Supplemental Information for:

PLK1 and NOTCH Positively Correlate in Melanoma and their Combined Inhibition Results in Synergistic Modulations of Key Melanoma Pathways

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Table S1. Antibodies used in this study

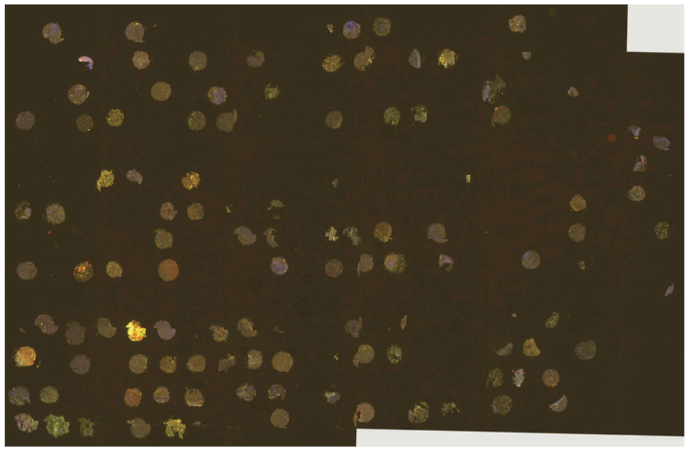
Antibody	Company	Catalog #	Dilution		Secondary	Company	Dilution
			IF	IB			
S100	Biocare Medical	CM164	1:50	N/A	None-bound to Zenon AlexaFluor 488	Thermo Fisher	1 part ea: S100, block, Zenon fAb
PLK1	Cell Signaling	4513	1:25	N/A	Goat α rabbit; AlexaFluor 647	Thermo Fisher	1:200
NOTCH1	Millipore	MAB5352	1:100	N/A	Goat α mouse; AlexaFluor 555	Thermo Fisher	1:200
Caspase 3*	Cell Signaling	9662	N/A	1:1000	Rabbit	Cell Signaling	1:2000
PARP*	Cell Signaling	9542	N/A	1:1000	Rabbit	Cell Signaling	1:2000
P53	Cell Signaling	9282	N/A	1:1000	Rabbit	Cell Signaling	1:2000
GAPDH	Proteintech	10494-1-AP	N/A	1:1000	Rabbit	Cell Signaling	1:2000
β-Actin	Cell Signaling	8457	N/A	1:1000	Rabbit	Cell Signaling	1:2000
B-tubulin	Cell Signaling	2146	N/A	1:1000	Rabbit	Cell Signaling	1:2000

*Antibody detects both full-length and cleaved forms of the protein.

Table S2. Mutation status of human melanoma cells used in this study

Cell Name	BRAF Status	NRAS Status	TP53 Status
A375	mt	wt	wt
SK-MEL-2	wt	mt	mt
SK-MEL-28	mt	wt	mt

A TMA Information



Parameter	Number
Melanoma	110
Benign nevus	16
Male	67
Female	53
Age < 50	29
Age > 50	78

