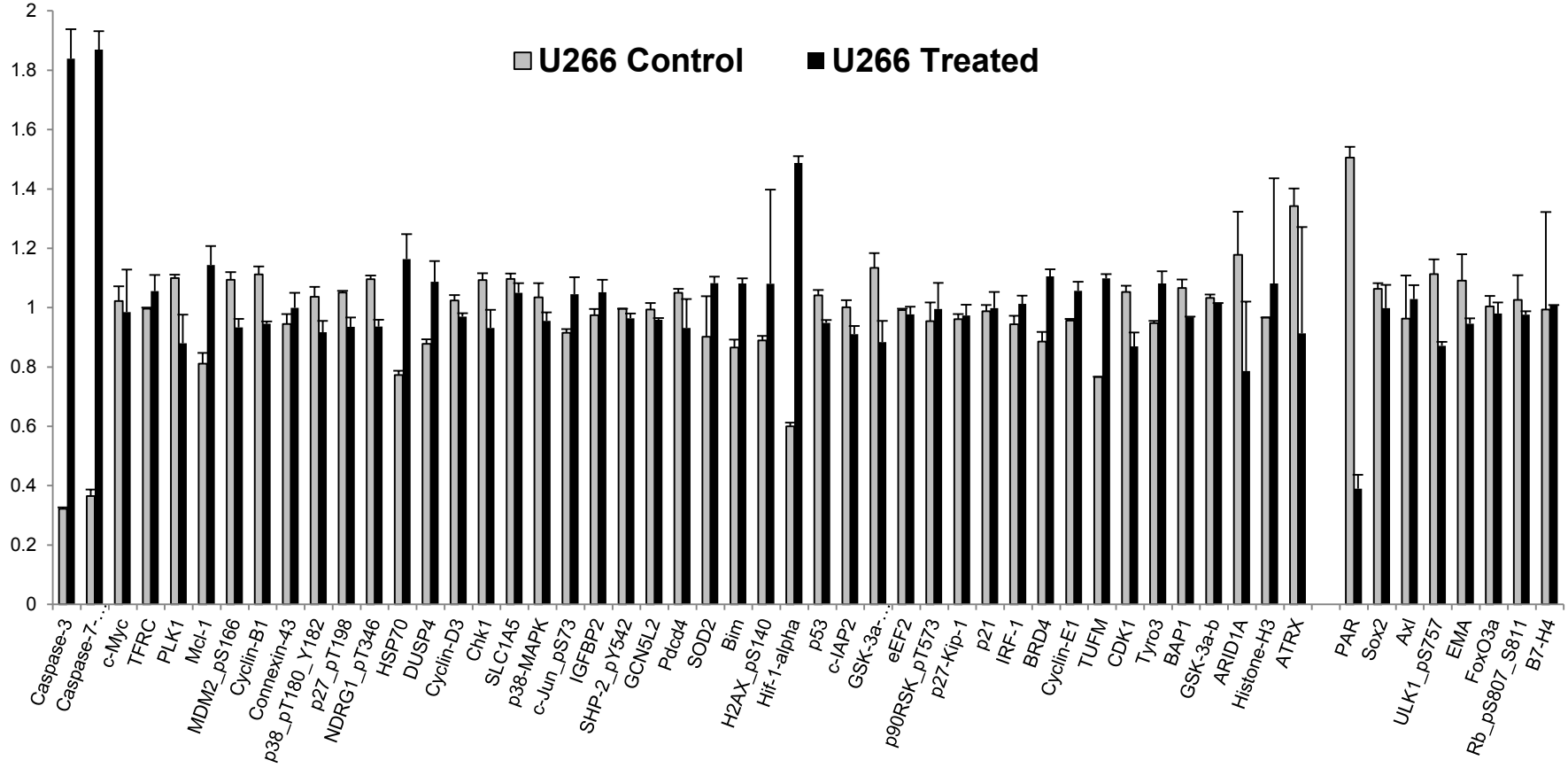

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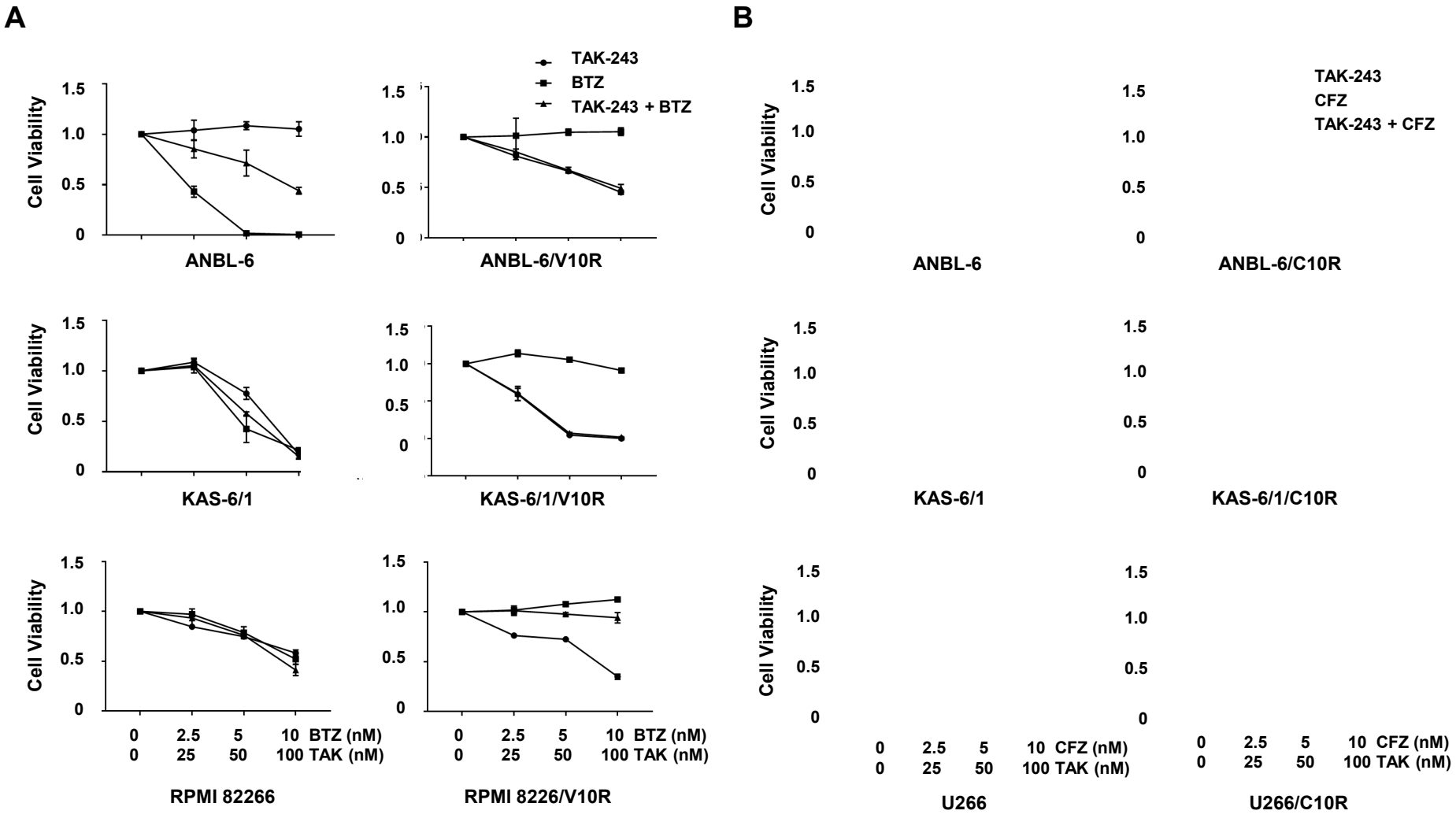

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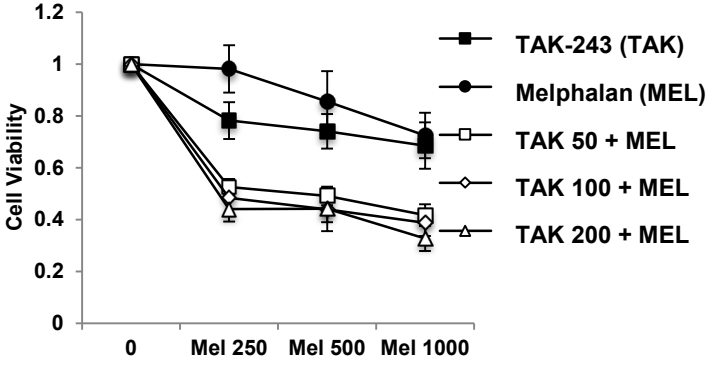

