

Supplementary Information

CDK2 Inhibitor BLU-222 Synergizes with CDK4/6 Inhibitors in Drug Resistant Breast Cancers through p21/p27 Induction

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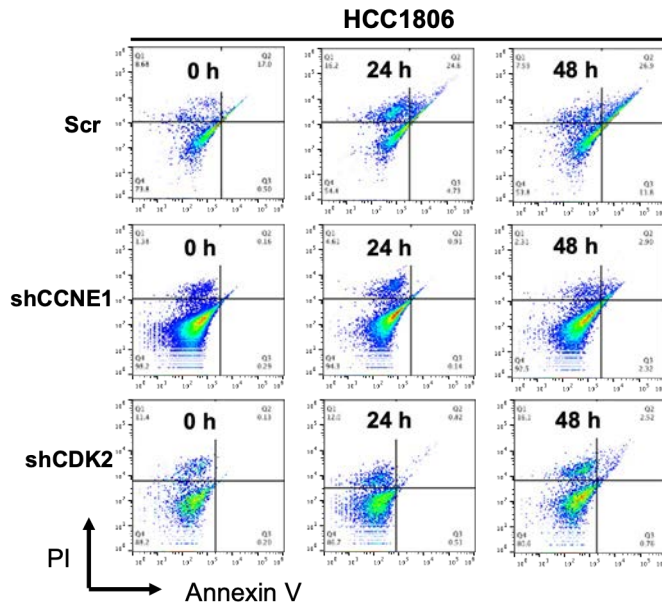
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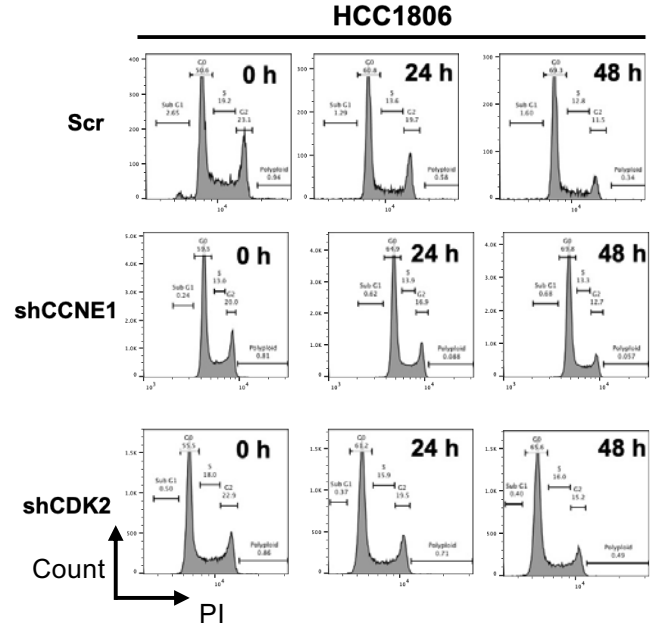
Running title: Synergistic Effects of BLU-222 with CDK4/6 Inhibitor

Supplemental Figure 1

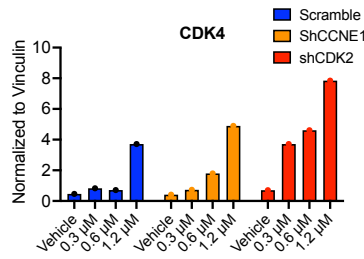
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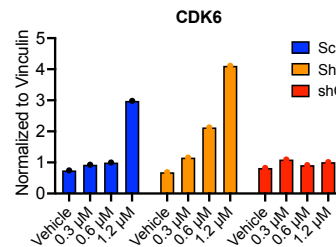
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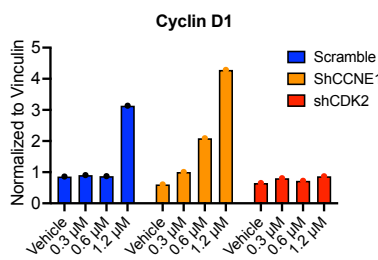
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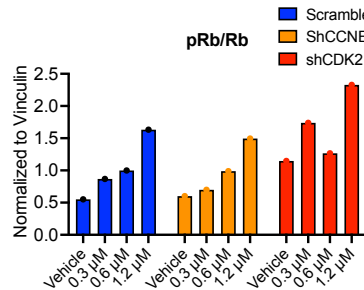
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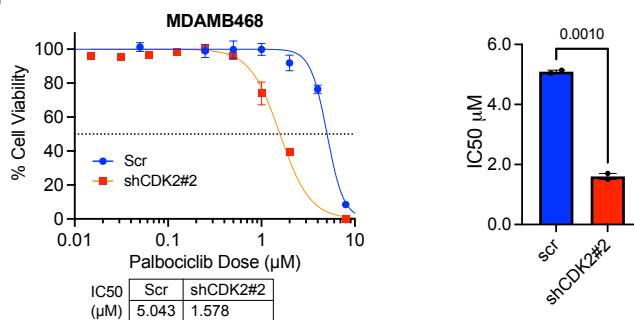
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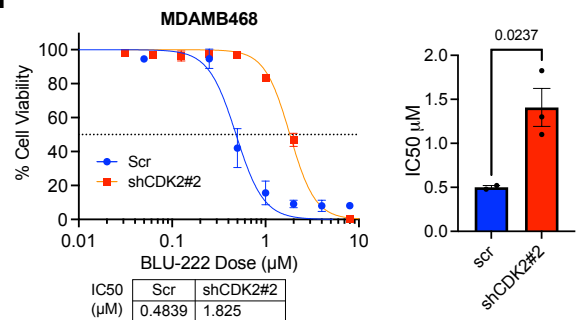
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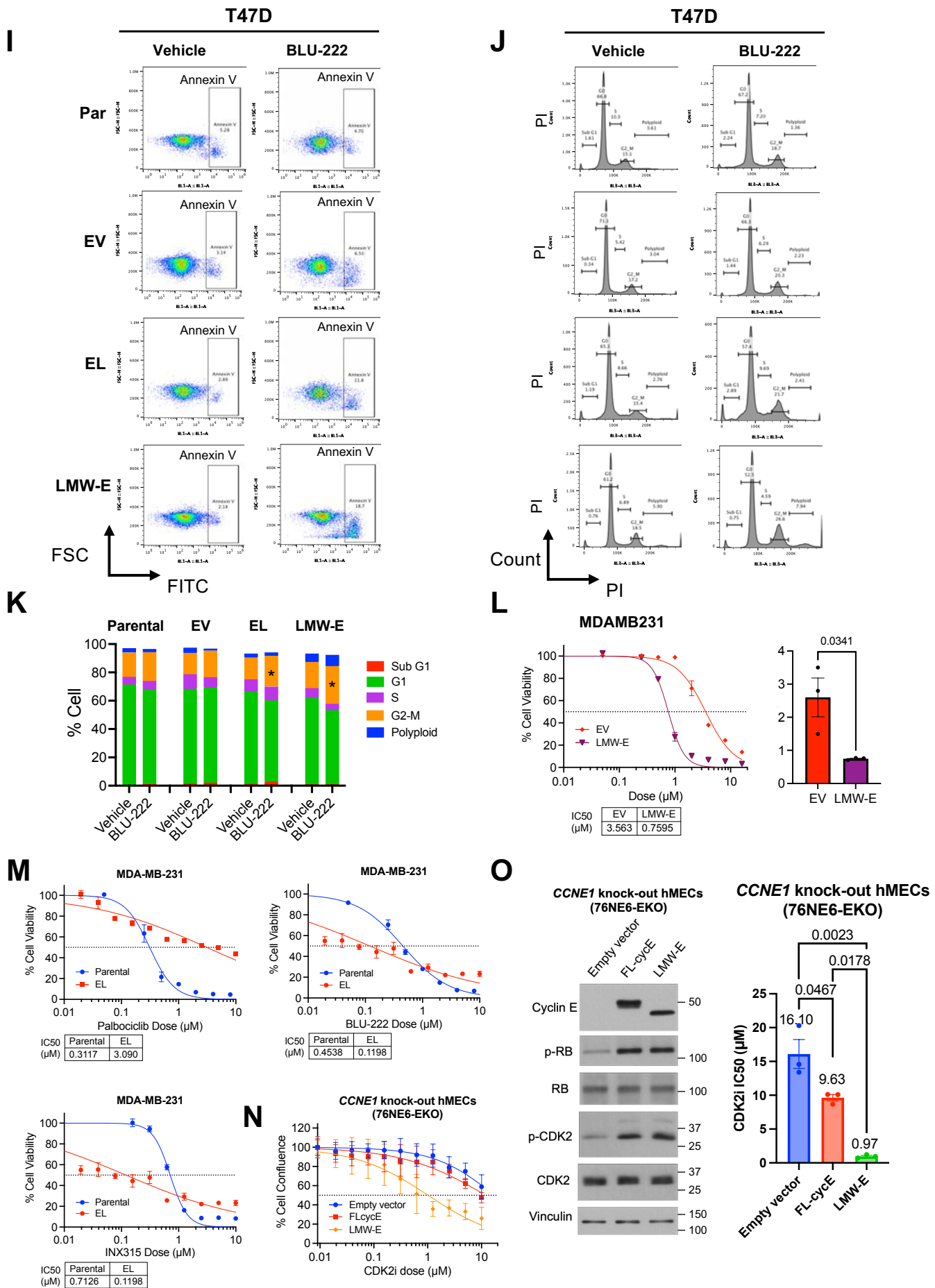
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Supplemental Figure 1

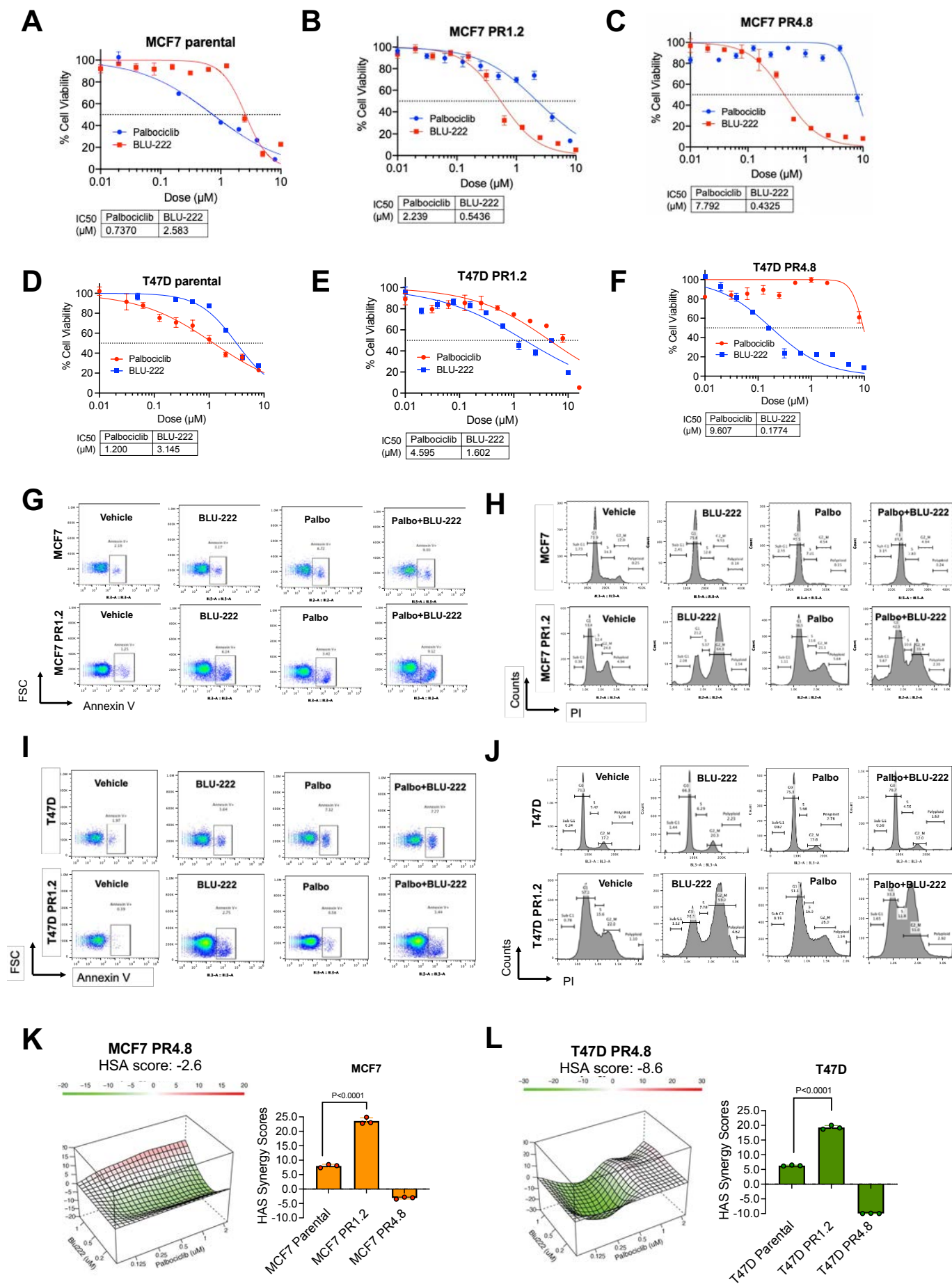


Supplemental Figure 1 | Cyclin E Overexpression Sensitizes CDK4/6 Inhibitor–Resistant Cells to BLU-222, a Selective and Potent CDK2 Inhibitor

(A) Representative flow cytometry gating of Annexin V (+) apoptotic HCC1806 panel cells treated with single-agent BLU-222 after 0, 24 and 48 hours. (B) Representative histogram of PI staining showing DNA content of HCC1806 panel cells treated with single-agent BLU-222 after 0, 24 and 48 hours. (C–F) Densitometry quantification of Western blots of (C) CDK4, (D) CDK6, (E) cyclin D1, and (F) pRb/Rb levels in HCC1806 panel cells treated with different doses of BLU-222 treatments for 3 days. (G) Representative dose-response curves and bar graph displaying the IC_{50} values for MDA-MB-468 scramble control and *CDK2* knockdown cell lines treated with various doses of (G) single-agent palbociclib and (H) single-agent BLU-222. Error bars represent standard error of the mean (SEM) from $n = 3$ biological replicates across 3 independent experiments. (I) Representative flow cytometry gating of Annexin V (+) apoptotic T47D parental cells and cells with overexpression of EL and LMW-E treated with vehicle or single-agent 0.3 μ M BLU-222. (J) Cell cycle distribution of T47D parental cells and cells with overexpression of EL and LMW-E treated with vehicle or single-agent 0.3 μ M BLU-222, measured by flow cytometry using PI staining. (K) Cell cycle distribution of T47D parental cells and cells with overexpression of EL and LMW-E treated with vehicle or single-agent 0.3 μ M BLU-222, measured by flow cytometry using PI staining. The experiments were done as 2 biological replicates. (L) Representative dose-response curves and bar graph displaying the IC_{50} values for MDA-MB-231 cells with empty vector control and cells with overexpression of LMW-E cyclin E treated with various doses of single-agent BLU-222. Error bars represent SEM from $n = 3$ biological replicates across 3 independent experiments. (*, $p < 0.05$; **, $p < 0.01$). (M) Dose–response curves showing the effect and IC_{50} values of palbociclib, BLU-222, and INX-315 on cell viability in MDA-MB-231 parental versus full-length cyclin E overexpression (EL) cells. Error bars represent SEM from $n = 3$ biological replicates across 3 independent experiments. (N) Dose-response curves of CDK2 inhibitor BLU-222 treatment in CCNE1 knock-out human mammary epithelial cells (hMECs;

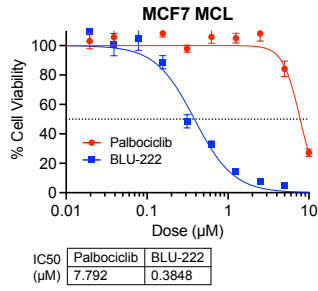
76NE6-EKO) expressing empty vector, full-length Cyclin E (FL-cycE), or low molecular weight Cyclin E (LMW-E). (●) Immunoblot analysis in CCNE1 knock-out hMECs expressing empty vector, FL-cycE, or LMW-E. Right: quantification of CDK2 inhibitor IC_{50} values from panel N. Bars represent mean $\pm$ SEM; statistical significance determined by one-way ANOVA with Tukey's post-test.

