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Supplemental Information

Identification of CDC25 as a Common Therapeutic Target for Triple-Negative Breast Cancer

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Pathway Analysis (GSEA)

Gene expression data were analyzed using GSEA (Subramanian et al., 2005) with parameters set to 2000 gene-set permutations and gene-sets size between 8 and 500. Genes were ranked according to the t values from the Partek t-test. The gene-sets included for the GSEA analyses were obtained from KEGG, MsigDB-c2, NCI, Biocarta, IOB, Netpath, HumanCyc, Reactome and Gene Ontology (GO) databases, updated November, 2012 (http://baderlab.org/GeneSets). An enrichment map (version 1.2 of Enrichment Map software was generated for each comparison using enriched gene-sets with a nominal p-value <0.005, FDR Q value < 0.05 and the overlap coefficient set to 0.5. By determining the significantly changed genes in the comparisons Pten/p53 vs. p53 and Rb/p53 vs p53 in both human BC and mouse models, lists of similarly regulated genes in human and mouse were generated.

Cell lines and Cultures

Pten:p53 tumor lines (RB400, RB401, TC53-120, & TC53-127), Rb:p53 tumor lines (TMCRP1 & TMCRP2), and CAMA1, MDA-MB-157, MDA-MB-231, and MDA-MB-468 human BC lines were maintained in DMEM containing 10% FBS and 1% PEST, at 37°C with 5% CO2. MDA-MB-361 cells were maintained in DMEM plus 20% FBS and 1% PEST. MDA-MB-436 cells were maintained in DMEM plus 10% FBS, 1% PEST, and 10 μg/ml insulin. HCC38, HCC3153, and Bt549 were maintained in RPMI, 10% FBS and 1% PEST. TMCRP1, TMCRP2, RB400, RB401, TC53-120, and TC53-127 are mouse lines generated in our lab. Bt549, MDA-MB-436, and MDA-MB-231 were kind gifts from Dr. Mona Gauthier. The remaining lines were purchased from the America Type Culture Collection (ATCC).

Transfection with Small Interfering RNA (siRNA)

siRNAs for CDC25A and CDC25B, siRNA transfection reagent, and Opti-MEM reduced-serum transfection medium were purchased from Dharmacon. A day before transfection, $2x10^5$ cells were seeded in each of 6-well plates with regular medium and incubated for 24 h. Transfection complexes were prepared with CDC25A and CDC25B siRNA and Lipofectamine RNAiMAX Reagent, diluted in Opti-MEM. After 20 min of incubation at room temperature, 500 μl of siRNA-lipofectamine complexes was added directly to the adherent cells, which were pre-washed in PBS. Six hours later, fresh regular medium was added. A scrambled siRNA (Dharmacon) was used as negative control. The cells were incubated at 37°C 3 days before analysis. For double transfection, $8x10^4$ were seeded and transfected as above. Three days later cells were washed in PBS, re-transfected, and incubated for three more days prior to analysis.

Western Blot Analysis

BT549 cultured in 6-well dishes were treated with 0.25% trypsin (Sigma), washed with PBS, pelleted, and lysed with Ripa lysis buffer (0.15 M NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM NaF, 0.5 mM Na_3VO_4, and 1:100 protease inhibitor cocktail [1 mg/mL leupeptin, 2 μg/mL aprotinin, and 100 mM PMSF]). Protein concentration was determined by Pierce Reagent (Thermo Scientific). About 20 μg of protein was

fractionated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk in phosphate-buffered saline containing 0.05% Tween 20 (PBST) at R.T. for 1 h, washed 3 × 5 min with PBST, and incubated at 4 °C overnight with following antibodies: rabbit anti-human CDC25A, rabbit anti-human CDC25B, rabbit anti-human p-CDK1 (Y15; #9111) or total CDK1 (#9112), PARP (#9542), P-AKT (473), AKT (# 9272), pcJUN (Ser 73, #3270) or rabbit anti-tubulin primary antibody (all from Cell Signaling Technologies). Membranes were washed with PBST buffer 3 × 5 min each and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (Cell Signaling) for 1 h. Primary antibodies were diluted 1:1000 in PBS (tubulin 1:2000) with 5% BSA; secondary antibody was diluted 1:2000.

MTT Viability Assay

BT549 cells were seeded in 96-well plates at 2–5 × 10^3 cells/well and treated the following day. Three days (72 h) 30 μL of 2 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, Sigma) was added to each well and incubated for about 3 h. MTT/media solution was aspirated and replaced with 100 μL DMSO and left at R.T. for 15–20 min to dissolve the formazan dye. After gentle agitation to ensure even mixture of the dye, a 96-well microplate reader (Molecular Devices) was used to determine the optical density (OD) of each well at 570 nm. Each assay was performed in 3x or 6x replica, and repeated at least 3 times.