B Correlation between PLK1/NOTCH1 and Breslow Thickness

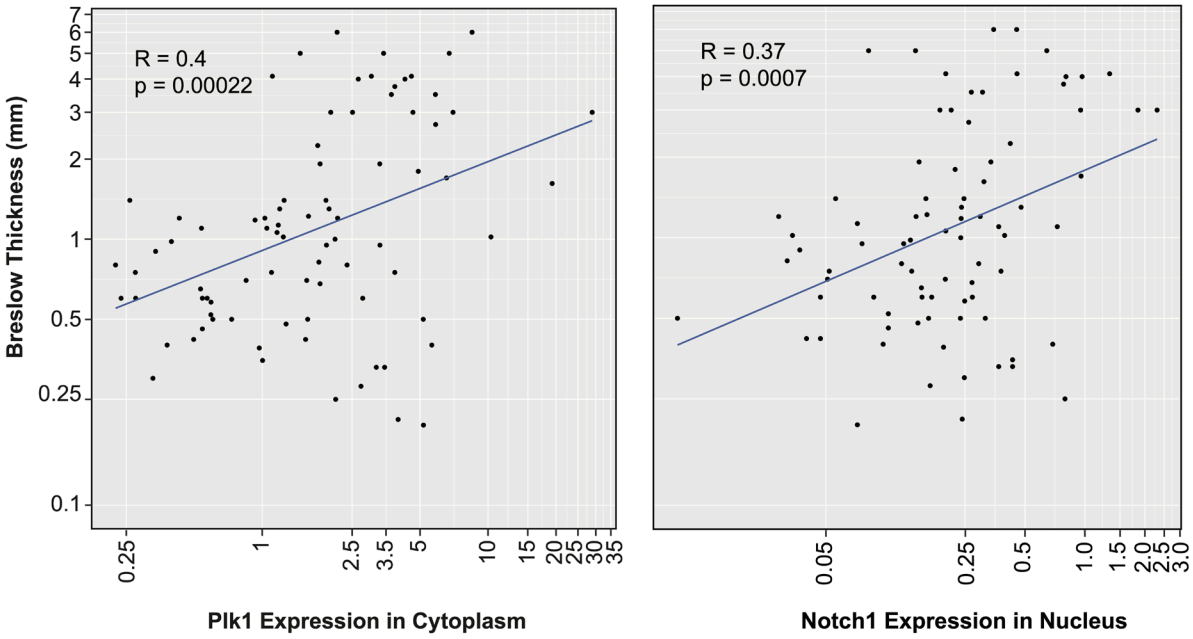


Figure S1. Tissue microarray (TMA) analysis. A, TMA information. The mosaic image shows the overlaid immunofluorescence images of the entire TMA, with compiled clinical information of the tissue cores used to make the TMA. **B,** Simple linear regression between PLK1/NOTCH1 and Breslow thickness. The plot indicates a significant positive correlation between Breslow thickness and PLK1/NOTCH1 expression.