Supplemental Figure 2

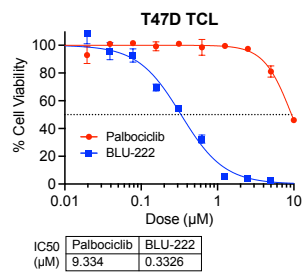


Supplemental Figure 2

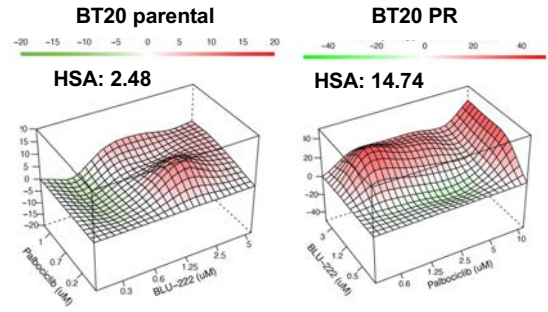
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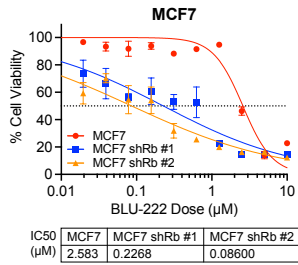
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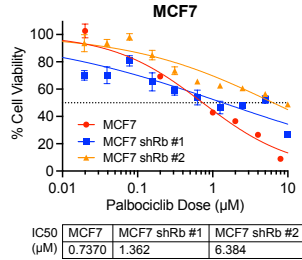
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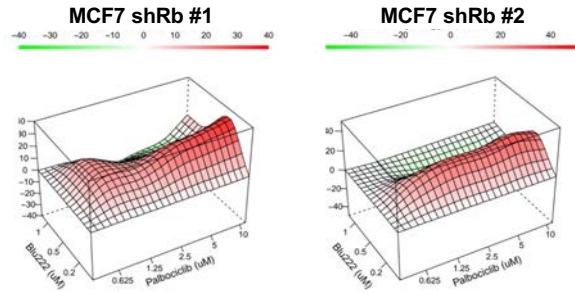
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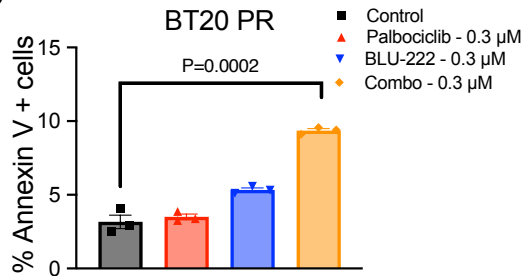
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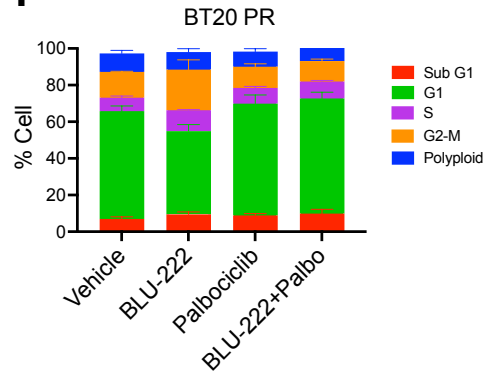
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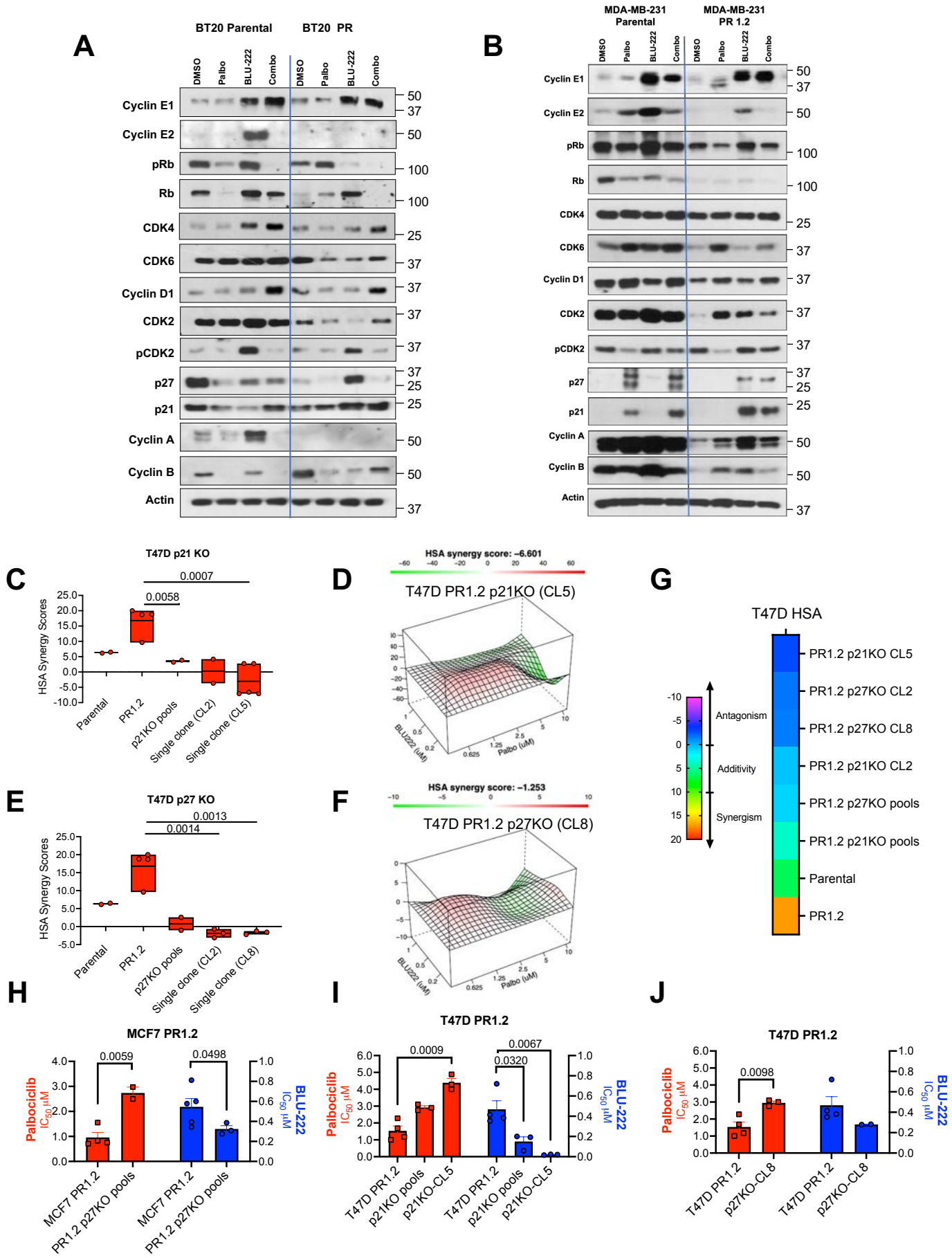


Supplemental Figure 2 | Palbociclib Resistance Sensitizes Cells to BLU-222

(A-C) Representative dose-response curves for MCF7 parental, PR1.2, and PR4.8 cell lines treated with various doses of palbociclib or BLU-222. (D-F) Representative dose-response curves for T47D parental, PR1.2, and PR4.8 cell lines treated with various doses of palbociclib or BLU-222. (G) Representative flow cytometry plots showing apoptotic Annexin V (+) cells in MCF7 parental and PR1.2 cells treated with a combination of 0.3 μ M palbociclib and 0.3 μ M BLU-222 or single agents for 3 days. (H) Representative histogram of PI staining showing DNA content in MCF7 parental and PR1.2 cells treated with a combination of palbociclib and BLU-222 or single agents for 3 days. (I) Representative flow cytometry plots showing apoptotic Annexin V (+) cells in T47D parental and PR1.2 cells treated with a combination of 0.3 μ M palbociclib and 0.3 μ M BLU-222 or single agents for 3 days. (J) Representative histogram of PI staining showing DNA content in T47D parental and PR1.2 cells treated with a combination of palbociclib and BLU-222 or single agents for 3 days. (J) Quantification of Annexin V (+) cells treated with single-agent and combination of BLU-222 and palbociclib in BT20 PR cells. Error bars represent SEM from $n = 3$ biological replicates across 3 independent experiments. (K) Representative 3D synergy plots illustrating the response of MCF7 PR4.8 cells to combination treatment with palbociclib and BLU-222. Bar graph illustrating the HSA synergistic scores for MCF7 parental, PR1.2, and PR4.8 cells treated with the combination of palbociclib and BLU-222. Error bars represent SEM from $n = 3$ biological replicates across 3 independent experiments. (L) Representative 3D synergy plots illustrating the response of T47D PR4.8 cells to combination treatment with palbociclib and BLU-222. Bar graph illustrating the HSA synergistic scores for T47D parental, PR1.2, and PR4.8 cells treated with the combination of palbociclib and BLU-222. (M-N) Representative dose-response curves for MCL and TCL cell lines treated with various doses of palbociclib or BLU-222. (O-P) Dose-response curves of MCF7 parental and shRNB clones (#1 and #2) treated with (O) BLU-222 and (P) palbociclib monotherapy. (Q) Synergy analysis of palbociclib and BLU-222 in BT20 parental and PR cells. Three-dimensional surface plots show drug interaction landscapes, with

synergy quantified using the HSA model. **(R)** Synergy analysis of BLU-222 and palbociclib in MCF7 cells with Rb knockdown (shRb #1 and shRb #2), quantified using the HSA model. **(S)** Annexin V (+) quantification in BT20PR cells treated with a combination of palbociclib and BLU-222, along with single-drug treatments. Cells were treated with 0.3 μ M of each drug for 3 days. Error bars represent SEM from n = 3 experimental replicates. **(T)** Cell cycle distribution of BT20PR cells treated with a combination of palbociclib and BLU-222, along with single-drug treatments, measured by flow cytometry using PI staining. Cells were treated with 0.3 μ M of each drug for 3 days. The experiments were done as 2 biological replicates.

Supplemental Figure 3



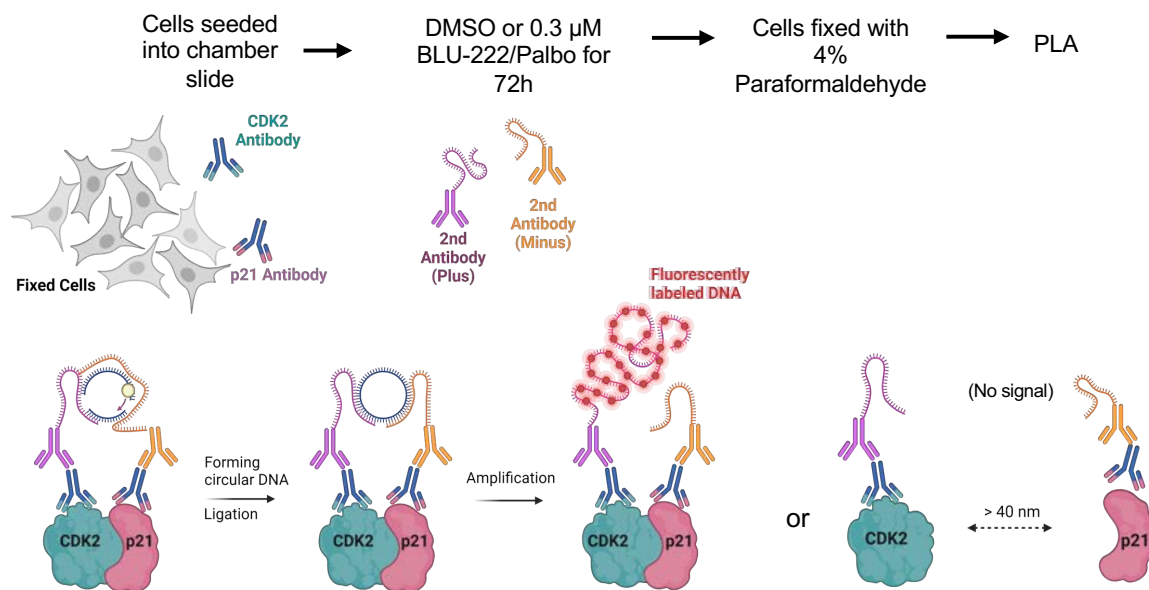
Supplemental Figure 3 | Induction of p21 and p27 by BLU-222 in PR Cells Mediates Synergistic Response to CDK4/6 Inhibitors

(A-B) Representative Western blots showing expression levels of cyclins E1, E2, A, B1, D1, CDK2, CDK4, CDK6, phosphorylated and total Rb, p21, p27 and actin in (A) BT20 parental and PR cell lines and (B) MDA-MB-231 parental and PR1.2 cell lines treated with 0.3 μ M of single agents or a combination of palbociclib and BLU-222 for 3 days. (C) Bar graph showing the HSA synergistic scores in T47D parental (n = 3), PR1.2 (n = 4) and p21 KO pooled (n = 3) and single clones of cells (CL2, n = 2; CL5, n = 5) treated with the combination of BLU-222 and palbociclib. (D) Representative 3D synergy plots illustrating the response of T47D PR1.2 p21 KO single clone (CL5) to combination treatment with different doses of palbociclib and BLU-222. (E) Bar graph showing the HSA synergistic scores in T47D parental (n = 2), PR1.2 (n = 4) and p27 KO pooled (n = 2) and single clones of cells (n = 3 for each cell line) treated with the combination of BLU-222 and palbociclib. (F) Representative 3D synergy plots illustrating the response of T47D PR1.2 p27 KO single clone (CL8) to combination treatment with different doses of palbociclib and BLU-222. (G) Heatmap showing HSA scores across T47D parental and PR1.2 CRISPR KO pools and single clones for p21 or p27. (H) Bar graph showing the IC₅₀ of MCF7 PR1.2 parental (n = 5) and CRISPR KO p27 pools (n = 3) treated with palbociclib or BLU-222 as single agents. (I) Bar graph showing the IC₅₀ of T47D PR1.2 parental (n = 4) and CRISPR KO p21 pools (n = 3) or single clone (CL5, n = 3) treated with palbociclib or BLU-222 as single agents. (J) Bar graph showing the IC₅₀ of T47D PR1.2 parental (n = 4) and CRISPR KO p27 single clone (CL8, n = 3) treated with palbociclib or BLU-222 as single agents. Error bars represent the SEM from n biological replicates performed across three independent experiments.