Apoptosis and Cell Cycle Analysis by Flow Cytometry

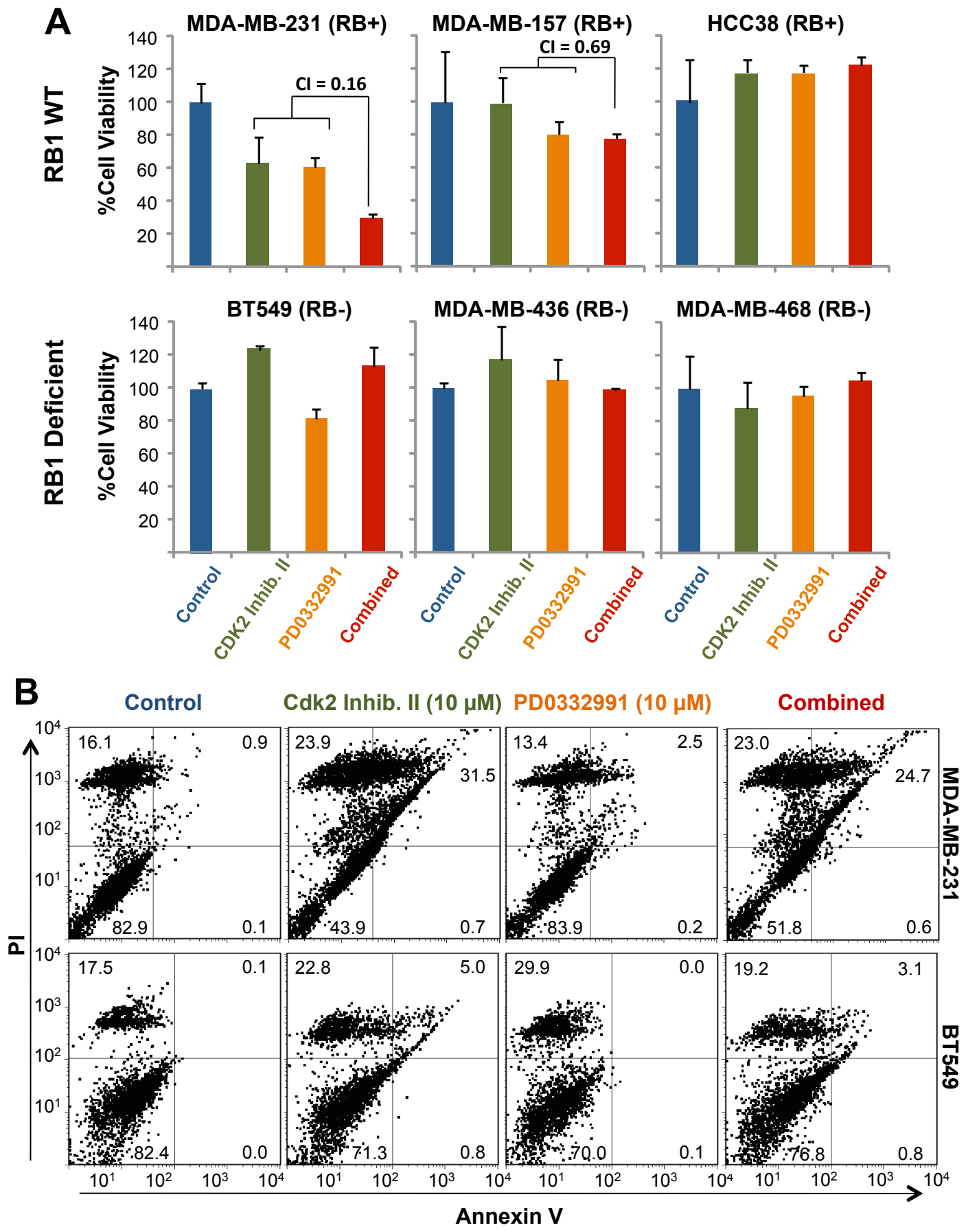
Apoptosis was detected using a PE-Annexin V Kit (BD Biosciences). MDA-MB-468 and BT549 cells were plated at optimal densities and co-transfected with CDC25A, CDC25B siRNAs. After double transfection, supernatants from each treatment group were collected before cells were trypsinized to single cell suspension. Trypsinized cells were added to their respective supernatants, washed with PBS, pelleted, resuspended in serum-free PBS, and counted. The cells were resuspended in 1× Annexin-V binding buffer: in a volume of 100 μL 1× Annexin-V binding buffer, 0.5–1.0 × 10^6 cells were incubated with 5 μL Annexin-V-PE and 5 μL PI in the dark, at R.T. for 15–20 min with occasional pulse vortexing. Then, an additional 400 μL 1× Annexin V binding buffer was added to each sample. Finally, cells were strained to single cells into 5 mL polystyrene round-bottom FACS tubes (BD Falcon), placed on ice, and processed on a FACSCaliber (Becton Dickinson) no longer than 1 h post-staining.