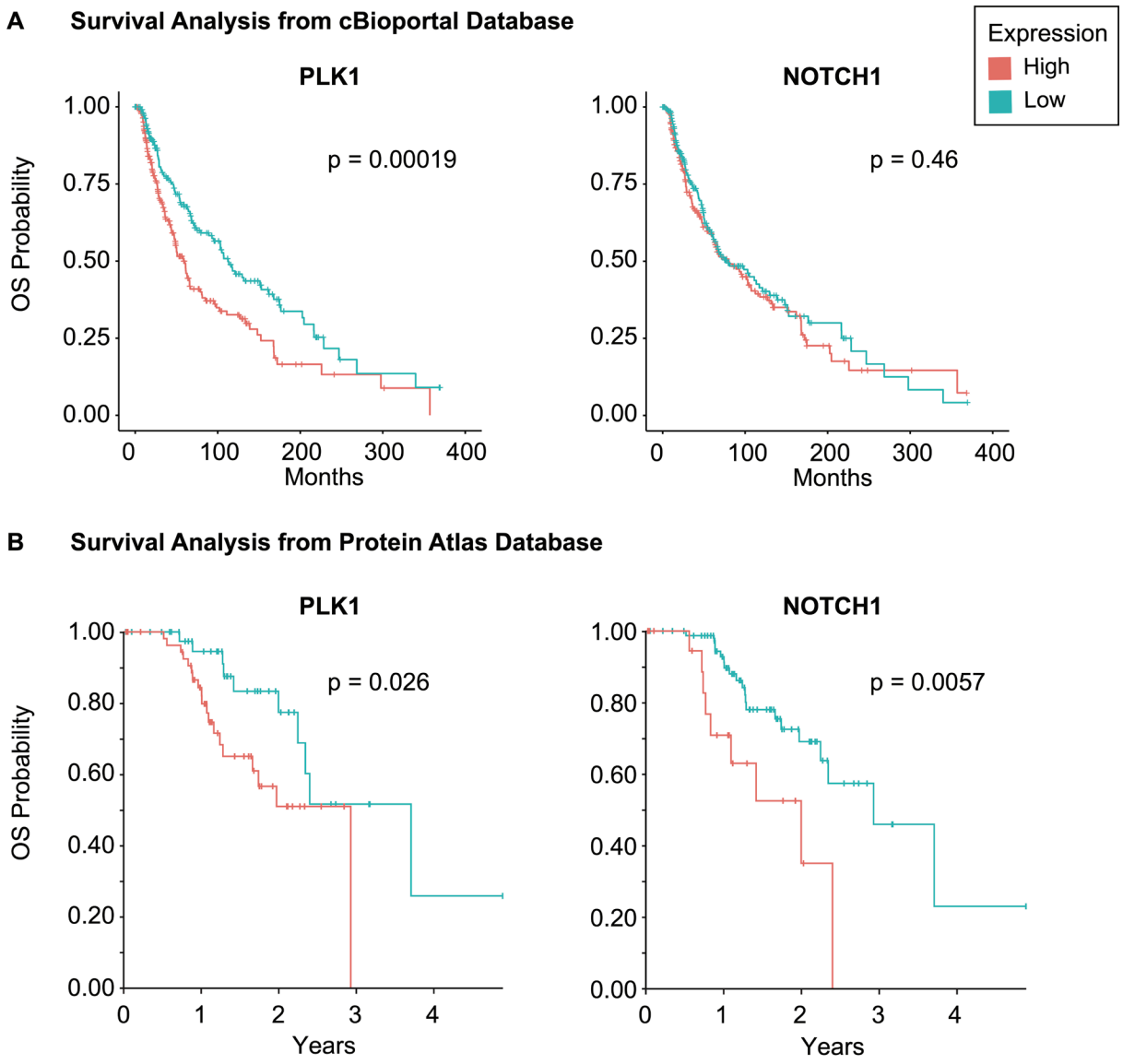


Figure S2. TCGA survival analysis. A, TCGA survival analysis using cBioPortal database. The overall survival of PLK1 or NOTCH1 was compared between high (red) and low (green) expression separated by median value in total 479 melanoma patients. **B,** TCGA survival analysis using the Human Protein Atlas database. The overall survival of PLK1 was compared between 57 high expression (red) and 45 low expression (green) melanoma patients. The overall survival in NOTCH1 was compared between 81 high expression (red) and 21 low expression (green) melanoma patients.