Supplemental Figure 4

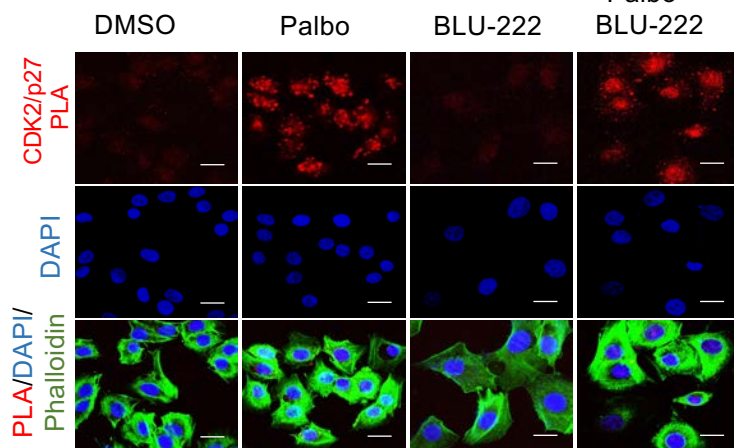
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Proximity Ligation Assay

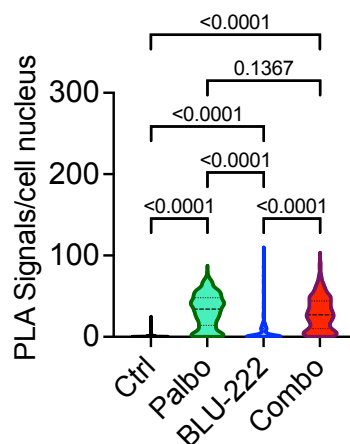


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MCF7 parental 72h

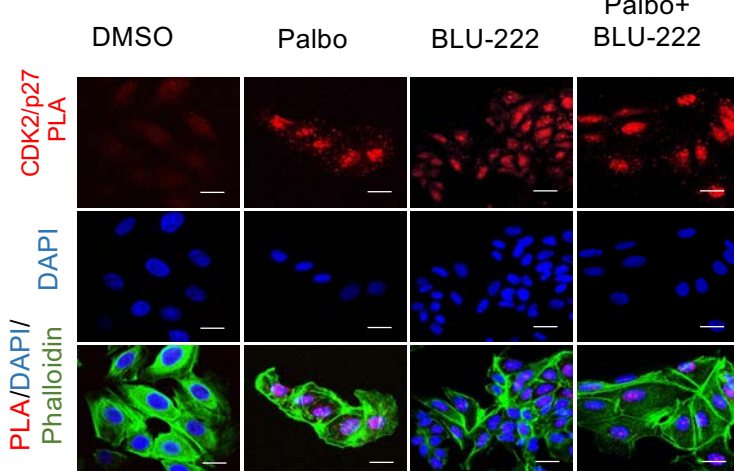


MCF7 Parental: CDK2-p27

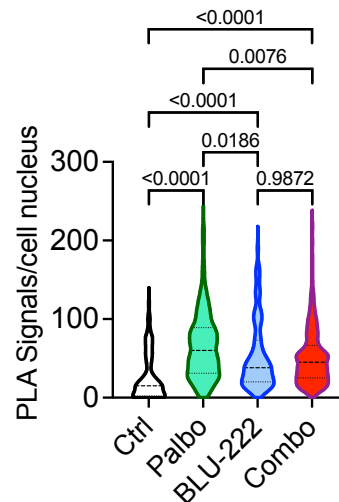


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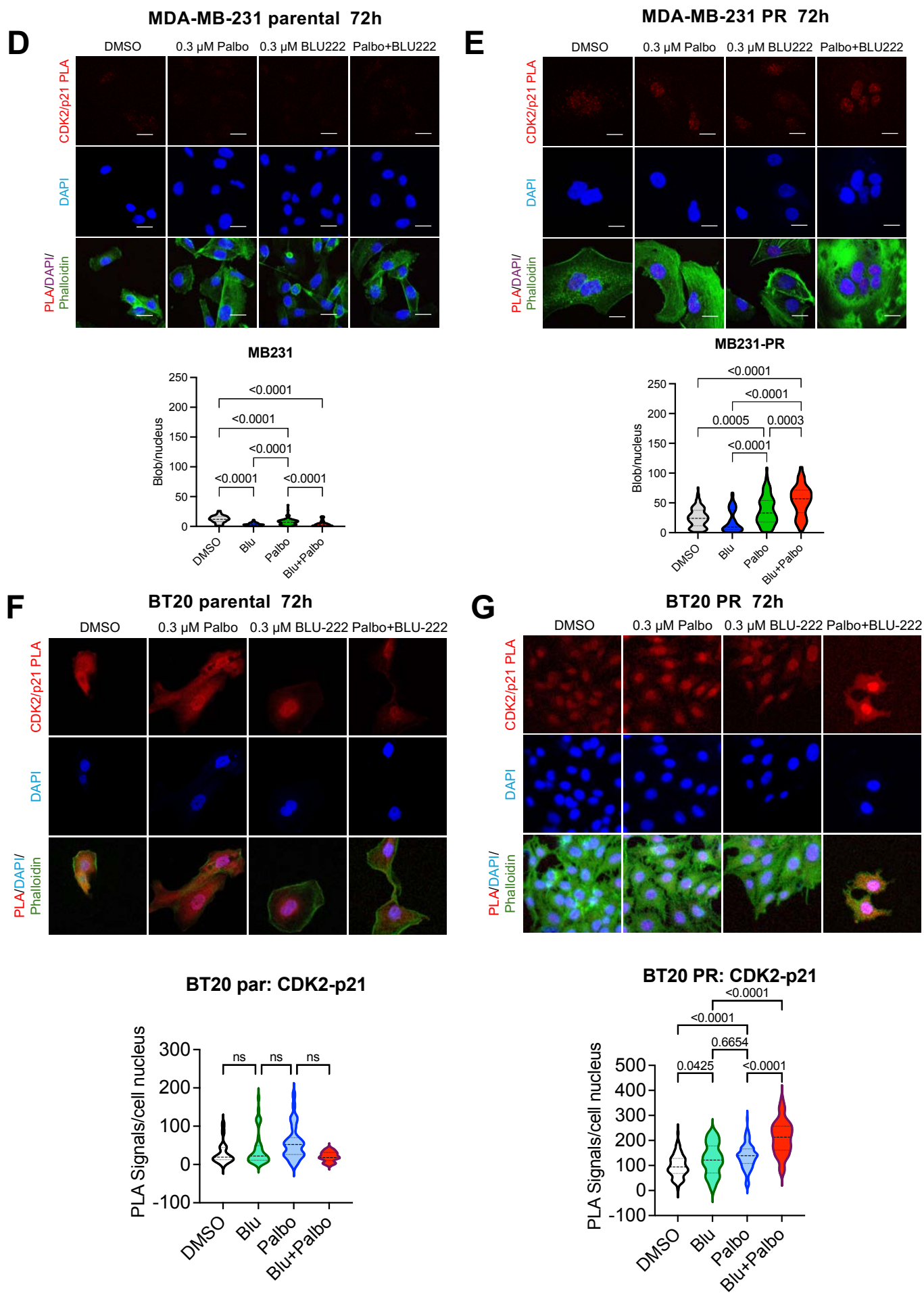
MCF7 PR1.2 72h



MCF7 PR1.2: CDK2-p27

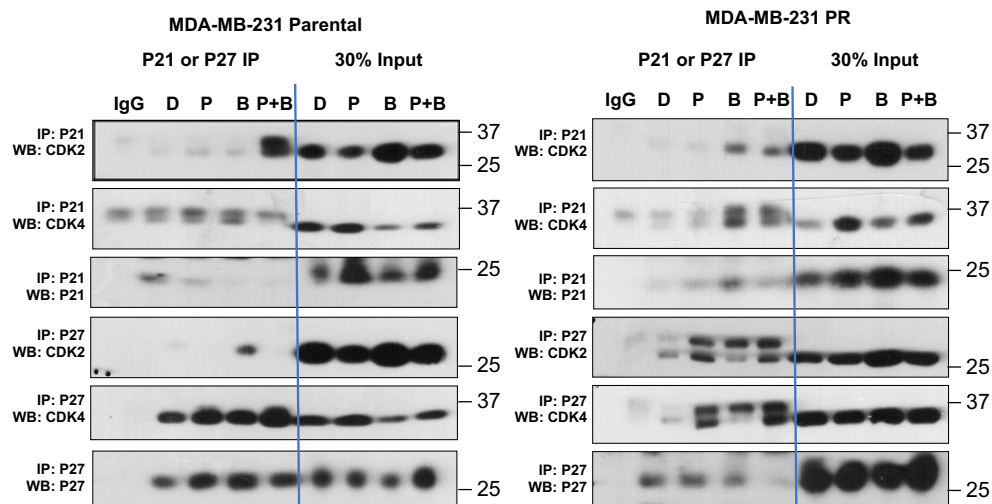


Supplemental Figure 4

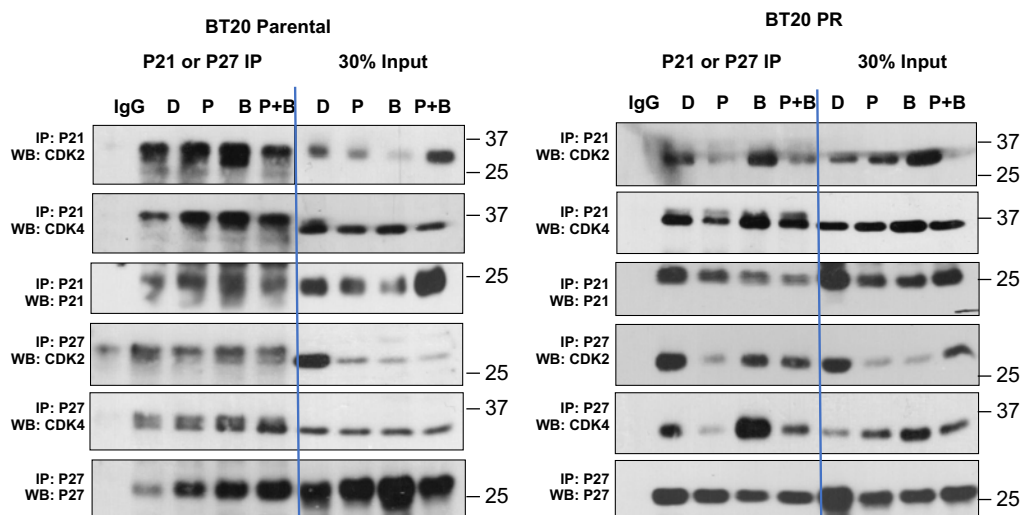


Supplemental Figure 4

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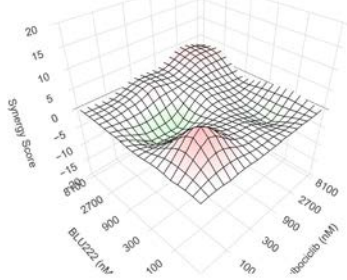


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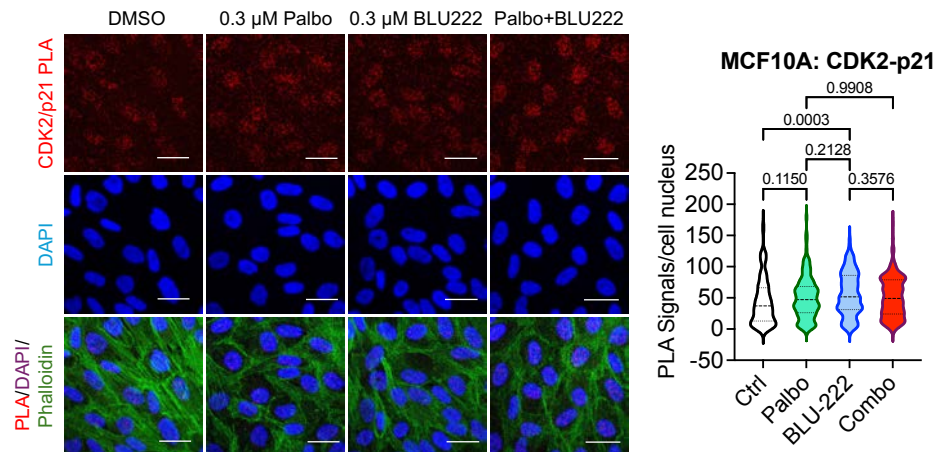


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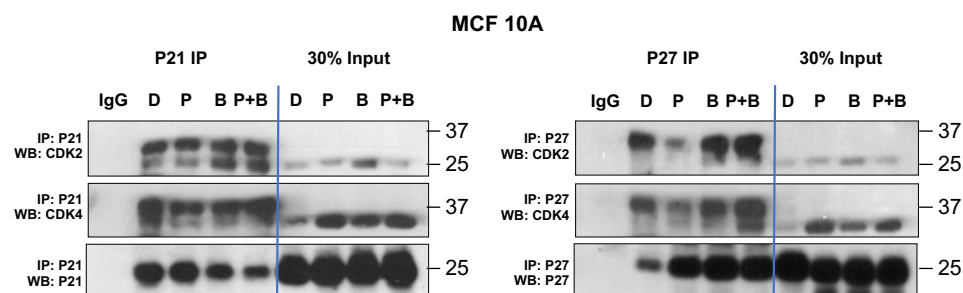
MCF10A
Synergistic Assay
HSA score = 0.73



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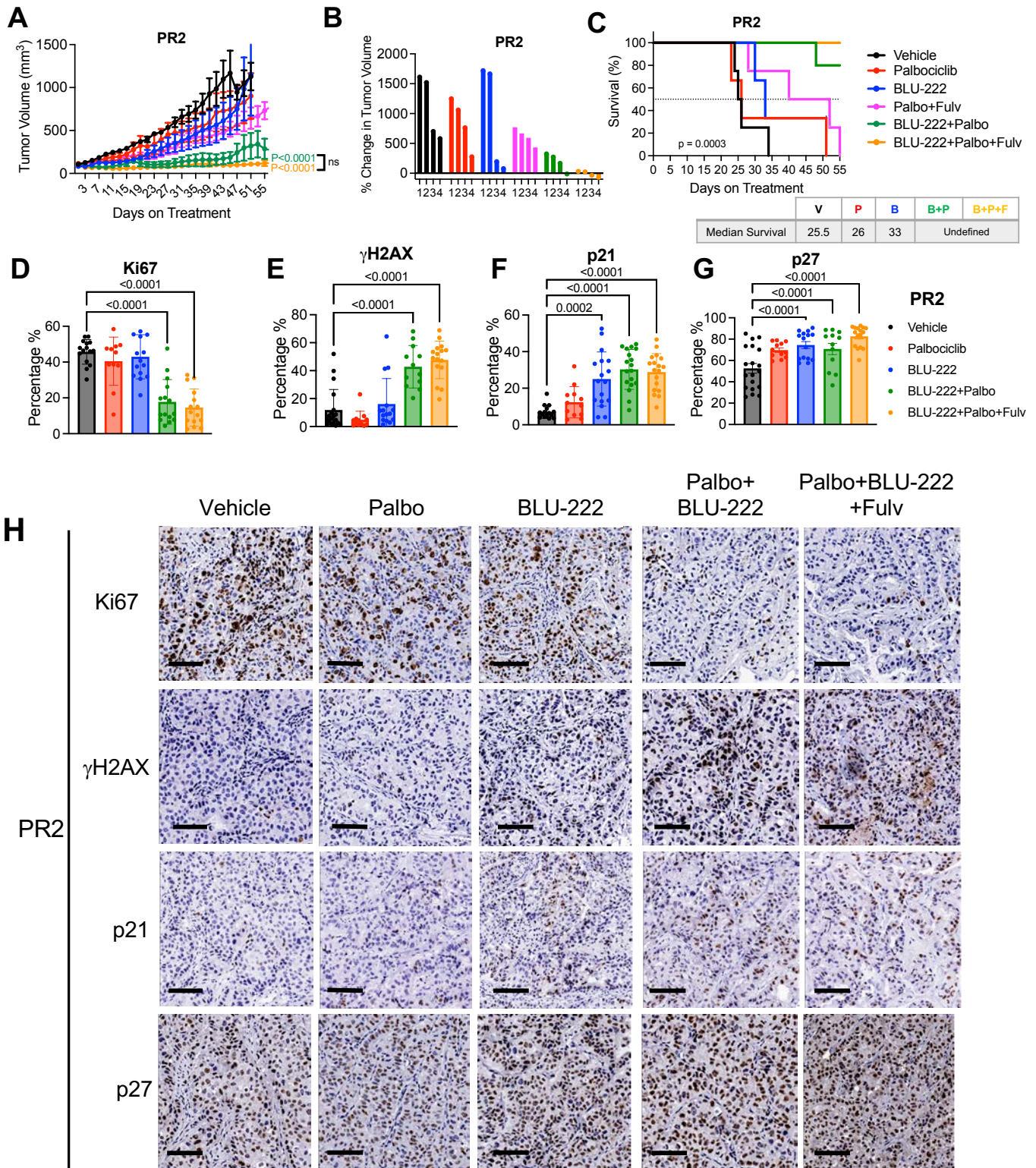


Supplemental Figure 4 | Role of p21 and p27 in Mediating Synergistic Response to CDK4/6 Inhibitor and BLU-222 Combination Treatment

