Cell Reports, Volume *25*

Supplemental Information

Non-overlapping Control of Transcriptome by Promoter- and Super-Enhancer-Associated Dependencies in Multiple Myeloma

Mariateresa Fulciniti, Charles Y. Lin, Mehmet K. Samur, Michael A. Lopez, Irtisha Singh, Matthew A. Lawlor, Raphael E. Szalat, Christopher J. Ott, Herve' Avet-Loiseau, Kenneth C. Anderson, Richard A. Young, James E. Bradner, and Nikhil C. Munshi

Figure S1: E2F1 and heterodimerization partner DP1 are required for MM cell growth *in vitro* and *in vivo*

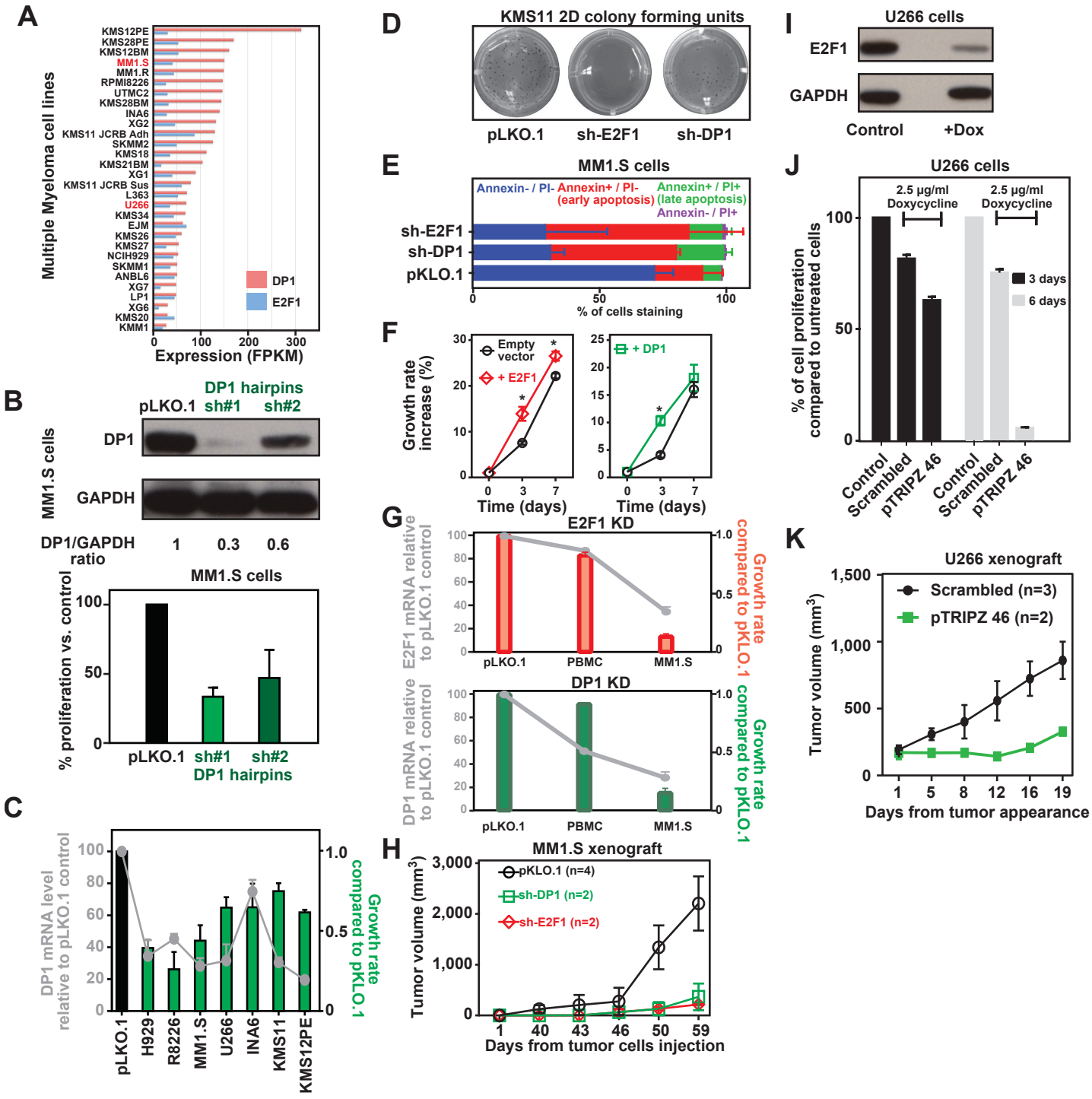


Figure S1 related to Figure 1. E2F1 and heterodimerization partner Dp1 are required for MM cell growth *in vitro* and *in vivo*. A) *TFDP1* (DP1 gene) and *E2F1* mRNA levels in a panel of 30 MM cell lines were evaluated by RNA-seq and presented as fragments per kilobase of exon per million reads mapped (FPKM). **B)** MM1.S cells were infected with either scrambled (pLKO.1) or 2 DP1-targeted shRNAs and selected with puromycin for 72 hours. Western blot analysis was performed to test knock down efficiency, using GAPDH as loading control. Transduced cells were analyzed for effect on cell growth by 3(H)-thymidine uptake. **C)** A panel of MM cell lines were infected with either scrambled (pLKO.1) or DP1-targeted shRNA and selected with puromycin for 72 hours. DP1 mRNA levels and cell growth were then evaluated by qPCR and Thymidine uptake respectively. The results are presented as mRNA (line) or cell growth (bars) changes from cells infected with pLKO.1. Data are shown as the mean values ± s.d. of triplicates. **D)** Representative phase contrast images for KMS11 colonies formed in semisolid methylcellulose medium at day 21 for control and knockdown (KD) cells. **E)** Apoptotic cell death was assessed in MM1.S cells infected with either scrambled (pLKO.1), DP1-targeted shRNA or E2F1-targeted shRNA #1 for 5 days after puromycin selection by flow cytometric analysis following Annexin V and propidium iodide (PI) staining. The % of Annexin V+/PI- (early apoptosis) and Annexin V+/PI+ (late apoptosis) cells are shown in the graph. **F)** Effect of E2F1 overexpression in MM1.S was evaluated over time by 3(H) thymidine uptake and presented as fold change increase compared to empty vector. **G)** PBMC from healthy individuals were infected with sh-E2F1 #1 (upper panel) or sh-DP1 #1 (lower panel) by spinoculation. mRNA levels and cell viability were evaluated after 3 days from puromycin selection by qPCR and cell titer glow respectively. The results are presented as mRNA (line) or cell viability (bars) changes from cells infected with pLKO.1. Data are shown as the mean values ± s.d. of triplicates. Effect of KD on MM1S cell viability was used as positive control. **H)** *In vivo* evaluation of the effects of DP1 and E2F1 knockdown on MM cells. Growth curve assess tumor size after injection of MM1.S KD or scrambled cells subcutaneously into the right posterior flank region of SCID mice. Data are shown as the mean values ± s.d **I)** Genetic depletion of E2F1 in U266 cells was achieved using tetracycline-inducible pTRIPz- Turbo-RFP vectors (Thermo Scientific, Pittsburg, PA) containing the target sequence or scramble control. Transfected U266 cells were plated in growth medium in the absence or presence of 5 μg ml−1doxycycline Doxycycline for 3 days before WB analysis to confirm decreased E2F1 protein expression in cells expressing inducible E2F1 shRNAs. **J)** U266 cellular proliferation was evaluated by ^{3}(H)thymidine uptake, and presented as percentage of cell proliferation compared to untreated cells. **K)** *In vivo* mouse xenograft studies was performed with U266 cells harboring doxycycline inducible shRNA hairpins targeting E2F1. Mice were treated with irradiated 0.0625% Doxycycline Diet continuously (1–6 mg of doxycycline per mouse/per day) after tumor appearance and monitored for tumor progression using caliper measurements.