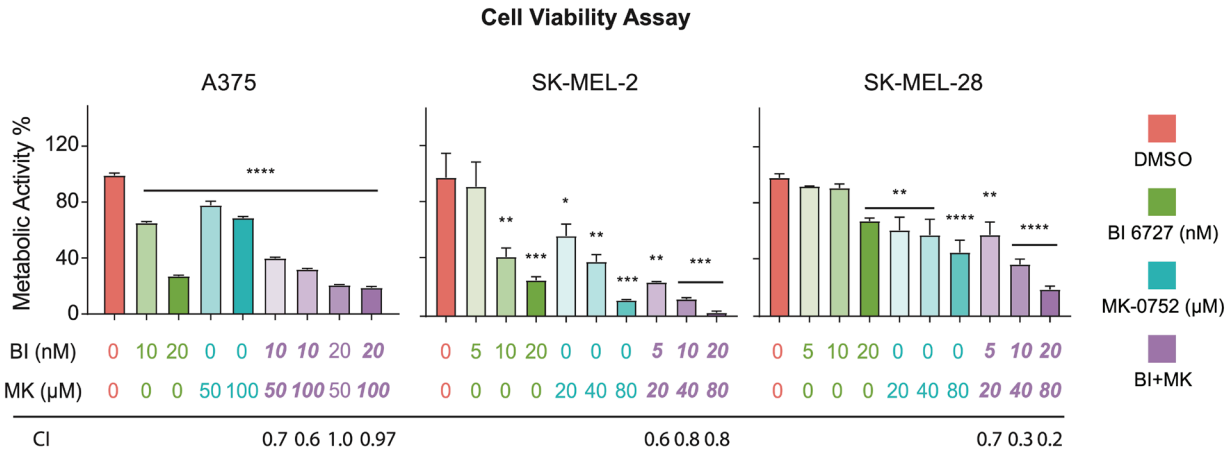


Figure S3. Synergistic response resulting from PLK1 and NOTCH inhibition in human melanoma cells. Cell viability assay**.** A375, SK-MEL-2, and SK-MEL-28 cells were treated with indicated doses of BI 6727, MK-0752, or a combination, and metabolic activity was determined of two biological replicates in triplicates. Combination Index (CI) was displayed under each corresponding combination treatment. CI < 1.0 indicates synergism, CI = 1.0 indicates additive effect. *P < 0.05, **P < 0.01, and ***P < 0.001. Error bars, standard error of the mean (SEM).

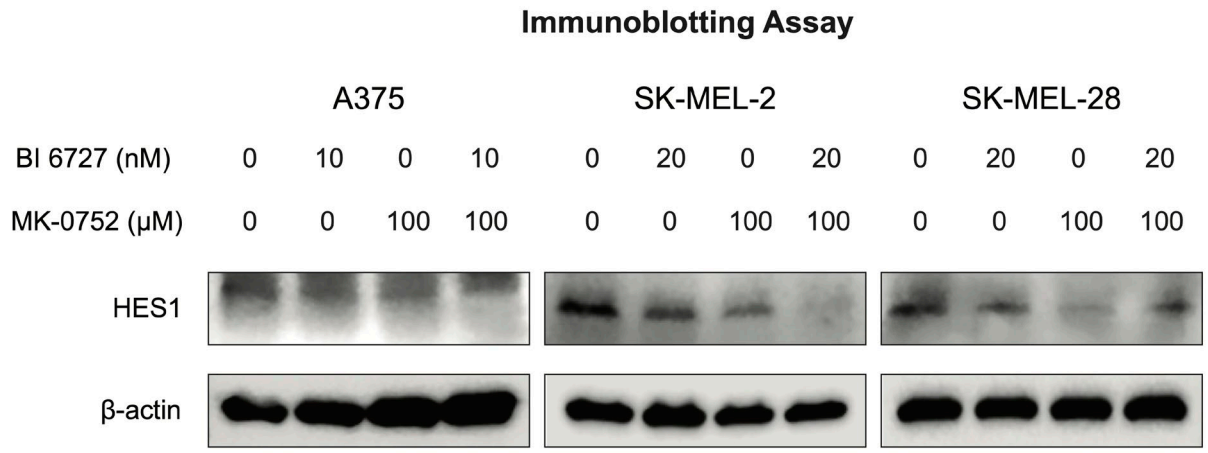


Figure S4. Target modulation of NOTCH1 downstream protein HES1. Immunoblotting assay. A375, SK-MEL-2, and SK-MEL-28 cells were treated with indicated doses of BI 6727, MK-0752, or a combination, and their HES1 expression was determined by immunoblotting with β-actin as control.