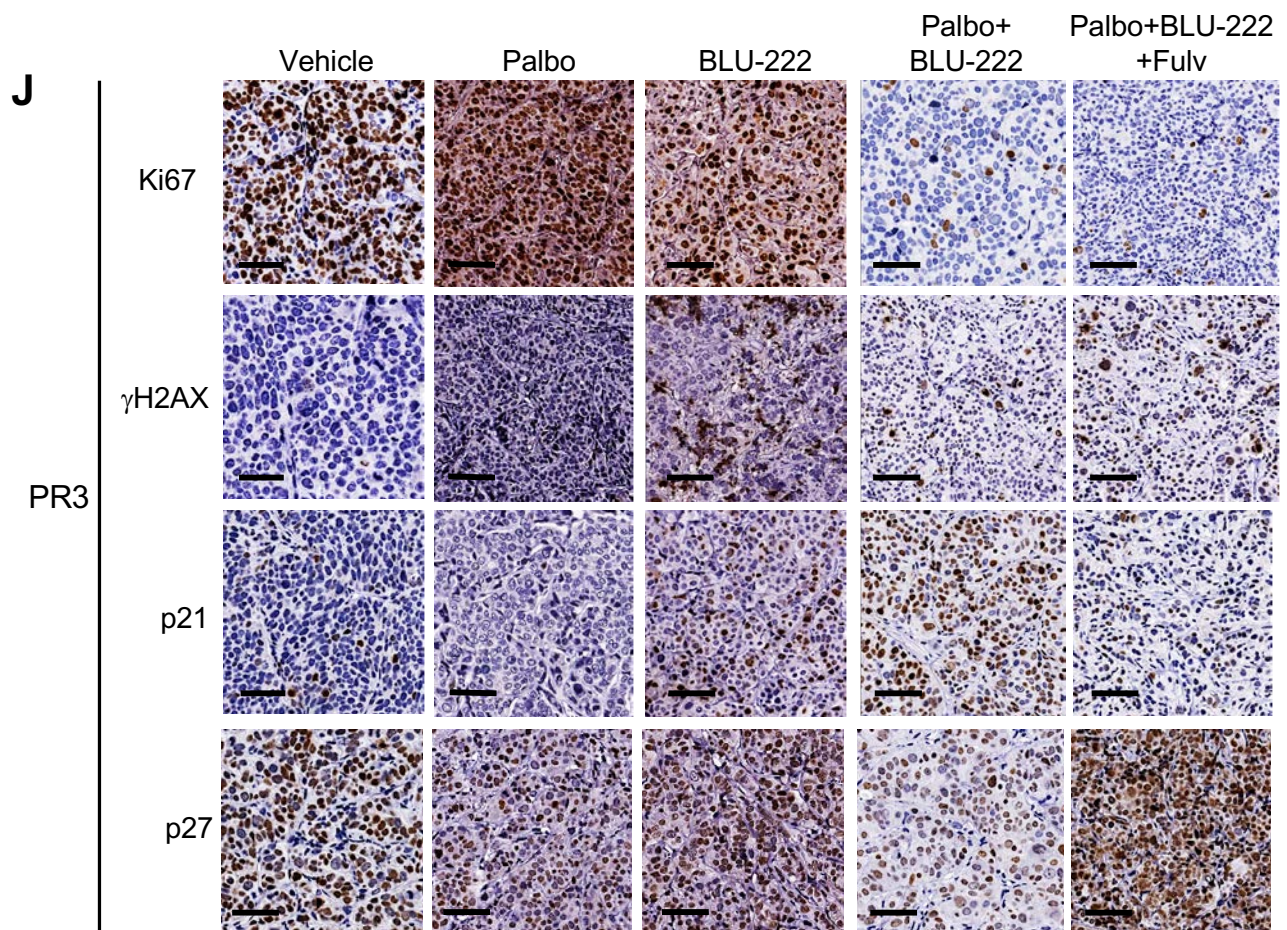
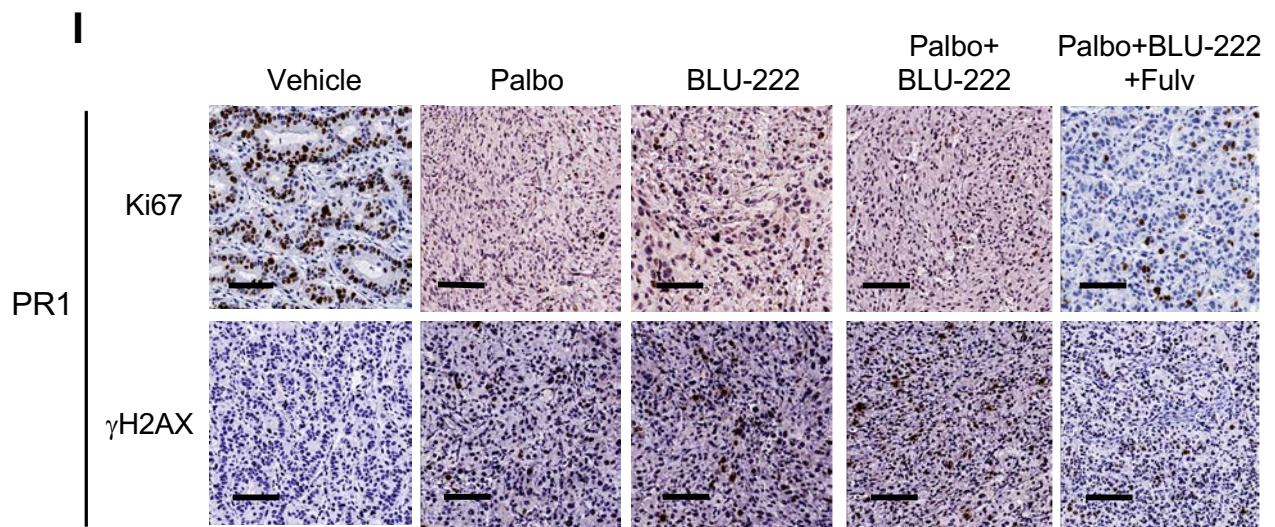
(A) Schema of workflow of proximity ligation assay (PLA). (B-C) PLA detecting CDK2-p27 interaction (red) in (B) MCF7 parental and (C) PR1.2 cells treated for 72 h with DMSO, palbociclib (0.3 μ M), BLU-222 (0.3 μ M), or the combination. Nuclei were stained with DAPI (blue) and actin filaments were visualized with phalloidin (green). Quantification of nuclear PLA signals per cell is shown. At least 180 cells were analyzed. Scale bars: 15 μ m. (D-E) PLA detecting CDK2-p21 interaction (red) in (D) MDA-MB-231 parental and (E) PR cells treated for 72 h with DMSO, palbociclib (0.3 μ M), BLU-222 (0.3 μ M), or the combination. Quantification of nuclear PLA signals per cell is shown below each panel. At least 40 cells were analyzed. (F-G) PLA detecting CDK2-p21 interaction (red) in (F) BT20 parental and (G) PR cells following 72 h treatment. Quantification of PLA blob counts per nucleus is shown below each image set. At least 10 cells were analyzed. (H) Co-immunoprecipitation (co-IP) analysis of p21 or p27 complexes in MDA-MB-231 parental (left) and PR (right) cells after 72 h treatment as in (D-E). Immunoprecipitants (IP: p21 or IP: p27) were probed by western blot (WB) for CDK2, CDK4, p21, and p27. IgG IP served as negative control; 30% input lysates were loaded for comparison. D, DMSO; P, palbociclib; B, BLU-222; P+B, combination. (I) Co-IP analysis of p21 or p27 complexes in BT20 parental (left) and PR (right) cells after 72 h treatment. immunoprecipitants were probed for CDK2, CDK4, p21, and p27, with IgG IP as negative control. 30% input lysates shown for comparison. (J) Representative 3D synergy plot showing the response of MCF10A cells treated with the combination of BLU-222 and palbociclib. (K) PLA detecting CDK2-p21 interaction in non-tumorigenic MCF10A mammary epithelial cells after 72 h treatment as in (D-E). Red, PLA signals; blue, DAPI; green, phalloidin. Quantification of nuclear PLA signals per cell is shown at right. At least 240 cells were analyzed. (L) Co-IP analysis of p21 or p27 complexes in non-tumorigenic MCF10A cells after 72 h treatment with DMSO, palbociclib (0.3 μ M), BLU-222 (0.3 μ M), or the combination. immunoprecipitants (IP: p21 or IP: p27) were probed by WB for CDK2, CDK4, p21, and p27. IgG IP served as a negative

control; 30% input lysates were loaded for comparison. Error bars represent SEM from $n = 3$ biological replicates across 3 independent experiments, one-way ANOVA with Tukey's post-test.

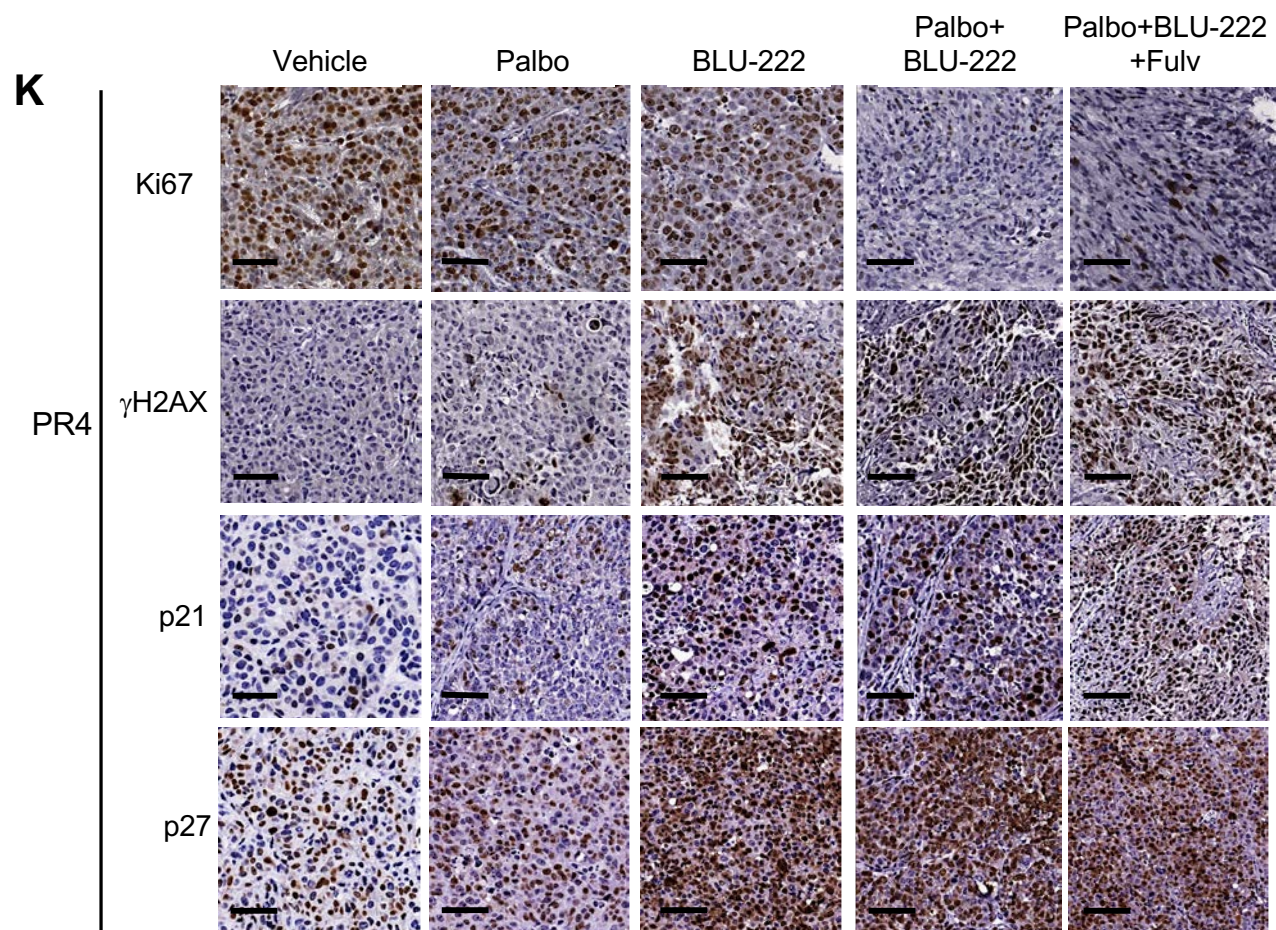
Supplemental Figure 5



Supplemental Figure 5



Supplemental Figure 5

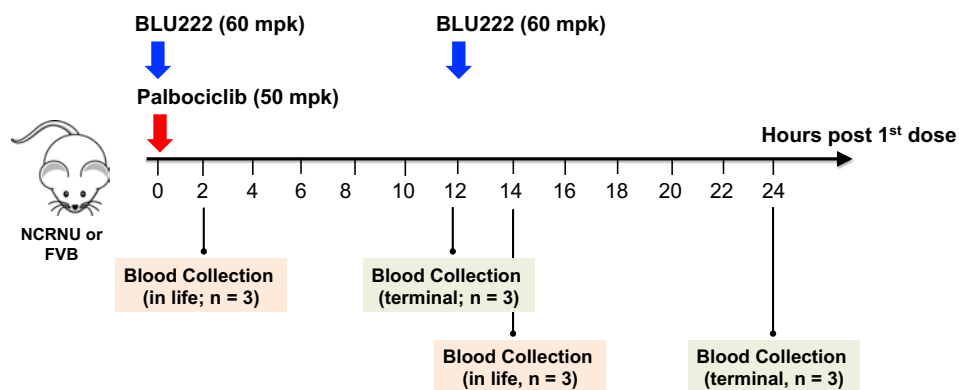


Supplemental Figure 5 | Combination Treatment with BLU-222 and Palbociclib Synergistic in HR+/HER2+ PDX Models That Progressed on Palbociclib Treatment

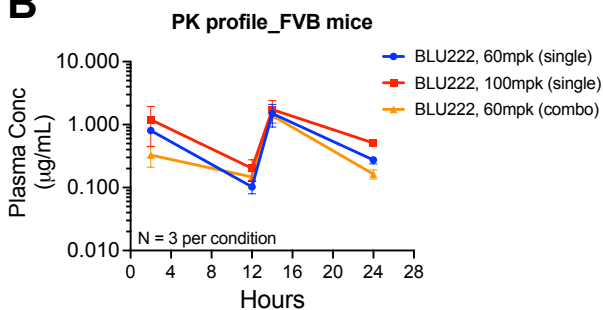
(A-C) Tumor growth curves and waterfall plots showing tumor volume changes and Kaplan-Meier survival curves of PR2 palbociclib-resistant HR+/HER2+ breast cancer model (vehicle, n = 5; BLU-222, n = 5; palbociclib, n = 4; BLU-222 + palbociclib, n = 5; BLU-222 + palbociclib + fulvestrant, n = 5; palbociclib + fulvestrant, n = 5). (D-G) IHC quantification of tumors collected from treated PR2 mice, staining for Ki67, γ H2AX, p21, and p27 percentage of positive staining cells (vehicle, n = 5; BLU-222, n = 5; palbociclib, n = 4; BLU-222 + palbociclib, n = 5; BLU-222 + palbociclib + fulvestrant, n = 5). (H) Representative IHC images of Ki67, γ H2AX, p21, and p27 expression in the PR2 model treated with vehicle, single agents, or a combination of palbociclib and BLU-222 +/- fulvestrant. (I) Representative IHC images of Ki67 and γ H2AX expression in the PR1 model. (J-K) Representative IHC images of Ki67, γ H2AX, p21, and p27 expression in the (J) PR3 and (K) PR4 model treated with vehicle, single agents, or a combination of palbociclib and BLU-222 +/- fulvestrant. Scale bar = 50 μ m. Error bars represent the SEM from n biological replicates.