Figure S2: Functional E2F dependency in primary MM cells

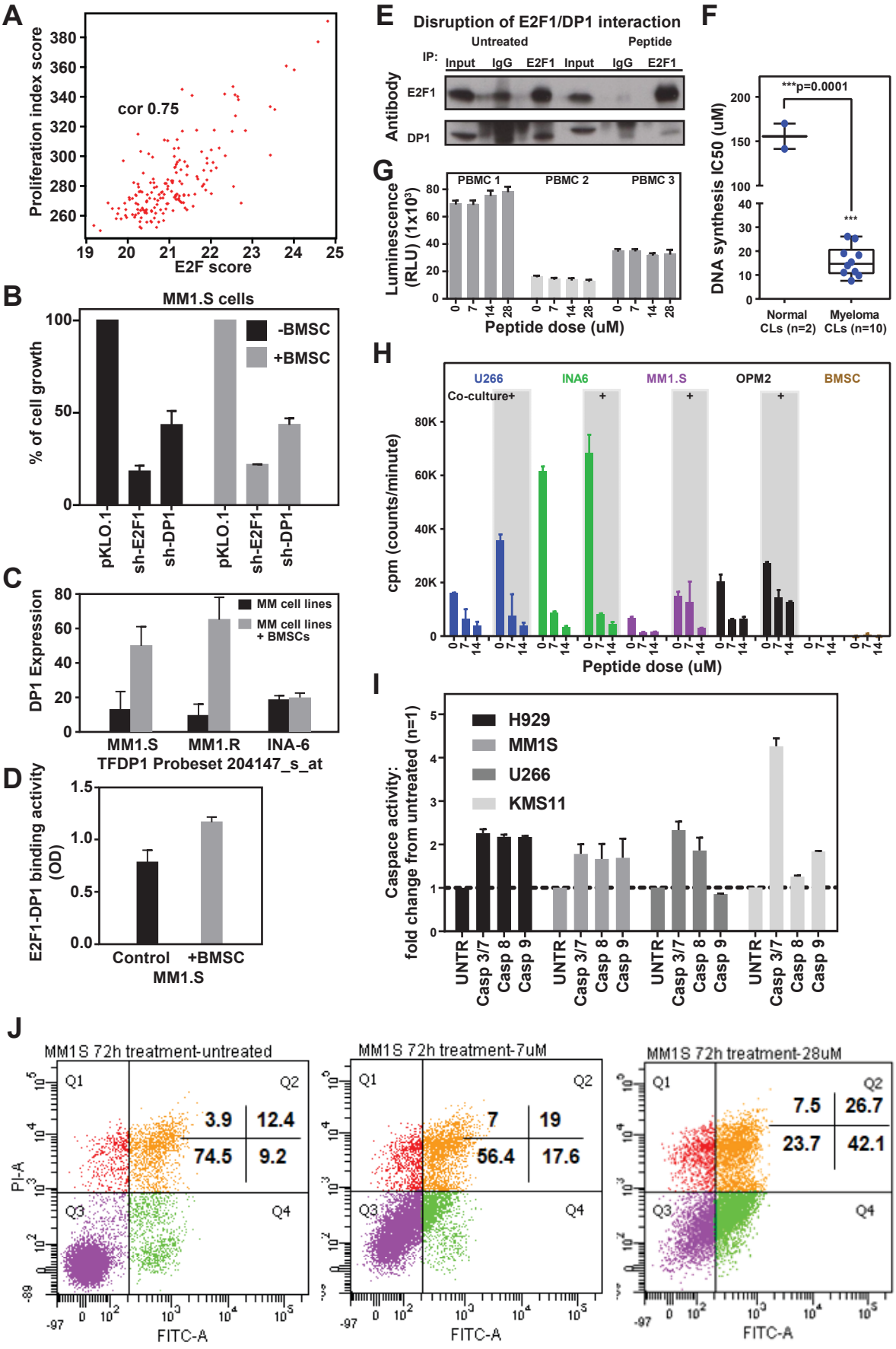