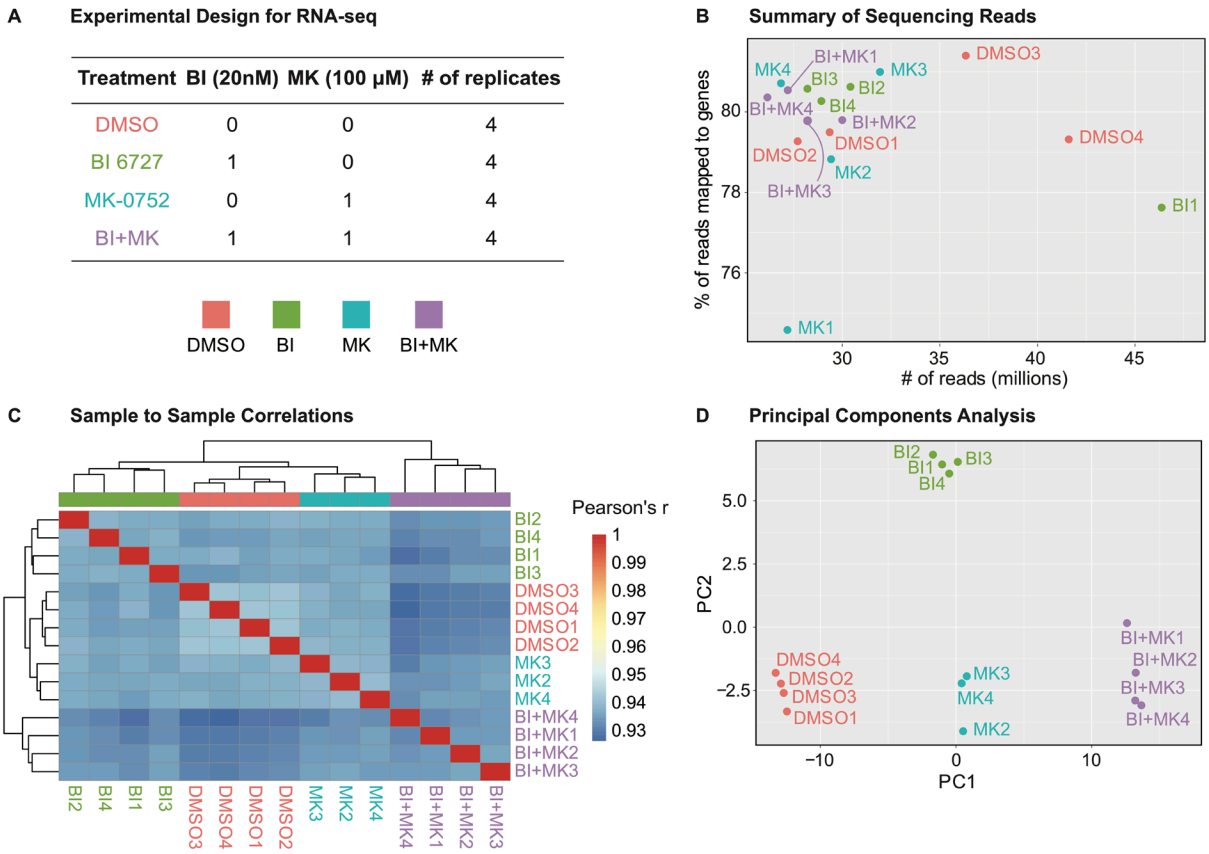


Figure S5. Experimental setup and quality control for RNA-seq results. A, Experimental setup for RNA-seq. There are 4 replicates in control, single drug treatment, and combination treatment groups. 0 and 1 represent without or with indicated drug added, respectively. **B,** Summary of sequencing reads and percent of reads mapped to human genome. **C,** Pearson correlation and hierarchical clustering for all samples except for MK1. **D,** Principle component analysis (PCA) for all samples except for MK1. Replicates within each treatment were closely clustered together.