Supplemental Figure 6

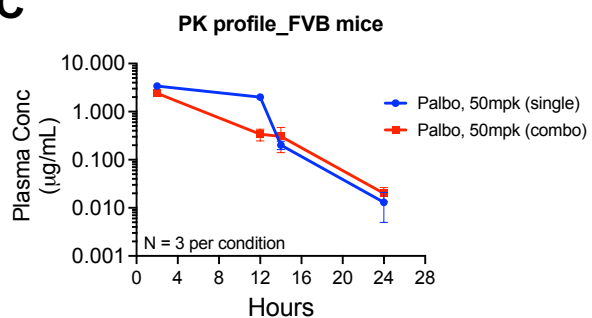
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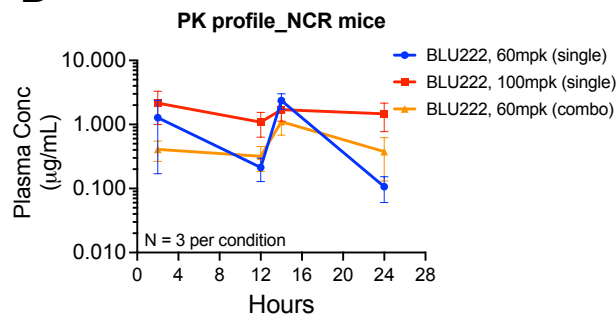
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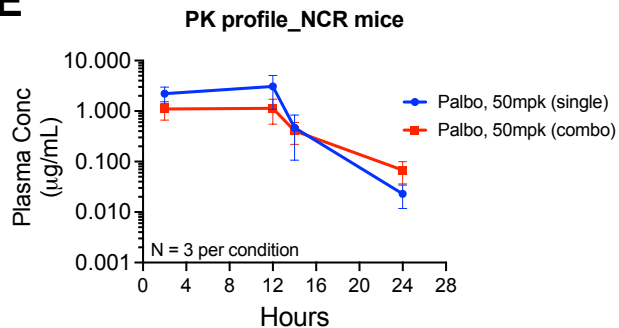
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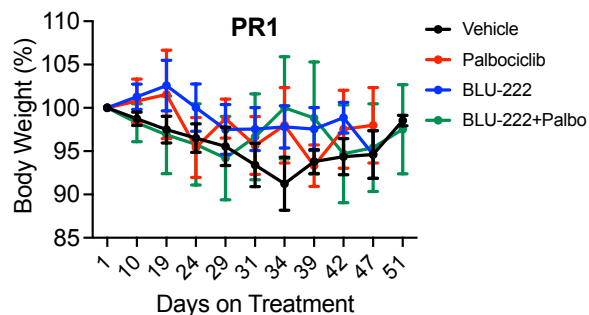


Supplemental Figure 6 | Pharmacokinetic Study in Mice

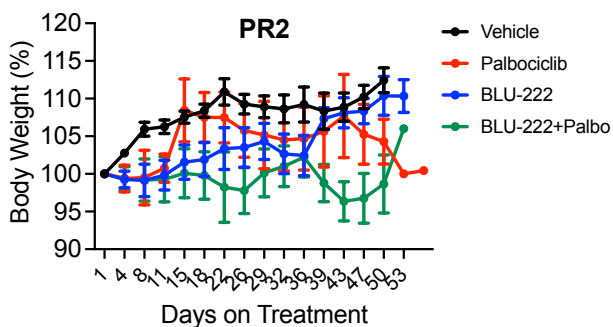
(**A**) The dosing schedule of the pharmacokinetic study. (**B**) Plasma concentration–time profiles of BLU-222, administered alone or in combination with palbociclib in immunocompetent FVB mice ($n = 3$). (**C**) Plasma concentration–time profiles of palbociclib, administered alone or in combination with palbociclib and BLU-222 in FVB mice ($n = 3$). (**D**) Plasma concentration–time profiles of BLU-222, administered alone or in combination with palbociclib in immunocompromised NCR mice ($n = 3$). (**E**) Plasma concentration–time profiles of palbociclib, administered alone or in combination with palbociclib and BLU-222 in NCR mice ($n = 3$). N indicates the number of biological replicates.

Supplemental Figure 7

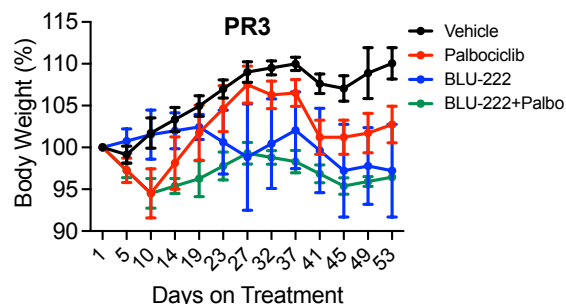
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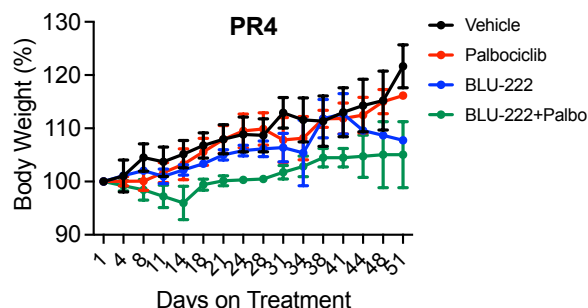
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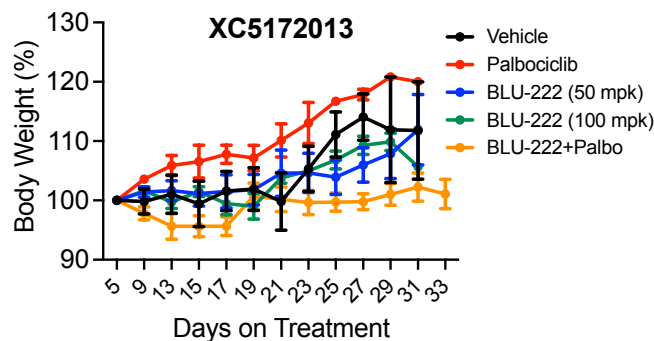
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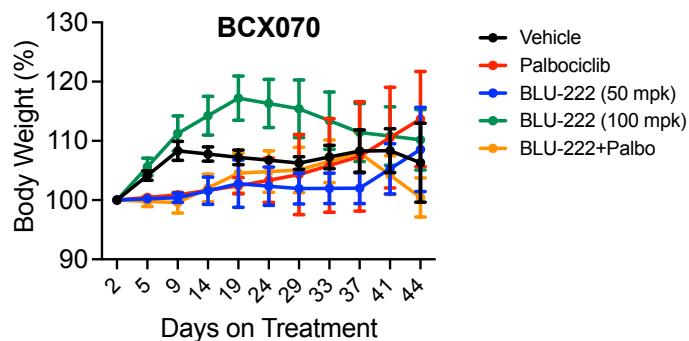
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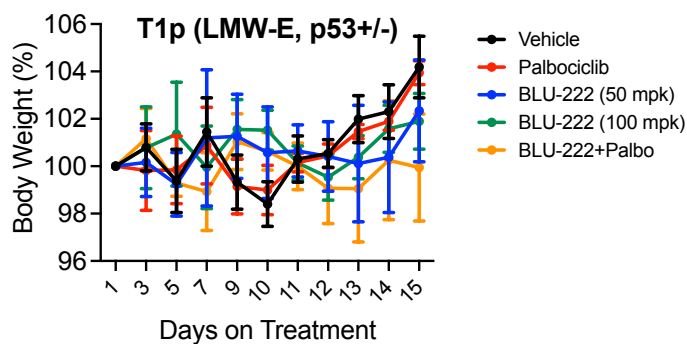
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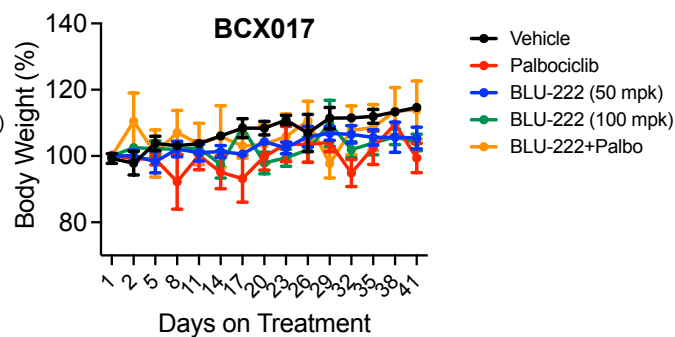
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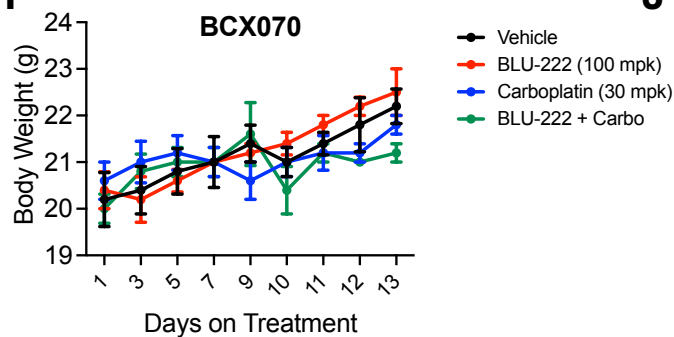
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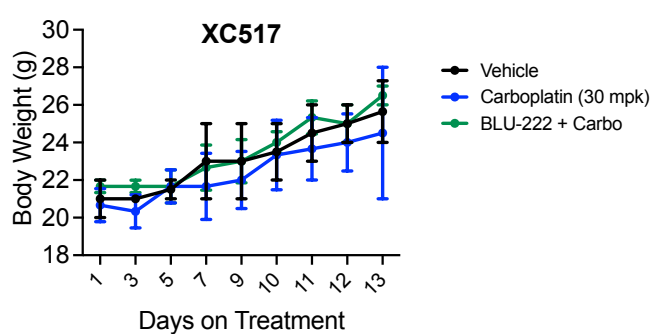
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I



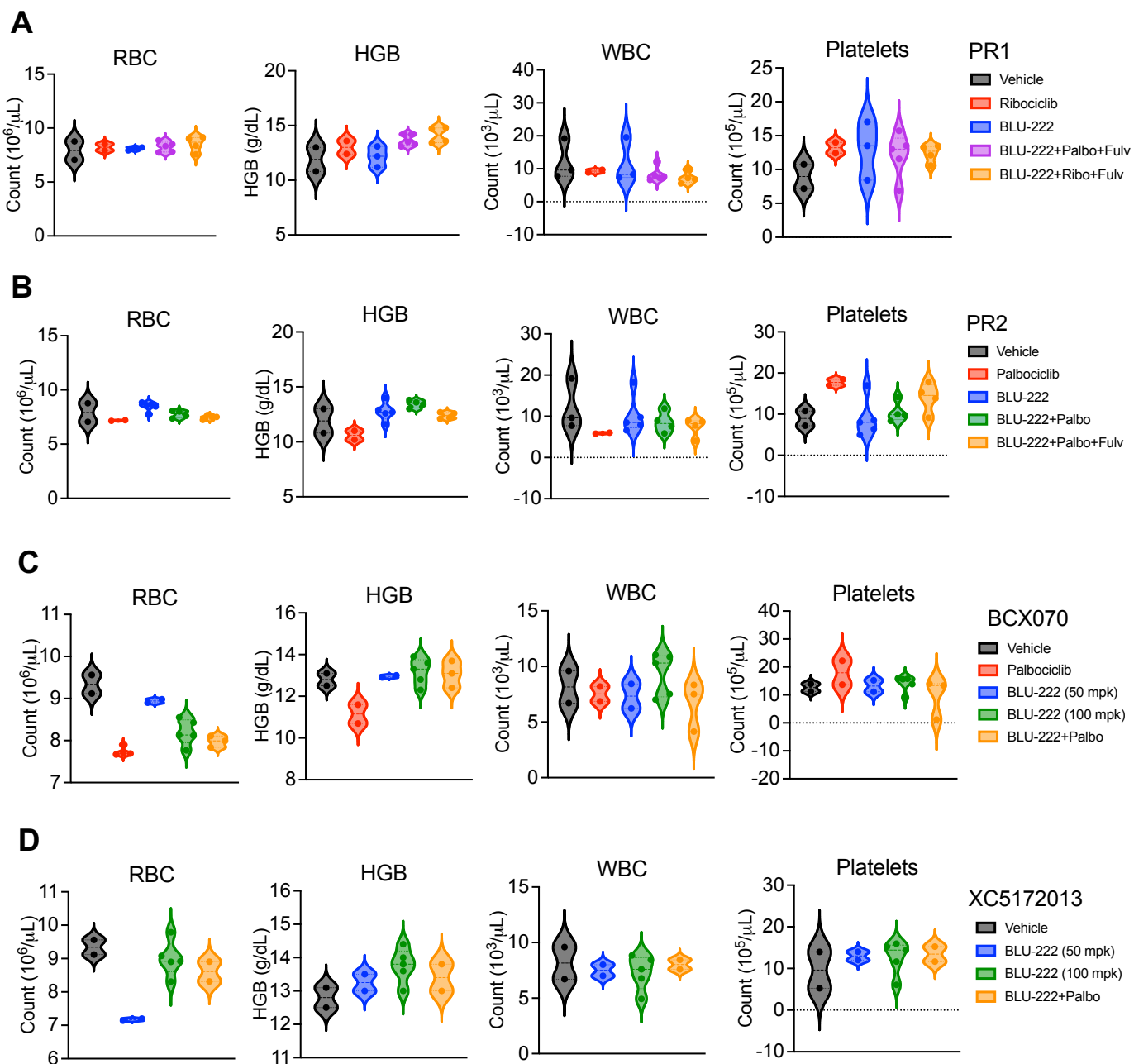
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Supplemental Figure 7 | Body Weight Changes in Mice Treated with Single Agents or Combination of BLU-222 and Palbociclib

(A-G) The percentage of body weight (%) was calculated 2-3 times per week throughout the treatment period in mouse models of **(A)** PR1 (vehicle, n = 5; palbociclib, n = 4; BLU-222, n = 5; BLU-222 + palbociclib, n = 5), **(B)** PR2 (vehicle, n = 4; palbociclib, n = 4; BLU-222, n = 5; BLU-222 + palbociclib, n = 5), **(C)** PR3 (vehicle, n = 4; palbociclib, n = 4; BLU-222, n = 4; BLU-222 + palbociclib, n = 4), **(D)** PR4 (vehicle, n = 5; palbociclib, n = 5; BLU-222, n = 5; BLU-222 + palbociclib, n = 5), **(E)** XC5172013 (vehicle, n = 5; palbociclib, n = 2; BLU-222 50mg/kg, n = 5; BLU-222 100mg/kg, n = 5), **(F)** BCX070 (vehicle, n = 5; palbociclib, n = 3; BLU-222 50mg/kg, n = 5; BLU-222 100mg/kg, n = 5), **(G)** T1p (vehicle, n = 4; palbociclib, n = 3; BLU-222 50mg/kg, n = 4; BLU-222 100mg/kg, n = 3; BLU-222 + palbociclib, n = 4), **(H)** BCX017 (vehicle, n = 5; palbociclib, n = 5; BLU-222 50mg/kg, n = 5; BLU-222 100mg/kg, n = 5; BLU-222 + palbociclib, n = 5), **(I)** BCX070 (vehicle, n = 5; BLU-222 100mg/kg, n = 5; carboplatin, n = 5; BLU-222 + carboplatin, n = 5), and **(J)** XC6517 (vehicle, n = 2; carboplatin, n = 3; BLU-222 + carboplatin, n = 3). The measurements were normalized to the body weight at the start of treatment. N indicates the number of biological replicates.

Supplemental Figure 8



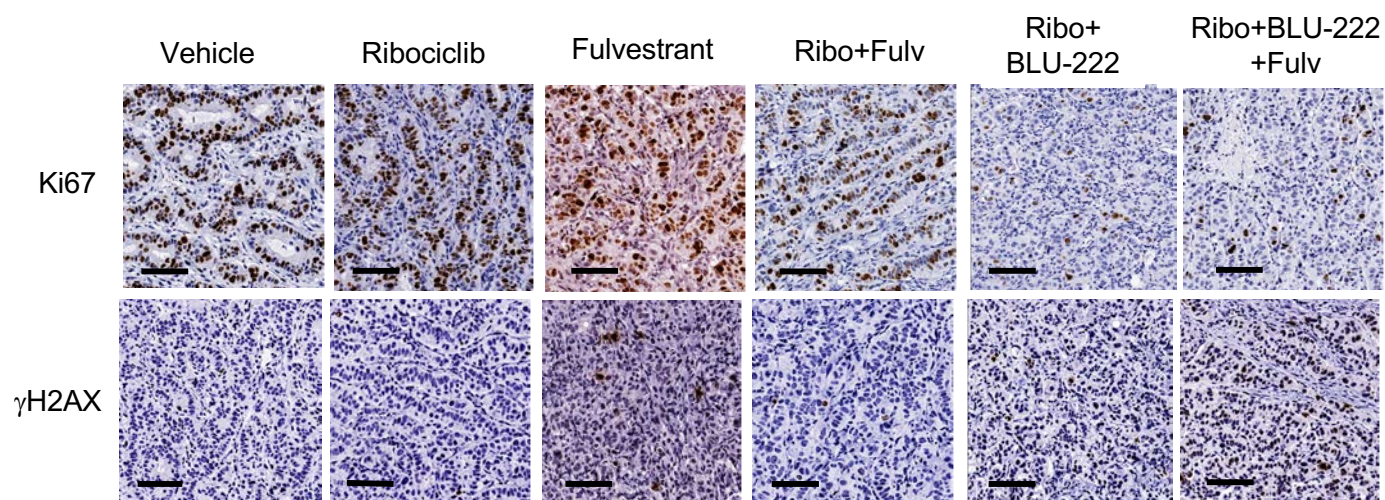
Supplemental Figure 8 | Complete Blood Count Analysis in Mice Treated with Single Agents or Combination of BLU-222 and Palbociclib

The parameters measured included red blood cell (RBC) count, hemoglobin (HGB), white blood cell (WBC) count, and platelet count, evaluated across different models including **(A)** PR1 (vehicle, n = 2; BLU-222, n = 3; ribociclib, n = 2; BLU-222 + ribociclib + fulvestrant, n = 4; BLU-222 + palbociclib + fulvestrant, n = 5), **(B)** PR2 (vehicle, n = 2; BLU-222, n = 5; palbociclib, n = 2; BLU-222 + palbociclib, n = 4; BLU-222 + palbociclib + fulvestrant, n = 4), **(C)** BCX070 (vehicle, n = 2; BLU-222 50 mg/kg, n = 2; palbociclib, n = 4; BLU-222 100 mg/kg, n = 5; BLU-222 + palbociclib, n = 3) and **(D)** XC5172013 (vehicle, n = 2; BLU-222 50 mg/kg, n = 2; BLU-222 100 mg/kg, n = 5; BLU-222 + palbociclib, n = 2). N indicates the number of biological replicates.