Cell cycle analysis was performed using PI/RNAse Staining Buffer (BD Sciences). Cells were treated with DMSO (vehicle control), WEE1 inhibitor (MK-1775), or CDC25 inhibitors (BN82002 and NSC663284). Due to different kinetics of cell killing, MK-1775 and NSC663284 treated cells were collected after 24 hours, whereas DMSO and BN82002 treated cells were collected after 48 hours of treatment. Prior to trypsinization, cell supernatants were collected. Supernatants were later combined with their respective trypsinized cell suspensions, pelleted, washed with PBS, and fixed in 66% Ethanol. Fixed cells were washed with PBS, resuspended in PI/RNAse staining buffer and incubated in the dark at 37ºC for 1 hour. Next cells were strained into a 5mL polysterene round-bottom FACS tubes (BD Falcon), and placed on ice. Samples were

processed on LSRII (BD Sciences) at the SickKids-UHN Flow and Mass Cytometry facility, and results were analyzed using FlowJo Software.

Additional Statistical Analysis

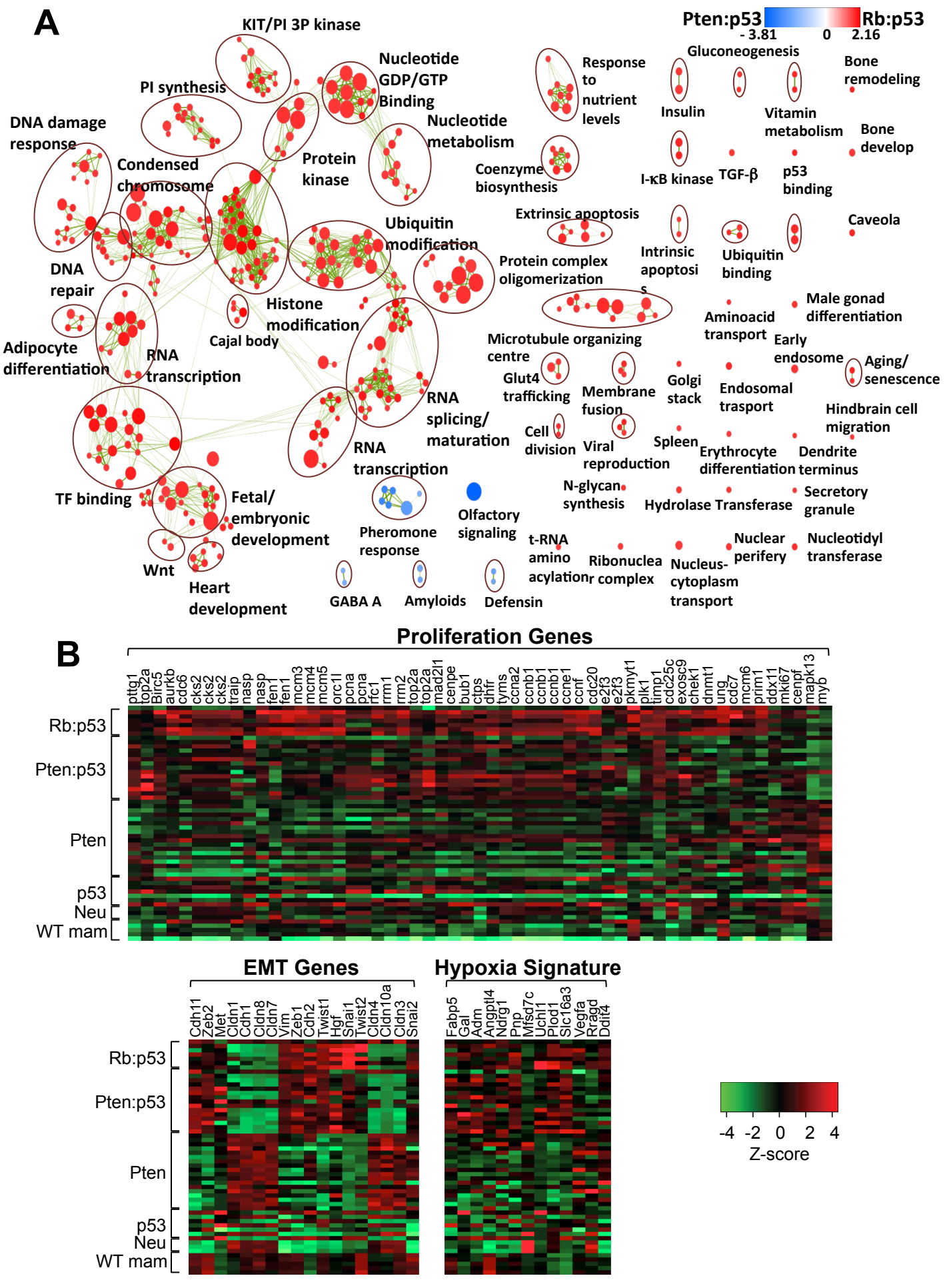
Significance of comparing multiple samples was calculated using ANOVA and the Tukey test for *post hoc* analysis. Chi-square test was used to compare two categorical variables.