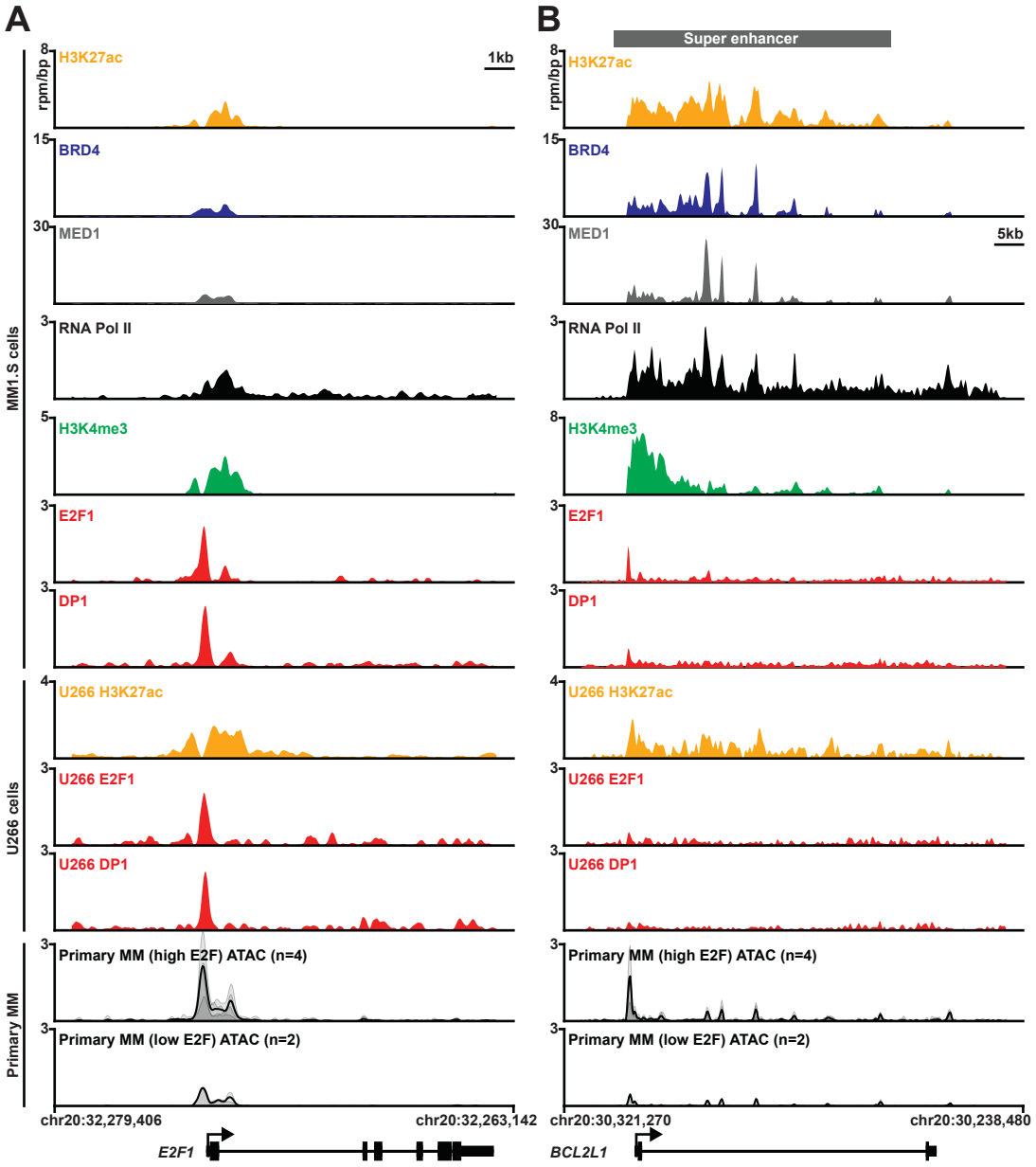
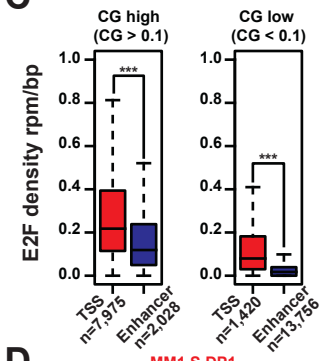
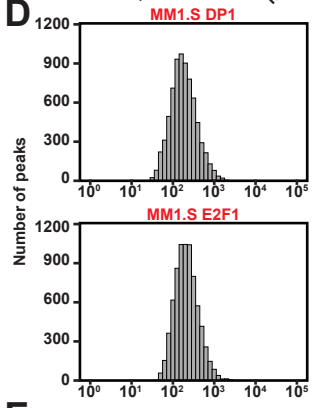