Supplemental Figure 9

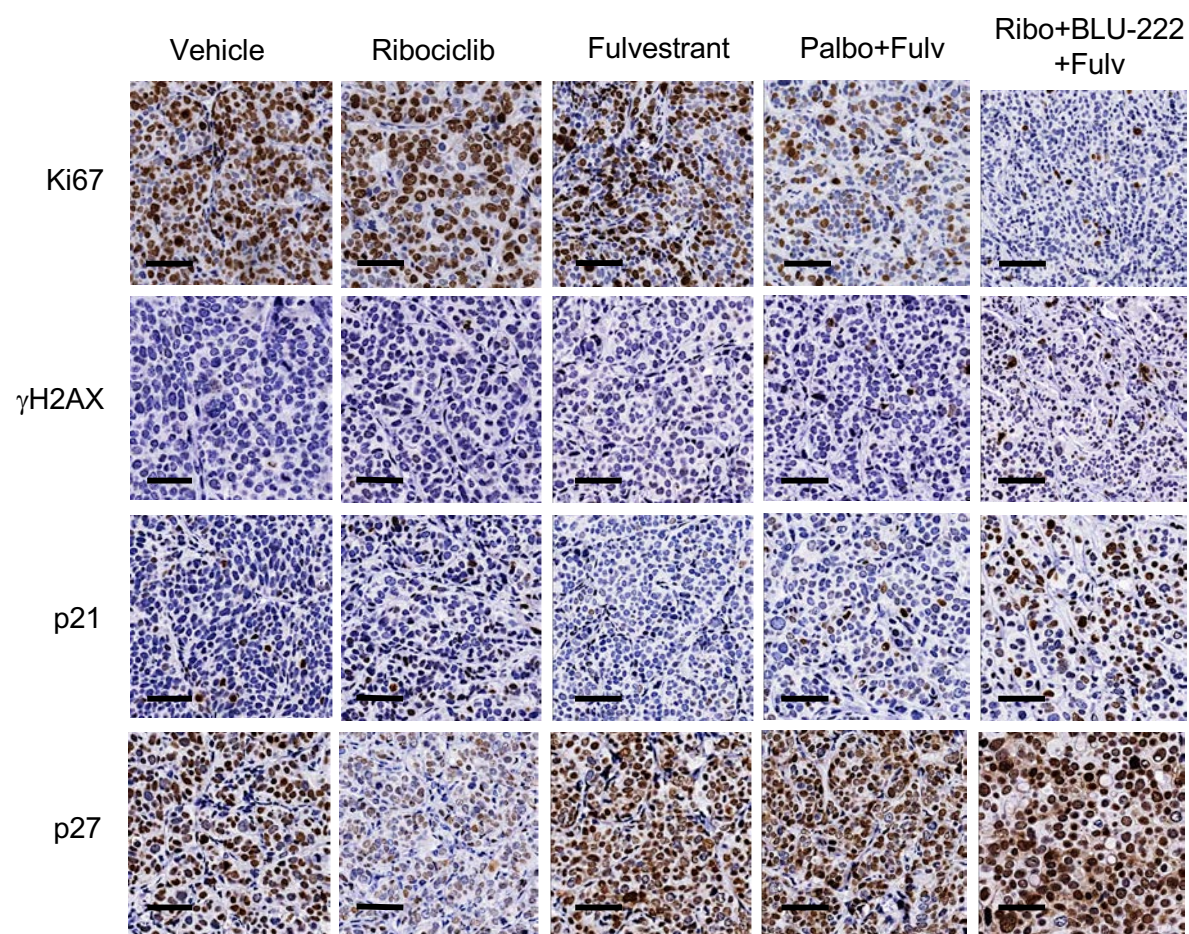
A

PR1



B

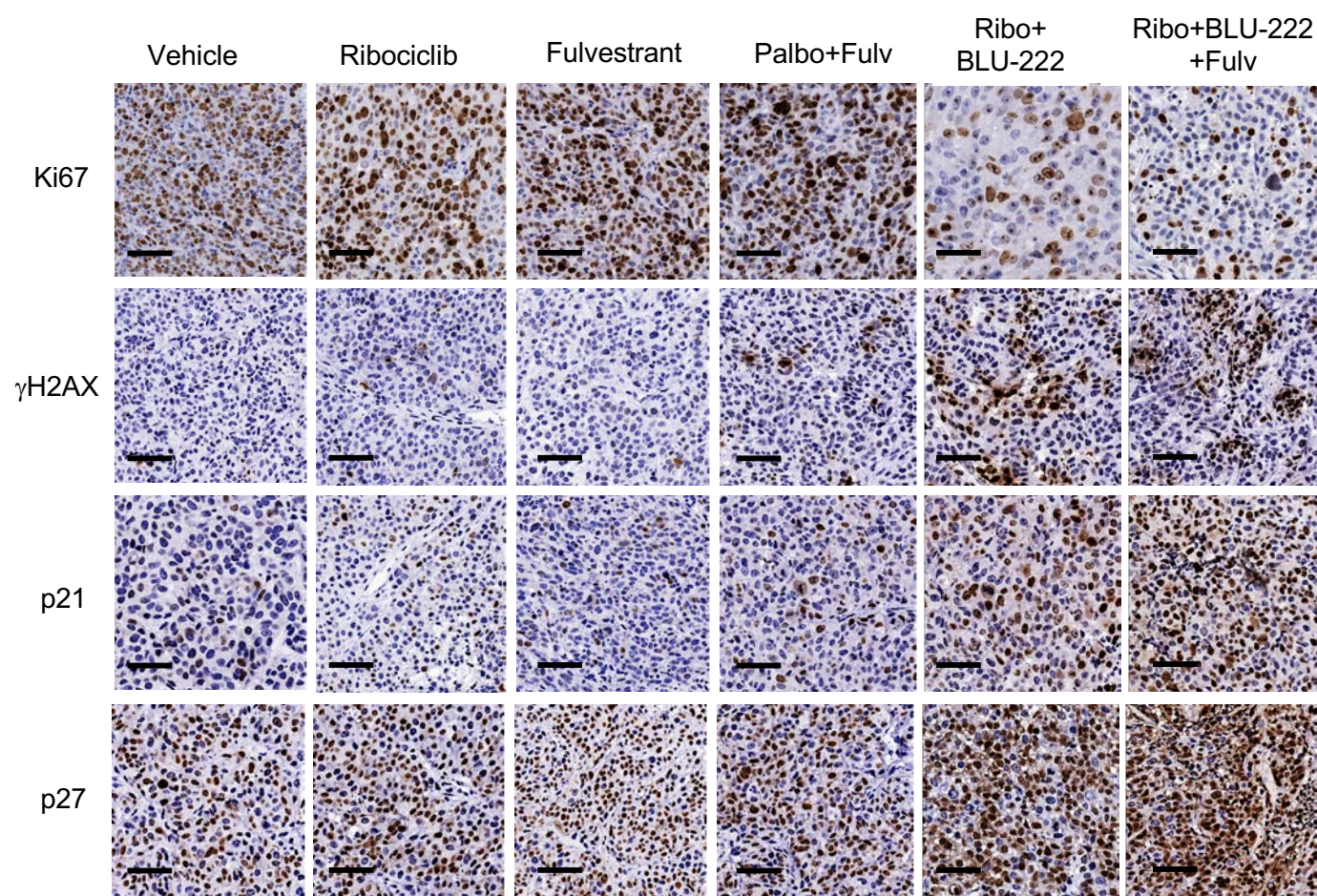
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Supplemental Figure 9

C

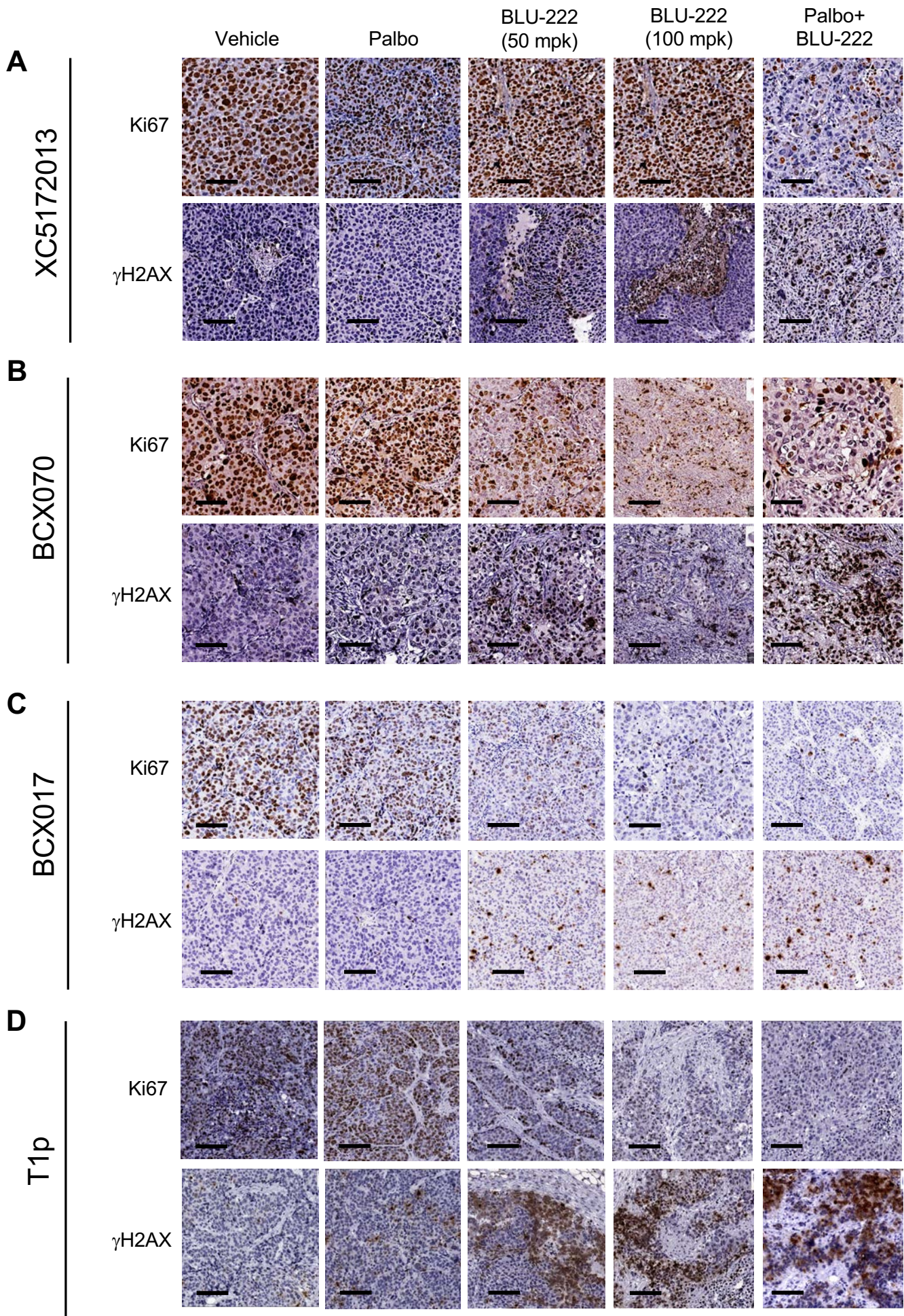
PR4



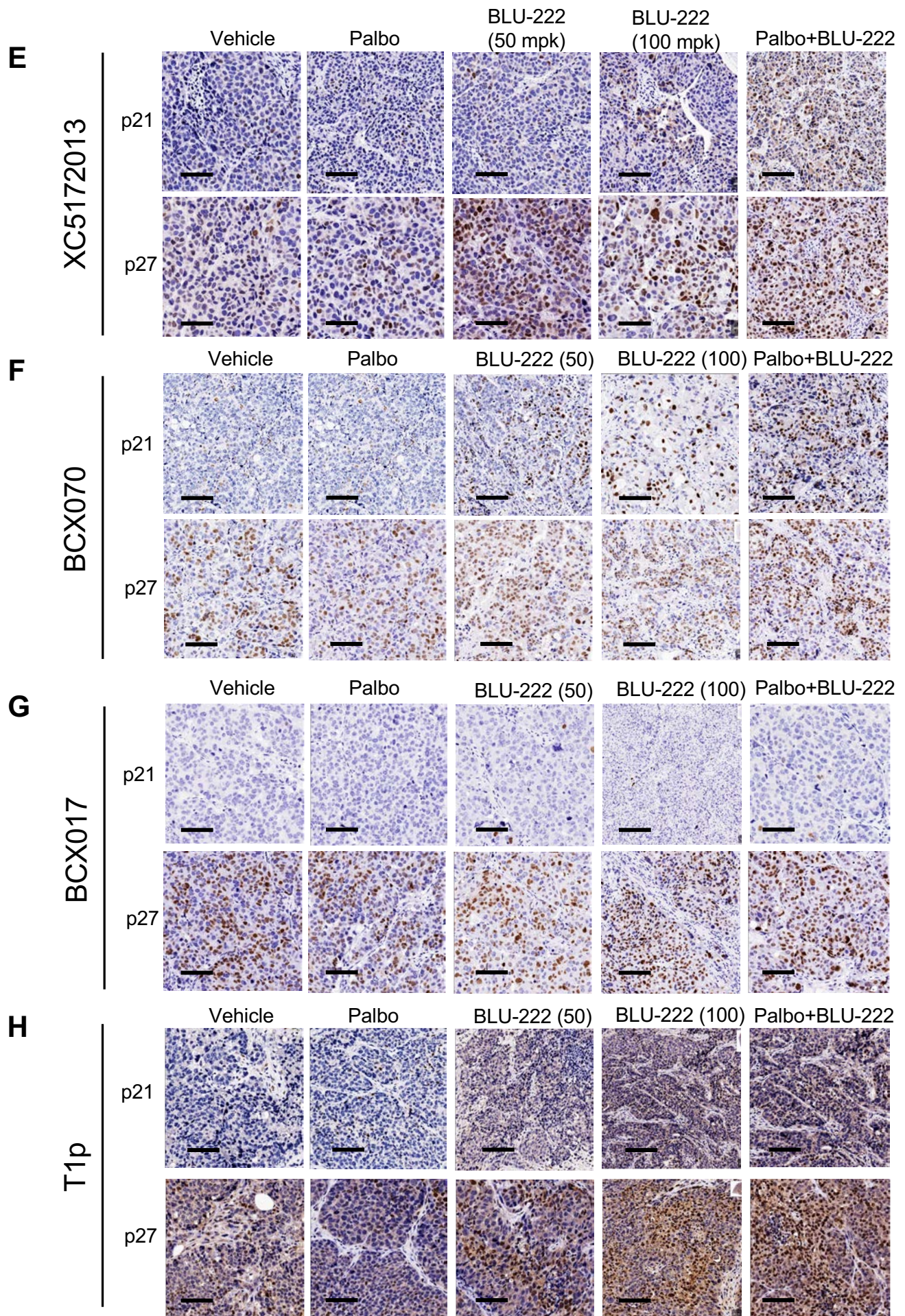
Supplemental Figure 9 | Combination Treatment with BLU-222 and Ribociclib Synergistic in ER+/HER2- PDX Models That Progressed on Palbociclib Treatment

(A-C) Representative IHC images of p21, p27, Ki67, and γ H2AX expression levels in the (A) PR1, (B) PR3, and (C) PR4 models treated with vehicle, single agents, or a combination of ribociclib and BLU-222 +/- fulvestrant. Scale bar = 50 μ m.