A
MDA-MB-231 (RB+)
MDA-MB-157 (RB+)
HCC38 (RB+)
RB1 WT
%Cell Viability
CI = 0.16
CI = 0.69
BT549 (RB-)
MDA-MB-436 (RB-)
MDA-MB-468 (RB-)
RB1 Deficient
Control
CDK2 Inhib. II
PD0332991
Combined
B
Control
Cdk2 Inhib. II (10 μM)
PD0332991 (10 μM)
Combined
MDA-MB-231
BT549
PI
Annexin V

Supplemental Figure S1: Combined Effects of CDK4/6 inhibitor PD0332991 plus CDK2 Inhibitor II on RB1$^+$ vs RB1$^-$ TNBC Cells. Related to Figures 1-2.

(A) Responses of RB1$^+$ (MDA-MB-231, MDA-MB-157, & HCC38) and RB1$^-$ (BT549, MDA-MB-436, & MDA-MB-468) cells to single or combination treatments with CDK2 Inhibitor II (1 μM) and/or PD0332991 (1 μM). Synergy between the two compounds was assessed by Combination Index (CI) using Compusyn Software.

(B) Apoptosis was assessed by Annexin V and PI staining followed by flow cytometry for the RB1$^+$ MDA-MB-231 vs RB1$^-$ BT549 cells treated with single or combination of CDK2 Inhibitor II (10 μM) and PD0332991 (10 μM).


A
Pten:p53
Rb:p53
- 3.81
0
2.16
KIT/PI 3P kinase
PI synthesis
Nucleotide GDP/GTP Binding
Nucleotide metabolism
Protein kinase
DNA damage response
Condensed chromosome
DNA repair
Adipocyte differentiation
RNA transcription
Cajal body
Histone modification
Ubiquitin modification
RNA splicing/ maturation
RNA transcription
TF binding
Fetal/ embryonic development
Wnt
Heart development
Pheromone response
Olfactory signaling
GABA A
Amyloids
Defensin
Response to nutrient levels
Coenzyme biosynthesis
Extrinsic apoptosis
Protein complex oligomerization
Microtubule organizing centre
Glut4 trafficking
Membrane fusion
Golgi stack
Endosomal trasport
Cell division
Viral reproduction
Spleen
Erythrocyte differentiation
N-glycan synthesis
Hydrolase
Transferase
t-RNA amino acylation
Ribonuclear complex
Nucleus-cytoplasm transport
Nuclear perifery
Gluconeogenesis
Insulin
Vitamin metabolism
Bone remodeling
I-κB kinase
TGF-β
p53 binding
Bone develop
Intrinsic apoptosis
Ubiquitin binding
Caveola
Aminoacid transport
Male gonad differentiation
Early endosome
Aging/ senescence
Hindbrain cell migration
Dendrite terminus
Secretory granule
Nucleotidyl transferase
B
Proliferation Genes
pttg1 top2a Birc5 aurkb cdc6 cks2 cks2 cks2 traip nasp nasp fen1 fen1 mcm3 mcm4 mcm5 orc1l pcna pcna rfc1 rrm1 rrm2 top2a top2a mad2l1 cenpe bub1 ctps dhfr tyms ccna2 ccnb1 ccnb1 ccnb1 ccne1 ccnf cdc20 e2f3 e2f3 pkmyt1 plk1 timp1 cdc25c exosc9 chek1 dnmt1 ung cdc7 mcm6 prim1 ddx11 mki67 cenpf mapk13 myb
Rb:p53
Pten:p53
Pten
p53
Neu
WT mam
EMT Genes
Cdh11 Zeb2 Met Cldn1 Cdh1 Cldn8 Cldn7 Vim Zeb1 Cdh2 Twist1 Hgf Snai1 Twist2 Cldn4 Cldn10a Cldn3 Snai2
Hypoxia Signature
Fabp5 Gal Adm Angptl4 Ndrg1 Pnp Mfsd7c Uchl1 Plod1 Slc16a3 Vegfa Rragd Ddit4
-4 -2 0 2 4
Z-score

Supplemental Figure S2: Complete GSEA and Gene expression Analysis of Pten/p53- and Rb/p53-Deficient Mammary Tumors. Related to Fig. 1.

(A) Complete GSEA shown in Fig. 1C.

(B) Expression of Proliferation, EMT and Hypoxia genes in mouse tumor models Pten/p53, Rb/p53, Pten only, p53 only, MMTV-Neu, and WT mammary glands.

Average Expression

	WTMam	Neu	p53	Pten	Pten:p53	Rb:p53
Cdc25a	811.64	920.39	898.83	825.11	793.54	1079.28
Cdc25b	438.83	488.12	626.28	393.20	550.86	647.76
Cdc25c	138.69	348.27	312.33	236.48	249.61	315.46

Standard Deviation

	WTMam	Neu	p53	Pten	Pten:p53	Rb:p53
Cdc25a	327.10	130.05	297.88	216.74	157.32	217.46
Cdc25b	31.29	105.40	281.43	71.01	143.63	251.22
Cdc25c	52.79	58.21	163.79	76.12	69.31	76.93

P-values of ANOVA with Tukey Posthoc Comparing Rb:p53 to:

	WTMam	Neu	p53	Pten	Pten:p53	Rb:p53
Cdc25a	0.335764	0.97396	0.674326	0.236644	0.189294	--
Cdc25b	0.230595	0.732946	0.998726	**0.002359**	0.840568	--
Cdc25c	**0.000683**	0.995889	0.991388	0.317711	0.603902	--

P-values of ANOVA with Tukey Posthoc Comparing Pten:p53 to:

	WTMam	Neu	p53	Pten	Pten:p53	Rb:p53
Cdc25a	0.999929	0.947293	0.988369	0.999896	--	0.189294
Cdc25b	0.660437	0.985783	0.977265	**0.00889**	--	0.840568
Cdc25c	**0.008522**	0.536102	0.941812	0.993662	--	0.603902

P-values of ANOVA with Tukey Posthoc Comparing p53 to:

	WTMam	Neu	p53	Pten	Pten:p53	Rb:p53
Cdc25a	0.983092	0.998623		0.997236	0.988369	0.674326
Cdc25b	0.413813	0.884677		**0.009594**	0.977265	0.998726
Cdc25c	**0.003675**	0.929119		0.745417	0.941812	0.991388

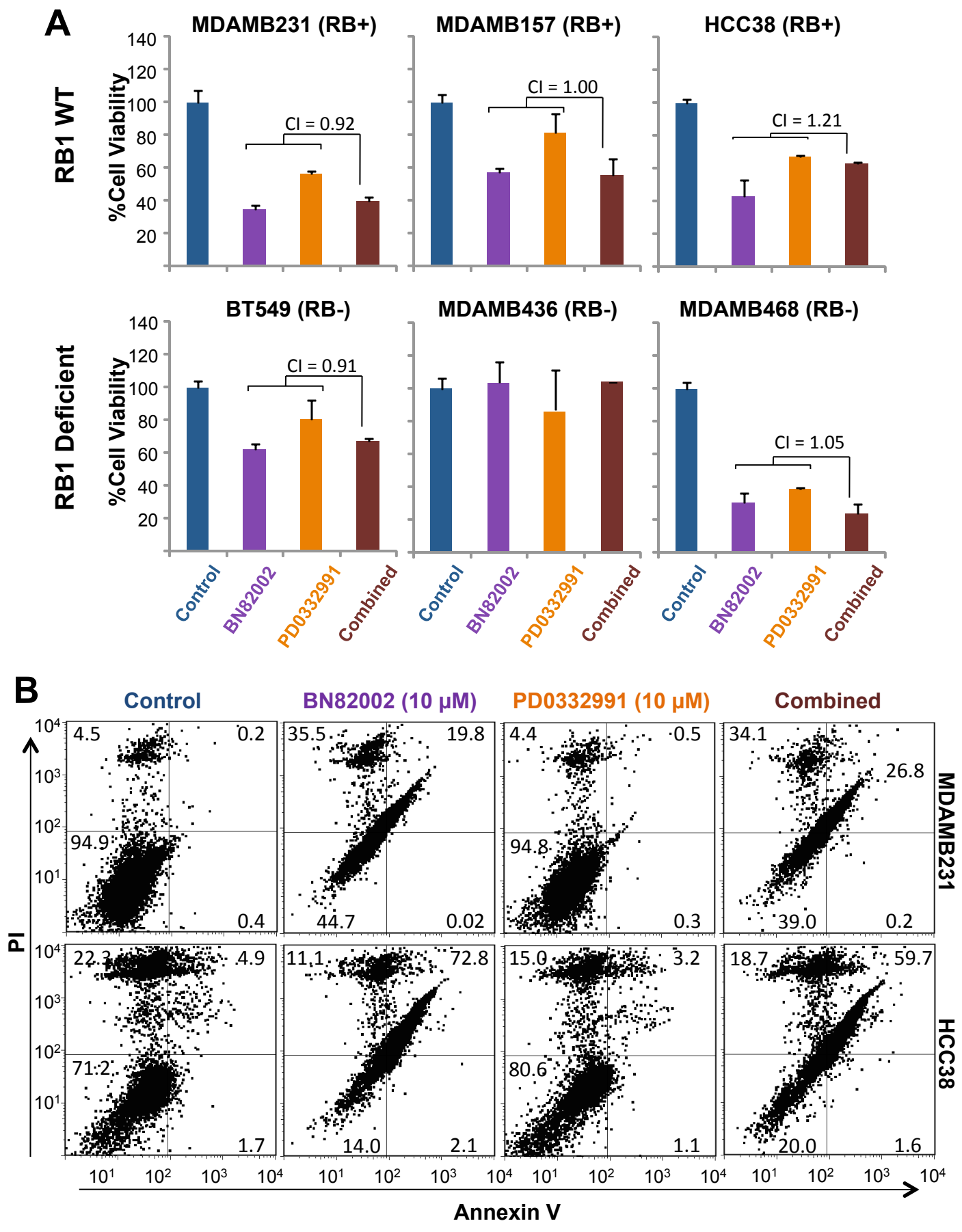
Supplemental Figure S3: Statistical analysis accompanying the heat map shown in Fig. 1D.