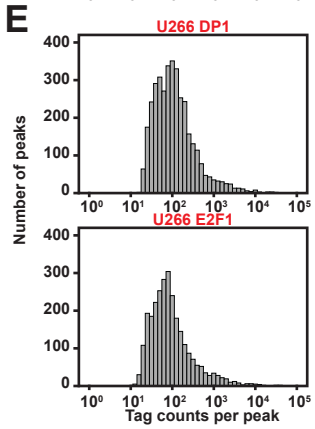
Figure S2 related to figure 2. Functional E2F dependency in MM cells. A) Proliferation index was calculated using the 50 genes from original publication [Hose D, Haematologica 2011]. E2F scores were calculated as sum of E2F gene expressions and correlation between E2F score and proliferation index was measured using pearson correlation. **B)** MM1.S KD or control cells were cultured in the absence or presence of a monolayer of patient-derived BMSCs for 2 days. Cell proliferation was evaluated by 3(H) thymidine uptake. **C)** Expression levels (linear scale) for TFDP1 transcript were evaluated using oligonucleotide microarray data for MM1.S, MM1R, and INA6 cells cultured in vitro in the presence or absence of HS-5 bone marrow stromal cells (GSE 20540).
D) MM1.S cells were cultured in the absence (control) or in the presence of BMSC for 6 h, harvested and nuclear extracts (15 μg) were subjected to ELISA to assess cell DNA binding activity. Absorbance was obtained with a spectrophotometer at 450 nm and presented as optical density (OD). **E)** Whole extracts from MM1.S cells untreated and treated with E2F/DP1 blocking peptide where immunoprecipitated using E2F1 Ab. Western blot analysis was performed using E2F1 and DP1 Abs. **F)** A panel of MM cell lines and normal human cell lines were treated with different concentration of blocking peptide for 72 hours. DNA synthesis was assessed by [3H] thymidine uptake. IC50 analysis was performed with the GraphPad software. **G)** PBMC from healthy donors were activated either with IL2 (50 U/ml) or PHA (20 ug/ml). Cell viability was assessed by cell titer glow. **H)** A panel of MM cell lines were cultured in the absence or presence (+) of BMSC at different concentration of peptide for 72 hours. Cell proliferation was assessed by ^{3}(H)thymidine uptake, and expressed as cpm (count per minute). Data represent means ± SD (n = 3). **I)** Indicated caspase activities were evaluated in 4 MM cell lines after treatment with blocking peptide for 72 hours using luminescence assay. **J)** MM1S cells were cultured in the absence or presence of peptide, and apoptotic cell death was assessed by flow cytometric analysis following AnnexinV and DAPI staining. The % of AnnexinV-/DAPI- (live), AnnexinV+/DAPI- (early apoptosis), AnnexinV+/DAPI+ (late apoptosis) and AnnexinV-/DAPI+ (necrosis) cells are shown in the graphs.