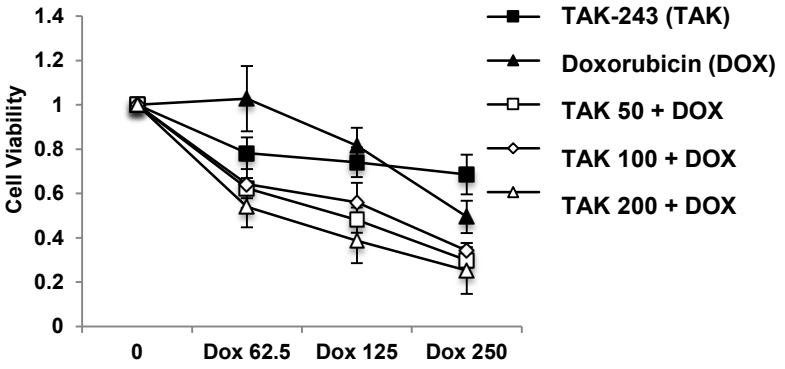

Supplementary Figure 9

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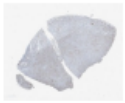
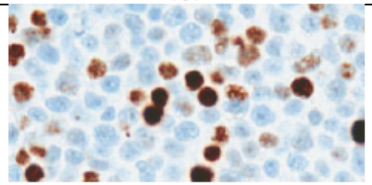
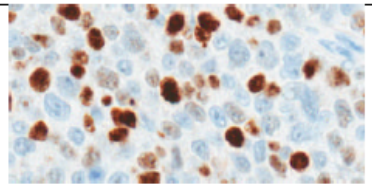
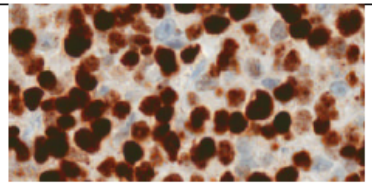
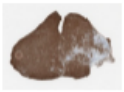
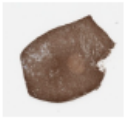
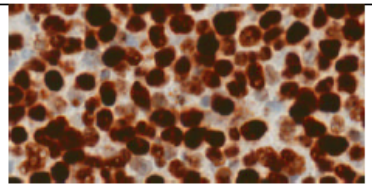
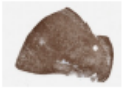
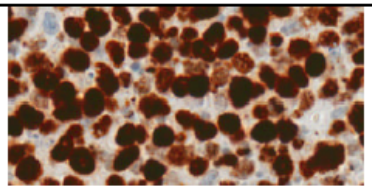
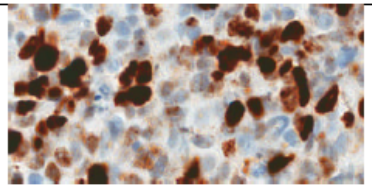
 UAE049527		 UAE049529	 UAE049529	CPUA-16-PD01 10% HPbCD IV (1 dose)~Acute 24hr
 UAE049530	 UAE049531	 UAE049532	 UAE049531	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 1hr
 UAE049533	 UAE049534	 UAE049535	 UAE049534	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 2hr
 UAE049536	 UAE049537	 UAE049538	 UAE049537	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 4hr
 UAE049539	 UAE049540	 UAE049541	 UAE049540	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 8hr
 UAE049542		 UAE049544	 UAE049542	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 24hr

Supplementary Figure 10

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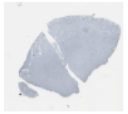
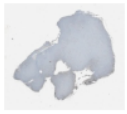
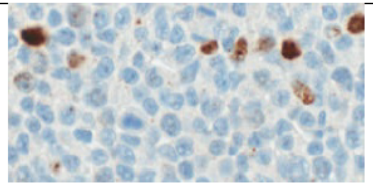
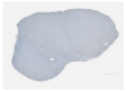
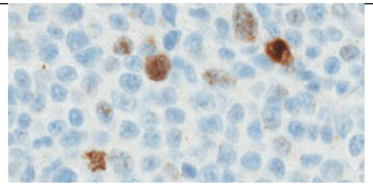
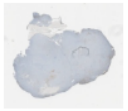
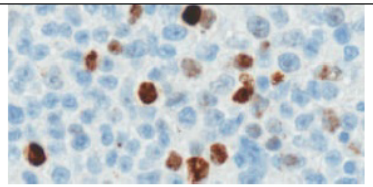
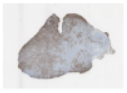
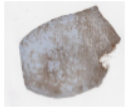
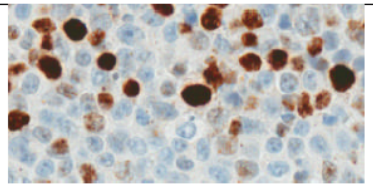
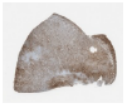
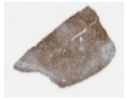
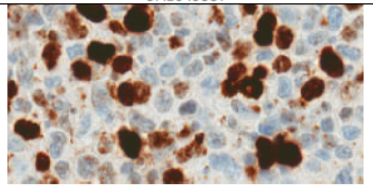
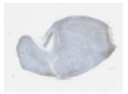
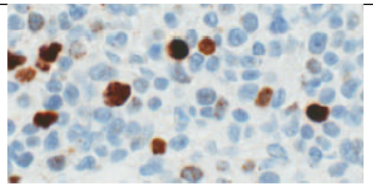
 UAE049527		 UAE049529	 UAE049529	CPUA-16-PD01 10% HPbCD IV (1 dose)~Acute 24hr
 UAE049530	 UAE049531	 UAE049532	 UAE049531	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 1hr
 UAE049533	 UAE049534	 UAE049535	 UAE049534	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 2hr
 UAE049536	 UAE049537	 UAE049538	 UAE049537	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 4hr
 UAE049539	 UAE049540	 UAE049541	 UAE049540	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 8hr
 UAE049542		 UAE049544	 UAE049544	CPUA-16-PD01 TAK-243-001-P IV (1 dose)~Acute 25.0 mg/kg 24hr