CDC25A vs. Oncogenes/ Tumor Suppressors	Normal-like		Luminal A		Luminal B		Basal-like		HER2	
	r	p	r	p	r	p	r	p	r	p
RB1 Loss	0.6406	0.0000	0.5220	0.0000	0.5122	0.0000	0.6813	**0.0000**	0.3733	0.0000
PTEN low	-0.2983	0.0002	-0.0937	0.0384	-0.0279	0.6188	-0.1673	**0.0147**	-0.0609	0.4965
P53 Pathway	-0.2739	0.0006	-0.2890	0.0000	-0.3401	0.0000	-0.3222	**0.0000**	-0.1277	0.1526
ER Pathway	0.3334	0.0000	0.1032	0.0226	0.1474	0.0082	-0.1377	**0.0452**	0.1191	0.1822

CDC25B vs. Oncogenes/ Tumor Suppressors	Normal-like		Luminal A		Luminal B		Basal-like		HER2	
	r	p	r	p	r	p	r	p	r	p
RB1 Loss	0.3232	0.0000	0.3193	0.0000	0.3995	0.0000	0.2599	**0.0001**	0.2088	0.0185
PTEN low	-0.0631	0.4366	-0.1768	0.0001	-0.1181	0.0344	-0.0961	0.1633	-0.1050	0.2402
P53 Pathway	-0.5733	0.0000	-0.3842	0.0000	-0.3210	0.0000	-0.2165	**0.0015**	-0.1984	0.0254
ER Pathway	-0.2305	0.0040	-0.0507	0.2638	0.0016	0.9770	-0.1377	**0.0452**	-0.1367	0.1255

CDC25C vs. Oncogenes/ Tumor Suppressors	Normal-like		Luminal A		Luminal B		Basal-like		HER2	
	r	p	r	p	r	p	r	p	r	p
RB1 Loss	0.7687	0.0000	0.7300	0.0000	0.5741	0.0000	0.6672	**0.0000**	0.3812	0.0000
PTEN low	-0.3253	0.0000	-0.2194	0.0000	-0.0823	0.1412	-0.2841	**0.0000**	-0.0591	0.5096
P53 Pathway	-0.2044	0.0110	-0.3499	0.0000	-0.2170	0.0001	-0.1857	**0.0067**	0.0408	0.6484
ER Pathway	0.3957	0.0000	0.1569	0.0005	0.1736	0.0018	0.0111	0.8721	0.2840	0.0012

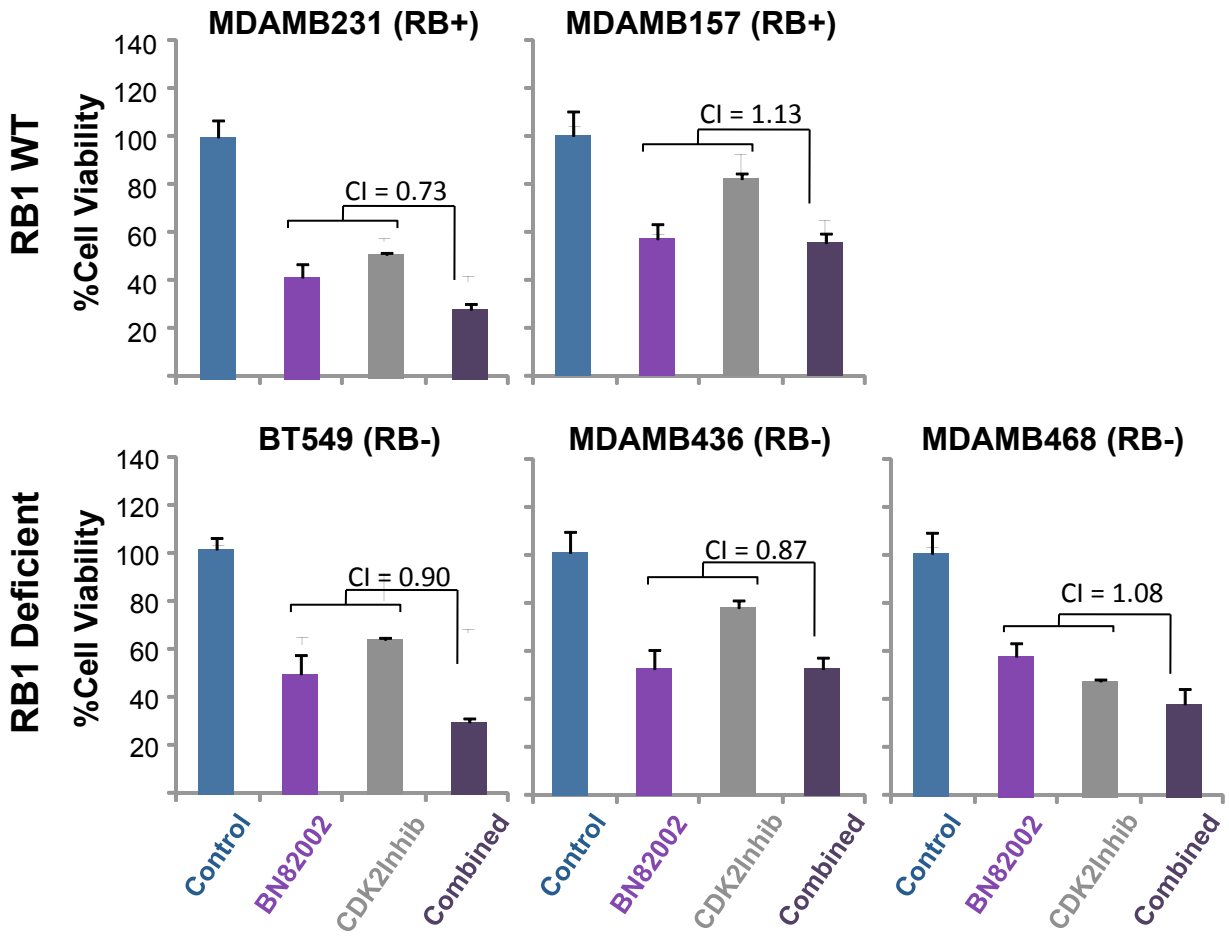
Supplementary Figure S4: Statistical Analysis Showing Correlation Between CDC25 Gene Family Expression and RB1, PTEN or TP53 Loss or ER Signature in Different Breast Cancer Subtypes. Related to Fig. 4B.