Figure S3: E2F binds to promoters of active genes in multiple myeloma





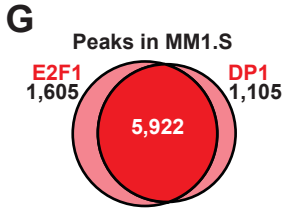


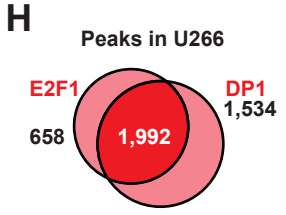


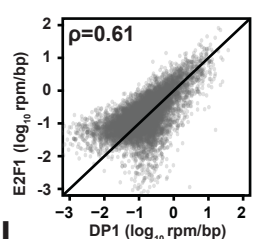
F

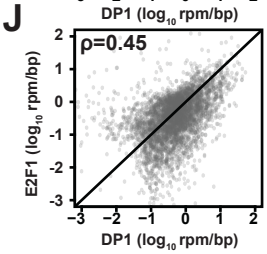
MM E2F ChIP-Seq metrics

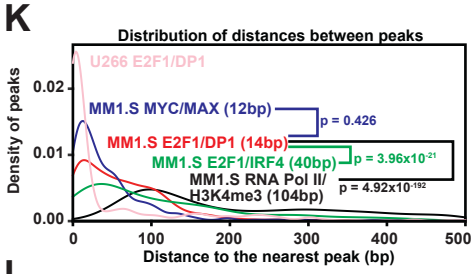
Dataset	# peaks	Total reads	Reads in peaks	% contained in peak	Tags/ peak	Peak GC%
MM1S E2F1	7,532	70.0M	2.03M	3	205	63
MM1S DP1	7,296	78.5M	1.73M	2	173	66
U266 E2F1	2,752	28.8M	1.13M	4	76	66
U266 DP1	3,656	43.2M	1.52M	4	97	66











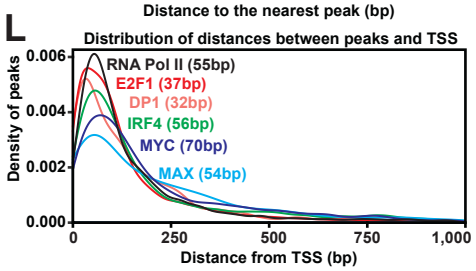
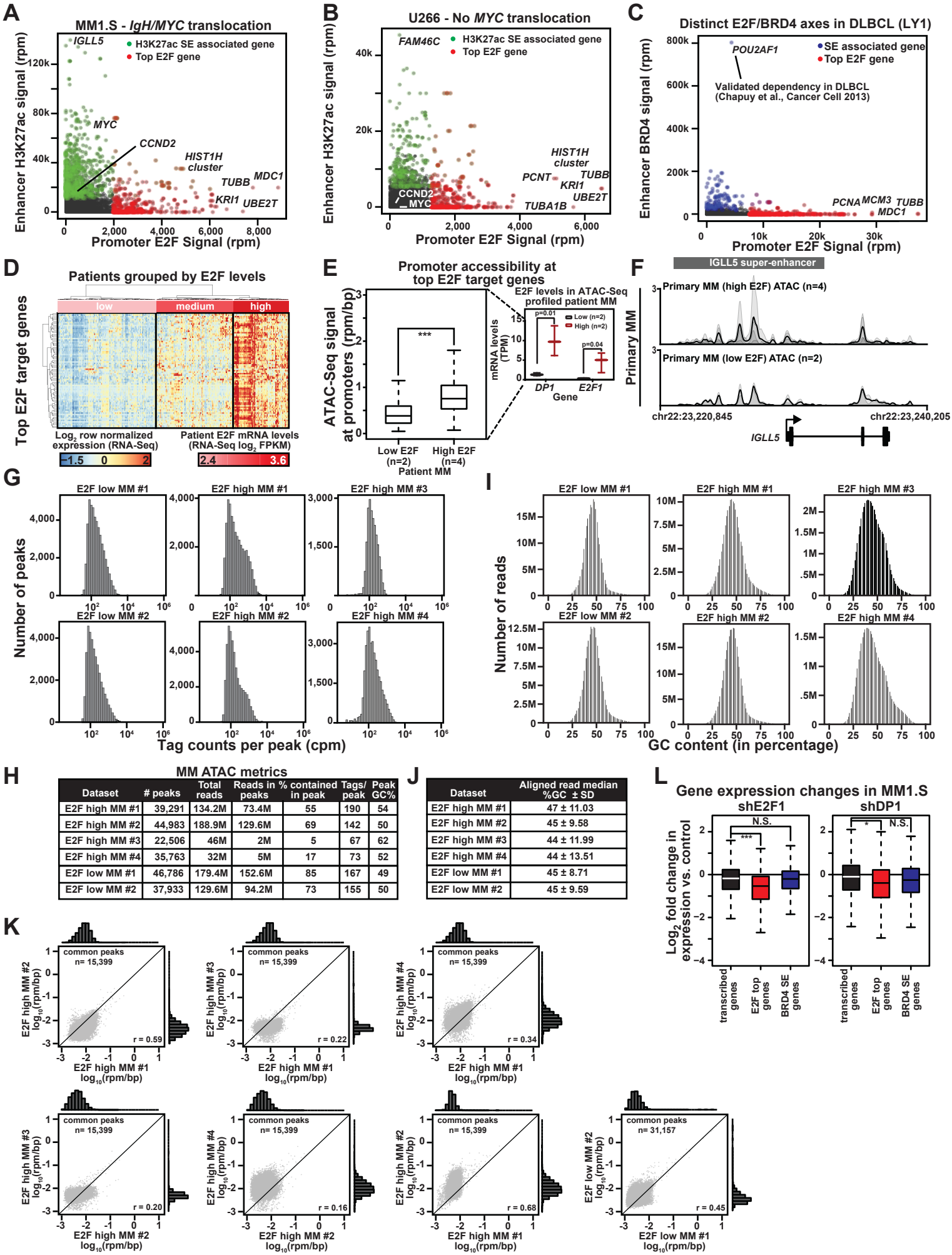