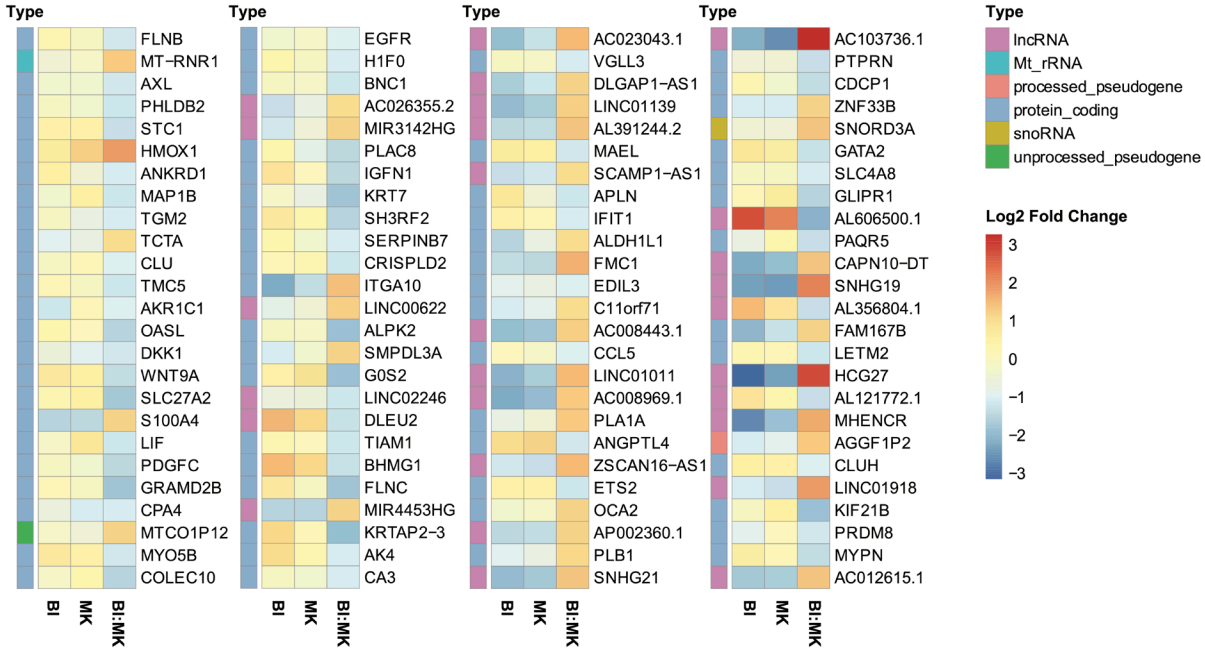


Figure S6. Heatmap of top 100 most significant DEGs with |log2 fold change| ≥1 affected by BI:MK interaction effects. The fold change for BI 6727 and MK-0752 single drug treatment, as well as the type of each gene, are also displayed.

Supplemental Methods

RealTime-Glo MT cell viability assay. SK-MEL-2 or SK-MEL-28 cells (500 cells per well) were plated in 96 well plate. 24 hours after seeding, RealTime-Glo reagent and drugs were added to the wells, and luminescence was measured at 72 h after seeding using the BioTek Synergy H1 plate reader. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test using the Prism software (GraphPad, San Diego, CA, USA). The data are expressed as the mean ± standard error of the mean with statistical significance (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$).The combination index (CI) was calculated using CalcuSyn software (Biosoft, UK).

MTT assay. A375 cells were plated at a density of 2.0×10^4 in a 12-well plate. 24 h after plating, the drugs were added to each well and the plate was incubated for 48 hours. After incubation, the media was removed and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich) reagent was added and the plate was incubated for 2 h at 37 C. After 2 h, the crystals were dissolved by mixing with dimethyl sulfoxide (DMSO) and the plate was read at 540 nm on a BioTek Synergy H1 plate reader. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test using the GraphPad Prism software. The data are expressed as the mean ± standard error of the mean with statistical significance (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$).

Survival analysis. Survival analysis of PLK1 or NOTCH1 was performed on data queried from two platforms: Protein Atlas and cBioPortal. For Protein Atlas, the survival curves of "PLK1" or "NOTCH1" were generated separately in these links (accessed on September 8, 2020): https://www.proteinatlas.org/ENSG00000148400-NOTCH1/pathology/melanoma, and https://www.proteinatlas.org/ENSG00000166851-PLK1/pathology/melanoma. For cBioPortal, the TCGA data containing the mRNA expression and clinical information of 479 melanoma patients was downloaded (accessed on April 6, 2020). According to whether the expression levels of PLK1 or NOTCH1 are greater than median expression, melanoma patients were divided into two groups. Using R, the OS between these two groups were analyzed by survival package (v2.43-3). The Kaplan–Meier curves between these two groups were plotted by survminer (v0.4.3) within R.