A
MDAMB231 (RB+)
MDAMB157 (RB+)
HCC38 (RB+)
RB1 WT
%Cell Viability
CI = 0.92
CI = 1.00
CI = 1.21
BT549 (RB-)
MDAMB436 (RB-)
MDAMB468 (RB-)
RB1 Deficient
CI = 0.91
CI = 1.05
Control
BN82002
PD0332991
Combined
B
Control
BN82002 (10 μM)
PD0332991 (10 μM)
Combined
MDAMB231
HCC38
PI
Annexin V
4.5
0.2
94.9
0.4
35.5
19.8
44.7
0.02
4.4
0.5
94.8
0.3
34.1
26.8
39.0
0.2
22.3
4.9
71.2
1.7
11.1
72.8
14.0
2.1
15.0
3.2
80.6
1.1
18.7
59.7
20.0
1.6

Supplemental Figure S5: Effects of Combined Treatment of TNBC Cells with BN82002 plus CDK4/6 inhibitor PD0332991. Related to Fig. 6.

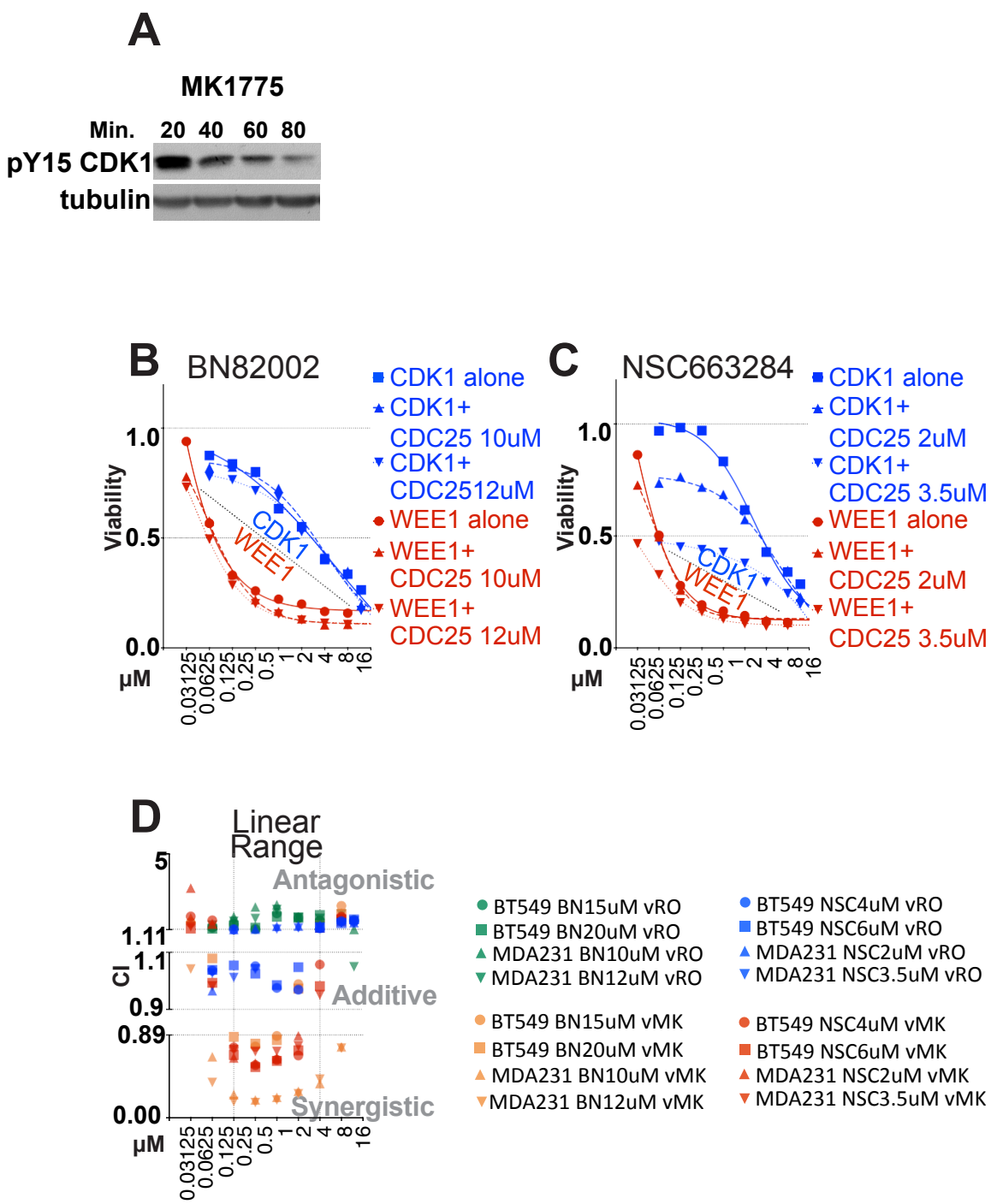
(A) Responses of $RB1^+$ (MDA-MB-231, MDA-MB-157, & HCC38) and $RB1^-$ (BT549, MDA-MB-436, & MDA-MB-468) TNBC cell lines to single or combination treatments with BN82002 (10 μM) and PD0332991 (1 μM). Levels of synergy between the two compounds were assessed by Combination Index (CI) using Compusyn Software.