Figure S3 related to figure 3. E2F binds to promoters of active genes in multiple myeloma. A,B) Gene tracks showing ChIP-Seq occupancy in MM1.S and U266 MM cell lines and ATAC-Seq signal in primary MM cells at individual loci for *E2F1* and *BCL2L1.* The x-axis displays genomic coordinates with gene models depicted below. The y-axis shows ChIP-Seq occupancy in units of rpm/bp. **C)** Box plots of E2F density (E2F1/DP1 background subtracted signal in units of rpm/bp) at TSS (+/- 1kb) or enhancer regions (as defined by BRD4) categorized by high (>0.1 CG) or low (< 0.1 CG) CG content. Statistical significance of the differences in the means of the distributions are shown using a twot ailed *t*-test (*** p<1e-9). **D,E)** Histograms showing tag counts per peak for E2F1 and DP1 ChIP-Seq datasets in MM1.S and U266 cells. **F)** The table provides summary metrics for ChIP-seq profile of E2F1 and DP1 in MM1.S and U266 cells, i) total number of peaks called by peak caller, ii) total number of tags with unique alignment, iii) total number of tags overlapping all the peaks, iv) fraction of total tags overlapping the peaks, v) median number of tags overlapping per peak and vi) median GC content of all peaks. **G,H)** Venn diagram showing the overlap of E2F1 and DP1 bound regions in MM1.S **(G)** and U266 **(H)**. **I,J)** Scatter plot showing the relationship between E2F1 and DP1 ChIP-Seq occupancy (rpm/bp) at all regions bound by either factor in MM1.S **(I)** and U266 **(J)**. The spearman correlation statistic is displayed in the upper left corner. **K)** Distribution functions showing the distribution of nearest ChIP-Seq peaks for pairs of ChIP-Seq marks including U266 E2F1/DP1 (pink), MM1.S MYC/MAX (blue), MM1.S E2F1/DP1 (red), MM1.S E2F1/IRF4 (green), and MM1.S RNA Pol II/H3K4me3 (black). The most frequent peak distance (mode) is annotated for each and the pairwise statistical differences are compared using a one-sided KS test. The top twenty percent high confidence peaks by signal for every factor were used. **L)** Distribution functions showing the distances between MM1.S ChIP-Seq peaks and the TSS for active genes. The most frequent peak distance (mode) is annotated for each dataset. The top twenty percent high confidence peaks by signal for every factor were used.