Supplemental Figure 10



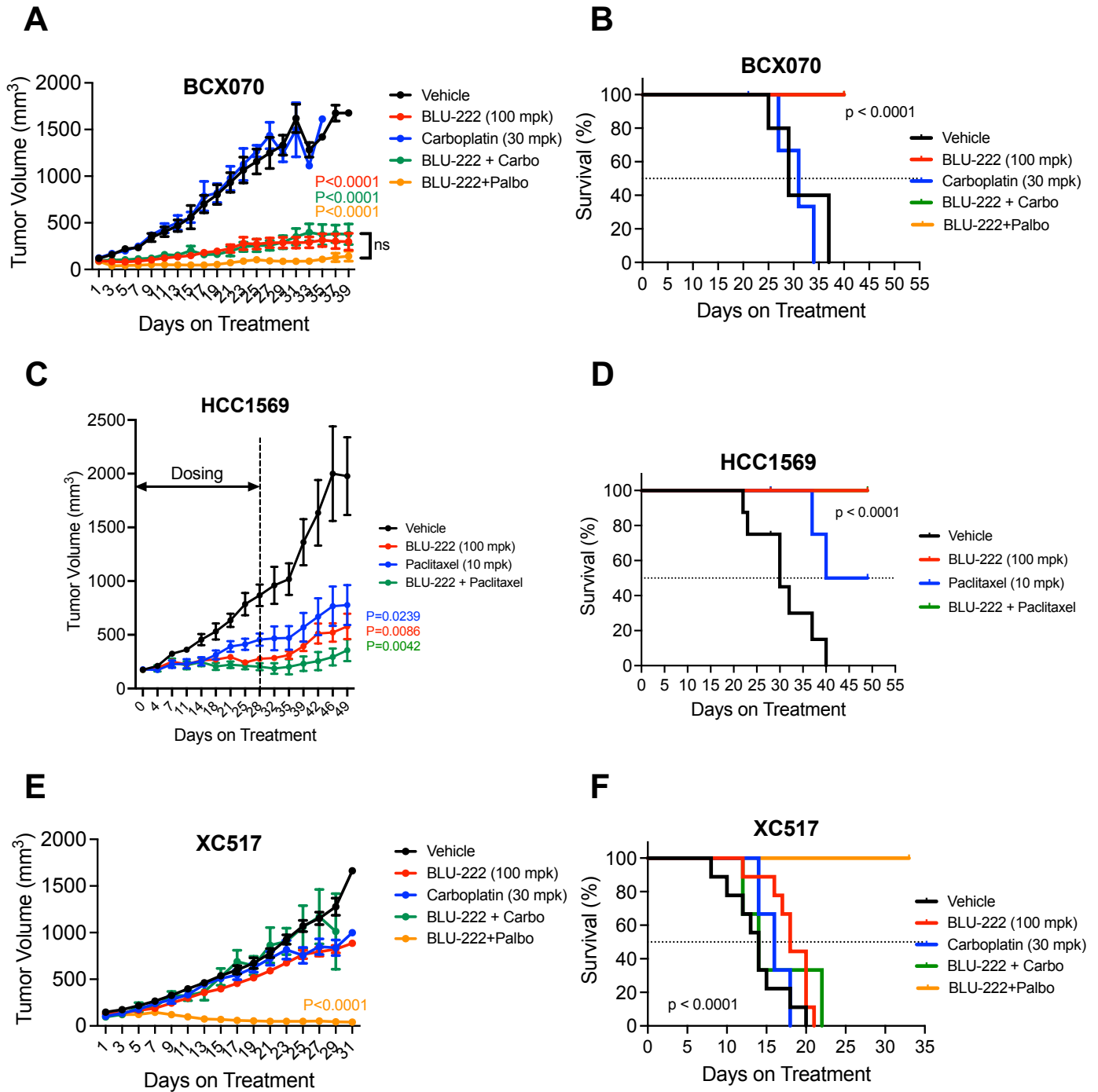
Supplemental Figure 10



Supplemental Figure 10 | Combination Treatment with BLU-222 and CDK4/6 Inhibitor Synergistic in TNBC PDX Models That Progressed on Palbociclib Treatment and in Transgenic Mice with Overexpression of LMW–Cyclin E

(A-D) Representative IHC images of Ki67 and γ H2AX expression in the (A) XC5172013, (B) BCX070, (C) BCX017, and (D) T1p models treated with vehicle, single agents, or a combination of palbociclib and BLU-222 +/- fulvestrant. (E-H) Representative IHC images of p21 and p27 expression in the (E) XC5172013, (F) BCX070, (G) BCX017, and (H) T1p model treated with vehicle, single agents, or a combination of palbociclib and BLU-222 +/- fulvestrant. Scale bar = 50 μ m.

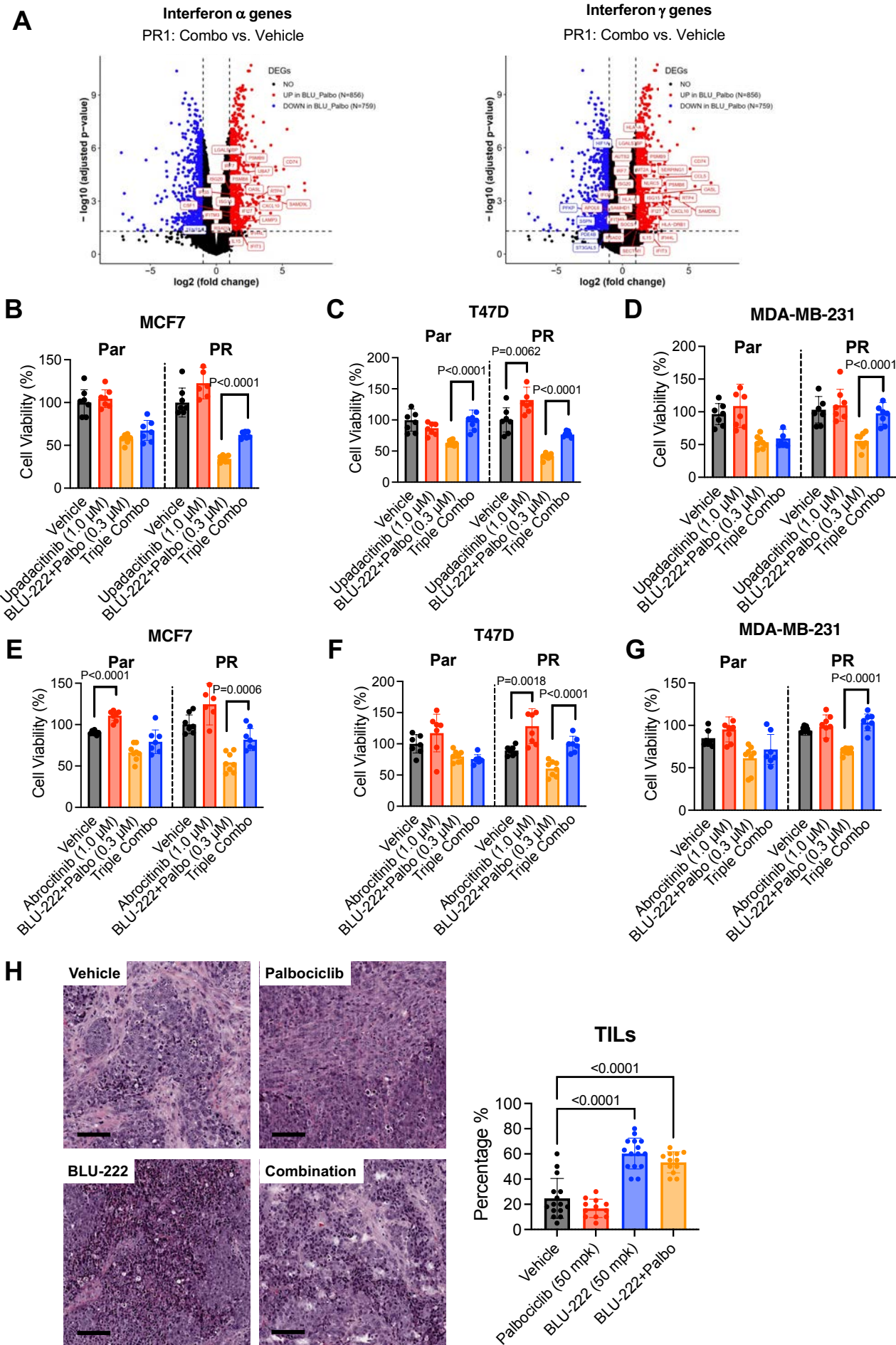
Supplemental Figure 11



Supplemental Figure 11 | Efficacy of BLU-222 chemotherapy combination treatments in TNBC PDX xenografts models

(A-B) Tumor growth curves and Kaplan–Meier survival analysis of BCX070 PDX model treated with vehicle (n = 5), BLU-222 (100 mg/kg, n = 5), carboplatin (30 mg/kg, n = 4), BLU-222 + carboplatin (n = 5), or BLU-222 + palbociclib (n = 4). Tumor volume data are presented as mean $\pm$ SEM; significance was determined by two-way ANOVA with Tukey's multiple comparisons test. Survival significance was calculated using the log-rank (Mantel–Cox) test. ***p < 0.001, ns = not significant. **(C-D)** Tumor growth curves and Kaplan–Meier survival analysis of HCC1569 xenografts treated with vehicle (n = 8), BLU-222 (100 mg/kg, n = 8), paclitaxel (10 mg/kg, n = 8), or BLU-222 + paclitaxel (n = 8). N indicates the number of biological replicates. Tumor volume data are mean $\pm$ SEM; significance determined as in (A-B). **(E-F)** Tumor growth curves and Kaplan–Meier survival analysis of XC517 PDX model treated with vehicle (n = 11), BLU-222 (100 mg/kg, n = 8), carboplatin (30 mg/kg, n = 3), BLU-222 + carboplatin (n = 3), or BLU-222 + palbociclib (n = 5). N indicates the number of biological replicates. Tumor volume data are presented as mean $\pm$ SEM; significance was determined by two-way ANOVA with Tukey's multiple comparisons test. Survival significance was calculated using the log-rank test.

Supplemental Figure 12

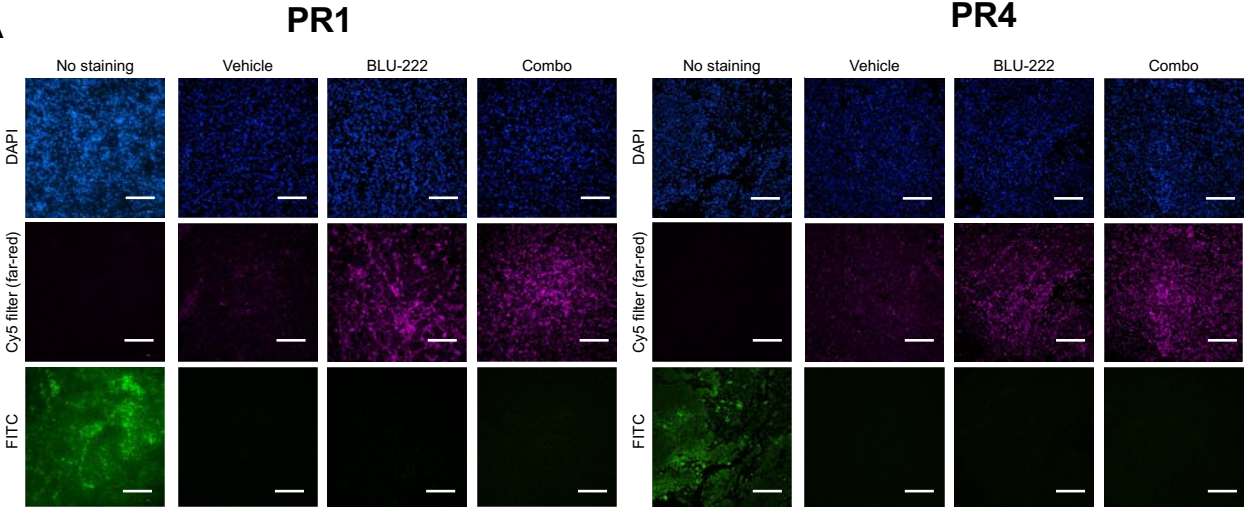


Supplemental Figure 12 | Interferon signaling, triple combination therapy effects, and tumor-infiltrating lymphocytes (TILs) in breast cancer models

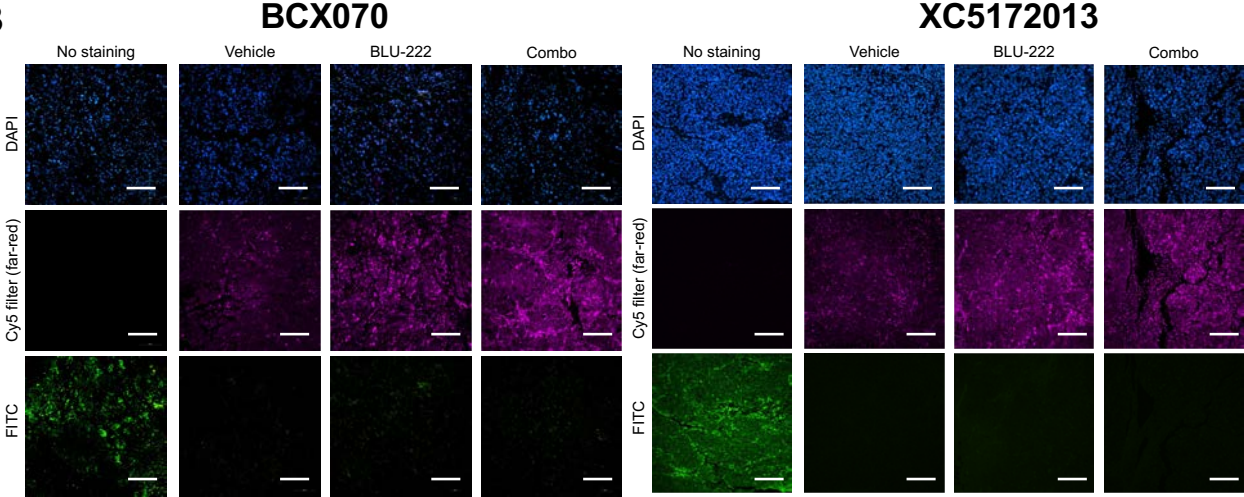
(A) Volcano plots showing differential expression of interferon α (left) and interferon γ (right) gene sets in PR1 tumors treated with BLU-222 + palbociclib (Combo) versus vehicle. Differentially expressed genes (DEGs) are highlighted in red (upregulated) or blue (downregulated), with selected genes labeled. FDR cutoff: 0.05; fold-change cutoff: 2. **(B-D)** Cell viability analyses in **(B)** MCF7 parental (Par) and PR, **(C)** T47D Par and PR, and **(D)** MDA-MB-231 Par and PR cells treated with vehicle ($n = 8$), upadacitinib (UPA; $1 \mu\text{M}$, $n = 8$), BLU-222 + palbociclib (Combo, $n = 8$), or UPA + Combo (Triple Combo, $n = 8$) for 5 days. Data are mean $\pm$ SEM; one-way ANOVA with Tukey's multiple comparisons test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. **(E-G)** Cell viability assays in **(E)** MCF7 Par and PR, **(F)** T47D Par and PR, and **(G)** MDA-MB-231 Par and PR cells treated with vehicle ($n = 8$), abrocitinib (ABR; $1 \mu\text{M}$, $n = 8$), Combo ($n = 8$), or ABR + Combo (Triple Combo, $n = 8$) for 5 days. **(H)** Representative H&E-stained tumor sections from PR1 xenografts treated with vehicle ($n = 5$), palbociclib ($n = 5$), BLU-222 ($n = 5$), or combination ($n = 4$) for the indicated duration. Quantification of tumor-infiltrating lymphocytes (TILs) is shown at right. Scale bar = $50 \mu\text{m}$. Error bars represent the SEM from biological replicates across three independent experiments using one-way ANOVA with Tukey's multiple comparisons test.