(B) Apoptosis was determined by Annexin V / PI staining and flow cytometry for the $RB1^+$ MDA-MB-231 and $RB1^-$ BT549 cells after single or combination treatments with BN82002 (10 μM) and PD0332991 (10 μM).



Supplemental Figure S6: Effects of Combined Treatment of TNBC Cells with BN82002 plus CDK2 Inhibitor II. Related to Fig. 6.

Responses of RB1^{+} (MDA-MB-231 & MDA-MB-157) and RB1^{-} (BT549, MDA-MB-436, & MDA-MB-468) cells to single or combinations of BN82002 (10 μM) and CDK2 Inhibitor II (10 μM).

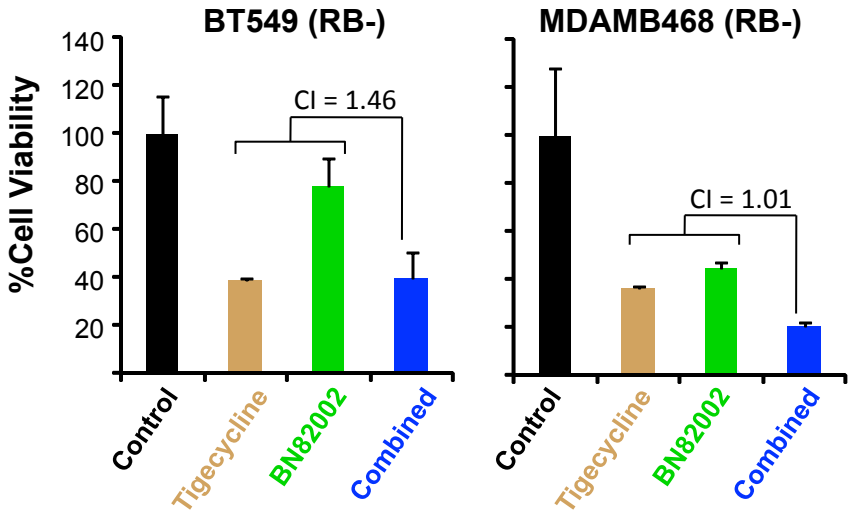


Supplemental Figure S7: Synergy between CDC25 and WEE1 inhibitors and detailed Compusyn analysis. Related to Fig. 6.

(A) Inhibition of CDK1 Tyr15 phosphorylation by the WEE1 inhibitor, MK1775.

(B-C) Combined CDC25 inhibitors (BN82002 or NSC663284) plus CDK1 inhibitor (RO3306) show an additive/antagonistic effects, whereas CDC25 plus the WEE1 inhibitor (MK1775) are mostly synergistic in MDA-MB-231 TNBC cells. Similar analysis in BT549 is shown in Fig. 6A-B.

(D) Detailed combination index calculated by Compusyn Software for the two CDC25 inhibitors plus CDK1 or WEE1 inhibitors in BT549 and MDA-MB-231, accompanying Fig. 6C.



Supplemental Figure S8: Responses of RB1$^-$ TNBC Cell Lines BT549 and MDA-MB-468 to BN82002 (10 μM) Alone or in Combination with tigecycline (10 μM). Related to Fig. 6.
Lack of synergy between the two compounds was uncovered by Combination Index (CI) using Compusyn Software. Similar analysis of MDA-MB-436 showing strong synergy in vitro and in vivo is depicted in Fig. 6F-I.