Figure S4: E2F and BRD4 super-enhancers establish distinct regulatory axes in multiple myeloma



Dataset	# peaks	Total reads	Reads in peaks	% contained in peak	Tags/ peak	Peak GC%
E2F high MM #1	39,291	134.2M	73.4M	55	190	54
E2F high MM #2	44,983	188.9M	129.6M	69	142	50
E2F high MM #3	22,506	46M	2M	5	67	62
E2F high MM #4	35,763	32M	5M	17	73	52
E2F low MM #1	46,786	179.4M	152.6M	85	167	49
E2F low MM #2	37,933	129.6M	94.2M	73	155	50

Dataset	Aligned read median %GC ± SD
E2F high MM #1	47 ± 11.03
E2F high MM #2	45 ± 9.58
E2F high MM #3	44 ± 11.99
E2F high MM #4	44 ± 13.51
E2F low MM #1	45 ± 8.71
E2F low MM #2	45 ± 9.59

Figure S4 related to figure 4. E2F and BRD4 super-enhancers establish distinct regulatory axes in multiple myeloma. **A,B**) Scatter plot showing the E2F promoter signal (x-axis) and H3K27ac enhancer signal (y- axis) for all actively transcribed genes in **A)** MM1.S and **B)** U266. E2F signal represents the average ChIP-Seq occupancy (units of reads per million, rpm) of E2F1 and DP1 in the +/- 5kb TSS region. H3K27ac enhancer signal represents the total H3K27ac ChIP-Seq occupancy (rpm) at all enhancers within 50kb of the TSS. Super-enhancer (SE) associated genes are colored in blue and the top 500 genes ranked by E2F promoter occupancy are shaded in red. Select genes are labeled. **C)** Scatter plot showing E2F promoter signal (x-axis) and BRD4 enhancer signal (y-axis) for all actively transcribed genes in the diffuse large B-cell lymphoma (DLBCL) cell line LY1. E2F signal represents the average ChIP-Seq occupancy (units of reads per million, rpm) of E2F1 in the +/- 5kb TSS region. BRD4 enhancer signal represents the total BRD4 ChIP-Seq occupancy (rpm) at all enhancers within 50kb of the TSS. Super-enhancer (SE) associated genes are colored in blue and the top 500 genes ranked by E2F promoter occupancy are shaded in red. Select genes are labeled. **D)** Heatmap of patient MM gene expression data for top 500 E2F ranked genes (as in Figure 4) across three groups of patient samples grouped by E2F expression levels (low, medium, high). The average expression of activating E2F members is shaded in red for each group of patients. Each row represents a single gene. Expression is colored in $\log_2$ row median normalized units. Rows and samples are hierarchically clustered. **E)** Boxplots showing ATAC-Seq chromatin accessibility signal (rpm/bp) at promoters of top E2F genes in patient MM cells with either high (n=4) or low levels of E2F (n=2). Inset: RNA-Seq expression levels in units of transcripts per million for E2F1 and DP1 in respective high and low patient E2F samples. The statistical significance between distributions of promoter ATAC-Seq signal or E2F & DP1 mRNA levels was determined using a Welch's two tailed *t*-test (*** p- value < 1e-9). **F)** Gene tracks showing ATAC-Seq signal at individual loci for the *IGLL5* region in MM patient cells with high (n=4) and low (n=2) E2F expression levels respectively. Individual replicates are plotted as translucent shapes and the plotted line represents the mean signal. **G)** Histograms showing tag counts per peak for ATAC-Seq datasets in primary patient MM. **H)** The table provides summary metrics for ATAC-Seq datasets in high and low E2F patient samples, i) total number of peaks called by peak caller, ii) total number of tags with unique alignment, iii) total number of tags overlapping all the peaks, iv) fraction of total tags overlapping the peaks, v) median number of tags overlapping per peak and vi) median GC content of all peaks. **I-J)** GC distribution **(I)** and total aligned read GC% **(J)** for ATAC-seq datasets. **K)** Scatter plots of shared peaks in E2F high or E2F low ATAC-seq samples along with their R^2. **L)** Box plots showing the $\log_2$ change in gene expression upon E2F1 or DP1 knockdown in MM1.S cells for all transcribed genes (black), top E2F bound genes (red), or BRD4 SE associated genes (blue). The statistical significance between distributions of gene expression changes was determined using a Welch's two tailed *t*-test (* p-value < 1e-3, *** p- value < 1e-9).

Figure S5: Significant myeloma growth inhibitory effects of dual E2F and BET inhibition

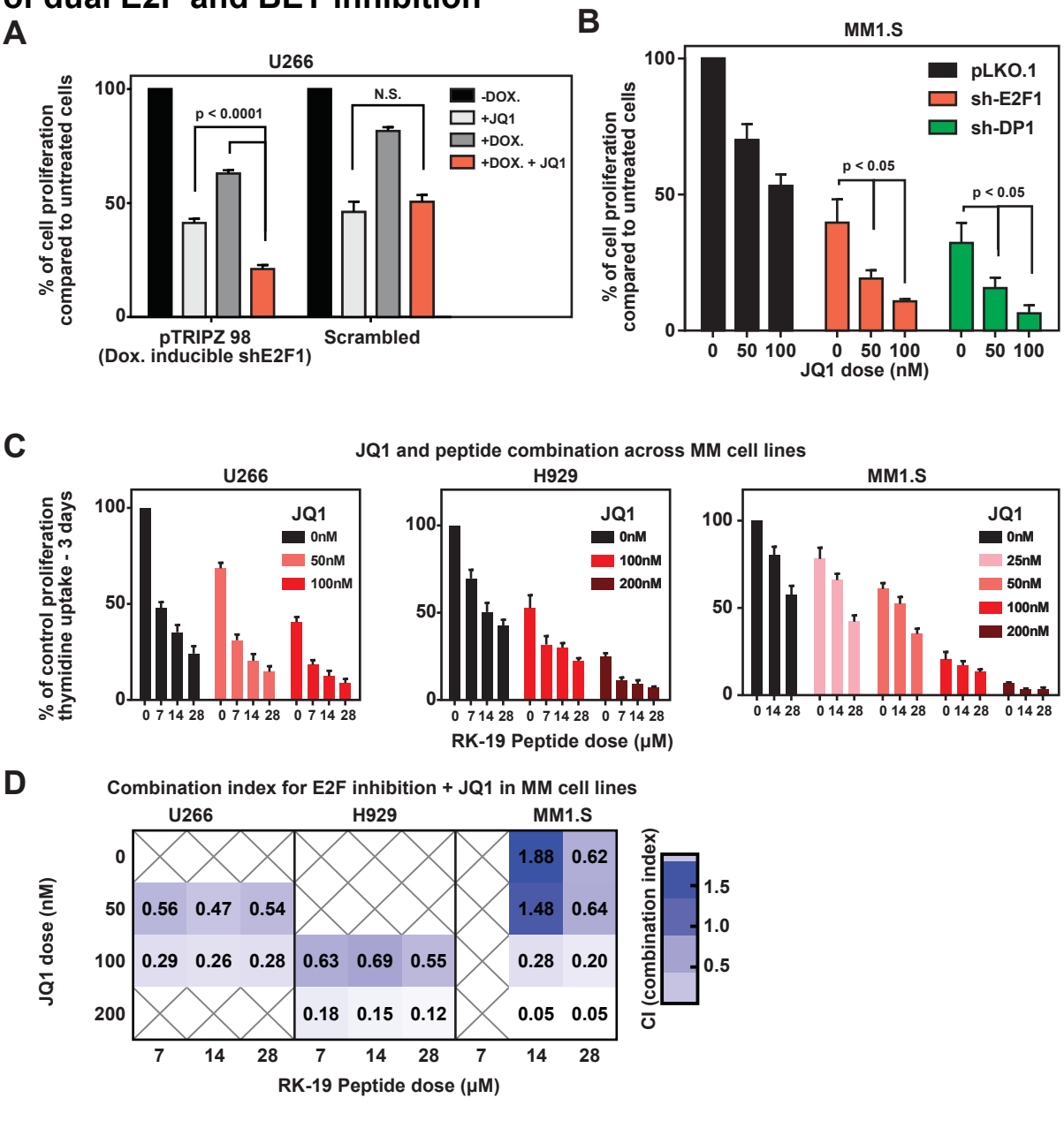


Figure S5 related to Figure 5. Dual chemical inhibition of BETs and E2F significantly impacts MM growth. **A)** Transfected U266 cells expressing tetracycline-inducible containing E2F1 sequence or scramble control were plated in growth medium in the absence or presence of 2.5μg ml−1 Doxycycline in combination with IC50 dose of JQ1 (100nM) for 3 days. Cell growth was evaluated by 3(H)-thymidine uptake and presented as % of cell proliferation compared to control cells. **B)** MM1.S scrambled and KD cells were cultured in the presence of different concentrations of JQ1. Cell growth assay was performed after 3 days of treatment. Results are presented as % of cell growth compared to control cells (scrambled and untreated). Data are representative of three independent experiments. **C)** MM cell lines were simultaneously treated with different concentration of blocking peptide and JQ1 for 72 h. Cell growth was assessed by [3H]thymidine uptake and presented as percentage of cell growth decrease compared to control cells (untreated cells). **D)** The interaction between blocking peptide and JQ1 was analyzed using the CalcuSyn software program (Biosoft, Ferguson, MO) based on the Chou-Talalay method. Isobologram analysis revealed strong synergism of the combination as compare to single agents. Combination index (CI) = 1 indicates additive effects. CI < 1 indicates synergism; CI > 1 indicates antagonism.