Supplemental Figure 13

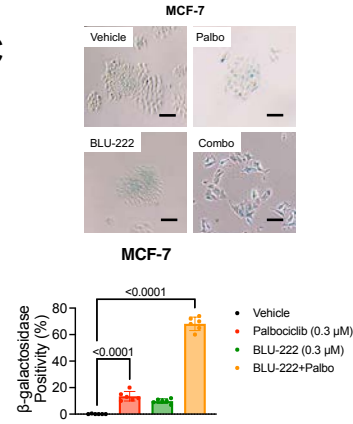
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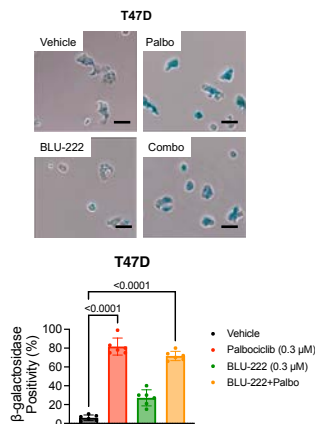
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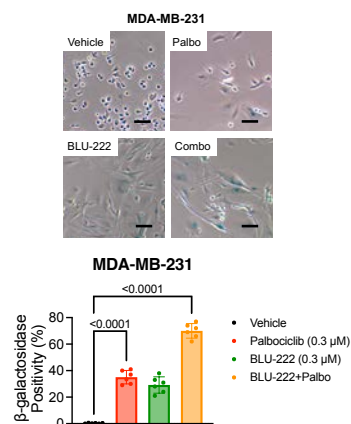
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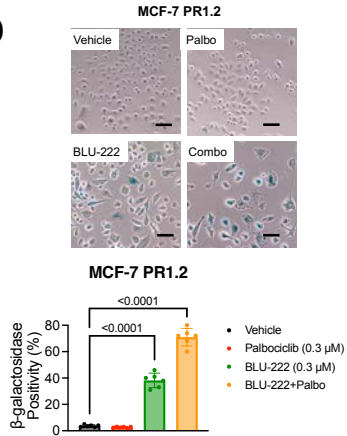
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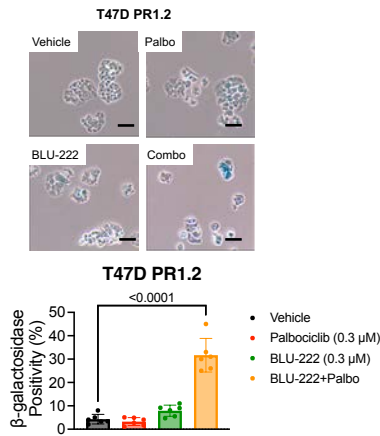
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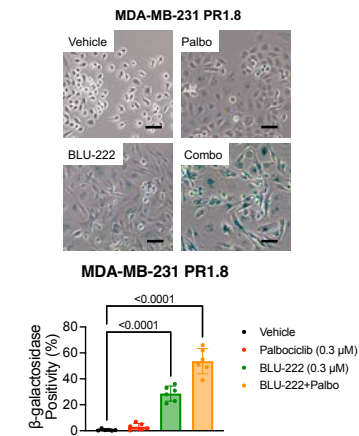
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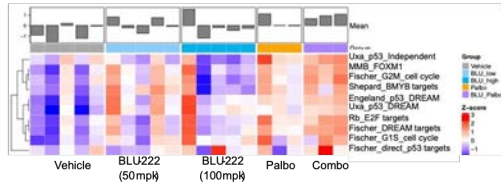


Supplemental Figure 13 | Senescence induction in breast cancer models following BLU-222 and palbociclib combination treatment

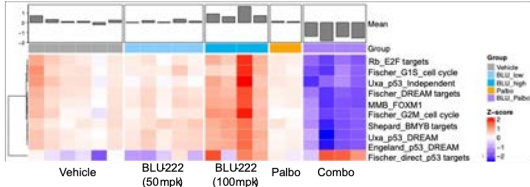
(A-B) Representative immunofluorescence images of lipofuscin accumulation detected by Sudan Black B (SBB) staining in (A) HR+/HER2- PDX models PR1, PR4, and (B) TNBC PDX models BCX070, and XC5172013 tumors treated with vehicle, BLU-222, or BLU-222 + palbociclib (Combo). Nuclei are stained with DAPI (blue), lipofuscin signal is detected in far-red channel (Cy5, magenta), and autofluorescent FITC signal is shown in green. No staining controls are included for reference. Scale bar = 50 μ m. (C-H) Senescence-associated β -galactosidase (SA- β -gal) staining in (C) MCF7, (D) MCF7 PR1.2, (E) T47D, (F) T47D PR1.2, (G) MDA-MB-231, and (H) MDA-MB-231 PR1.8 cells following 5 days of treatment with vehicle, palbociclib (0.3 μ M), BLU-222 (0.3 μ M), or combination (n = 6 for each condition). Representative images are shown (top), and quantification of SA- β -gal-positive cells (% total) is presented below. Scale bar = 15 μ m. Error bars represent the SEM from biological replicates across three independent experiments using one-way ANOVA with Tukey's multiple comparisons test.

Supplemental Figure 14

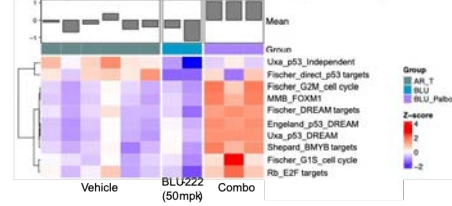
A BCX070_ssGSEA_DREAM pathway



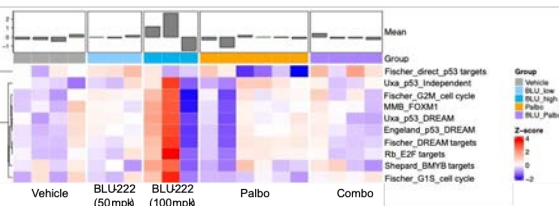
B XC517_ssGSEA_DREAM pathway



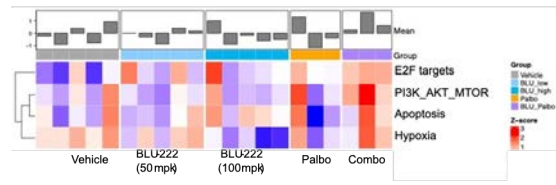
C PR1_ssGSEA_DREAM pathway



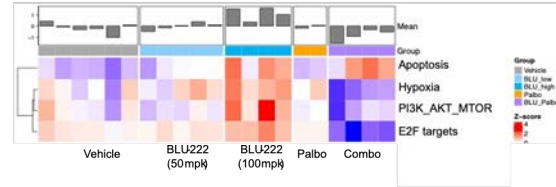
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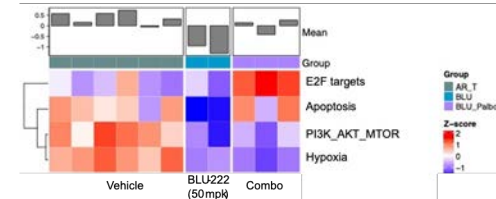
E BCX070_ssGSEA_Apoptosis and hypoxia



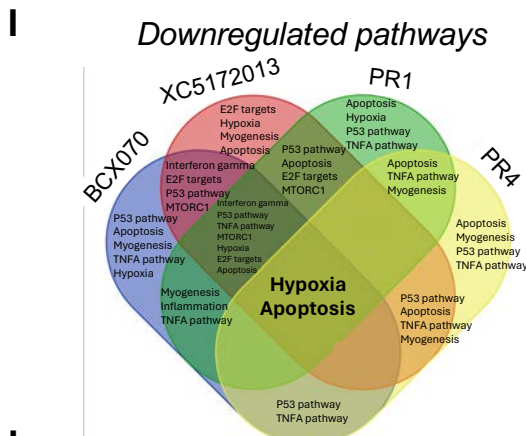
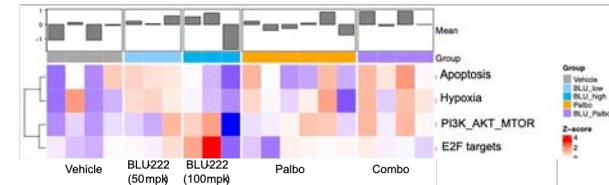
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G PR1_ssGSEA_Apoptosis and hypoxia



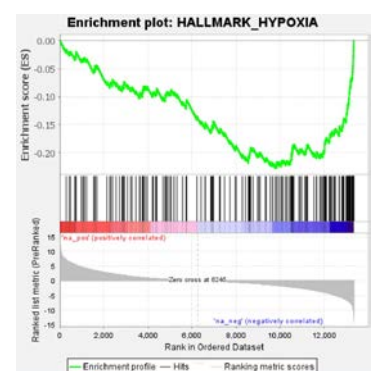
H PR4_ssGSEA_Apoptosis and hypoxia



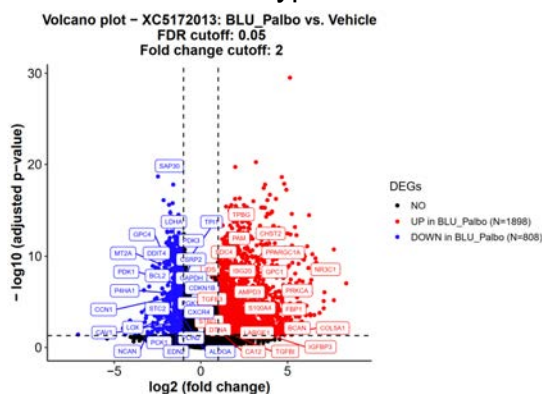
J XC5172013



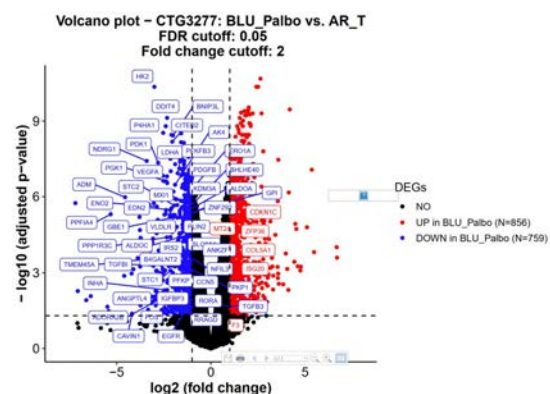
K PR1



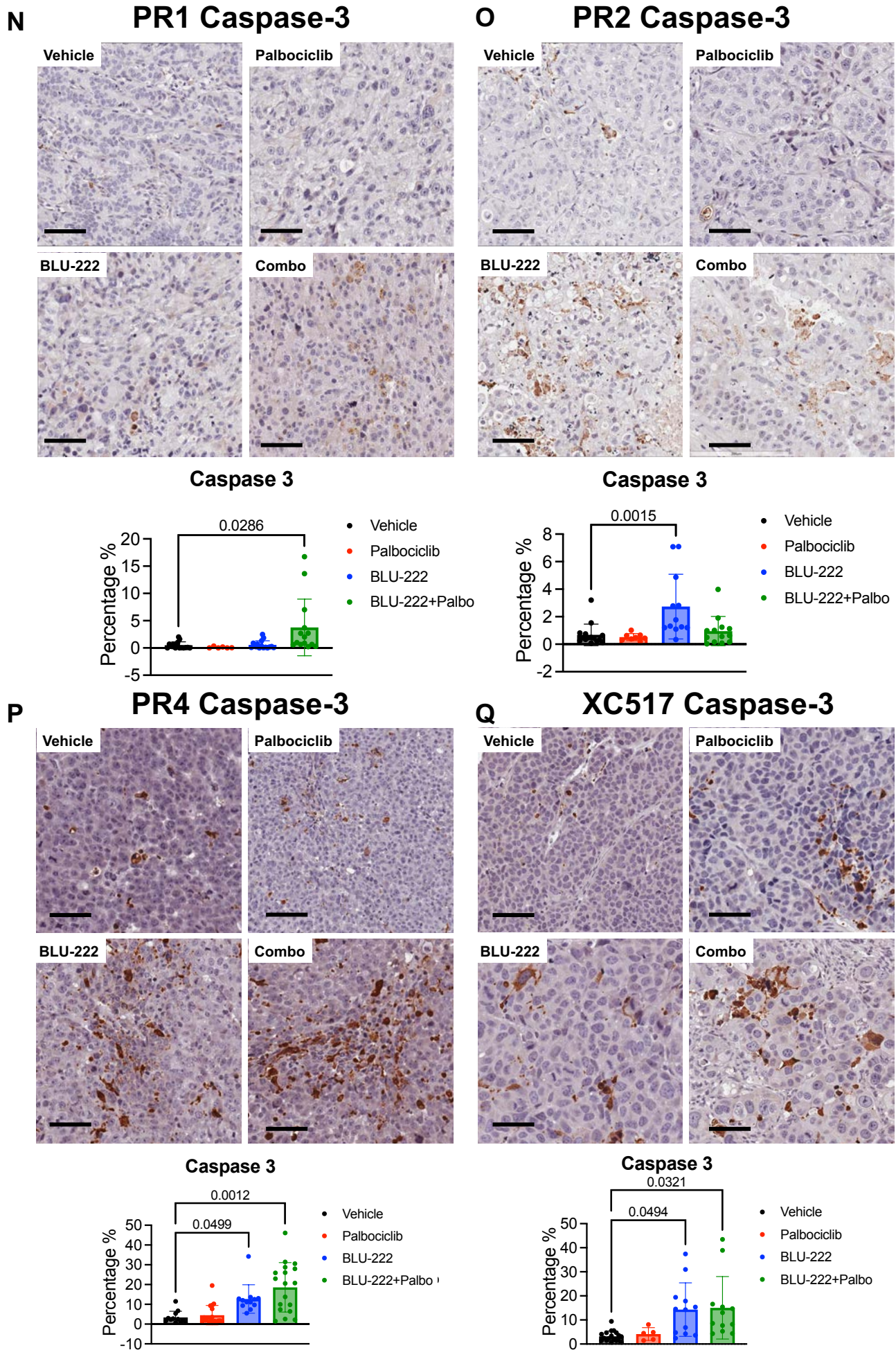
L XC517 - Hypoxia



M PR1 - Hypoxia



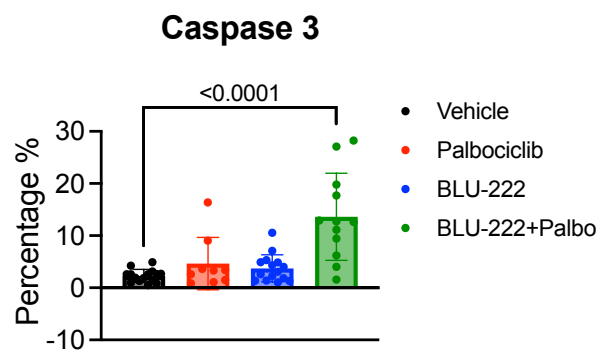
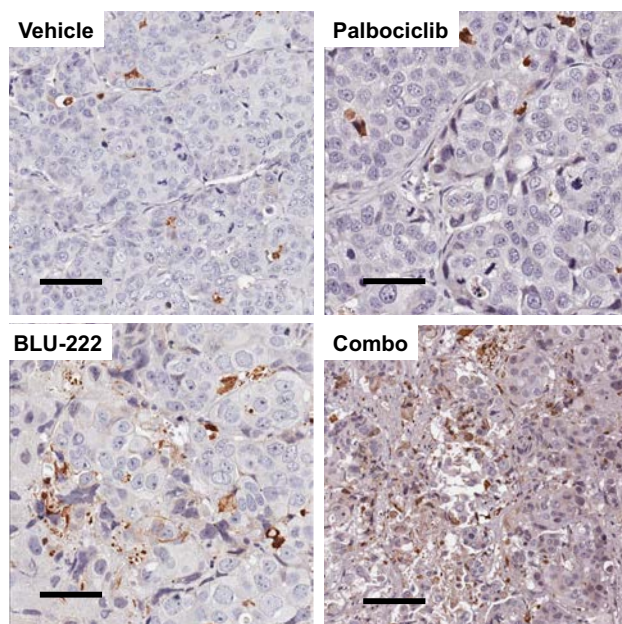
Supplemental Figure 14



Supplemental Figure 14

R

BCX070 Caspase-3



Supplemental Figure 14 | Apoptosis and Hypoxia Genes Downregulated After Combination Treatment with BLU-222 and CDK4/6 Inhibitor in TNBC and HR+/HER2- PDX Models That Progressed on Palbociclib Treatment

(A-D) Heatmaps showing the standardized ssGSEA scores for each sample of p21-DREAM-related pathways across 4 PDX models. Bar plots on top of each heatmap indicate the average value of the standardized ssGSEA scores for each sample. (E-H) Heatmaps showing the standardized ssGSEA scores for each sample of signatures related to apoptosis and hypoxia pathways across 4 PDX models. (I) Venn diagram showing common and unique downregulated genes associated with pathways upon combination treatment with BLU-222 and palbociclib across 4 PDX models. (J-K) Enrichment plots of the hypoxia gene set from GSEA performed on XC5172013 and PR1 tumors for the comparison of the treatment combination of BLU-222 and palbociclib versus vehicle treatment. (L-M) Volcano plots showing differentially expressed genes associated with the hypoxia pathway in (L) XC5172013 or (M) PR1 tumors treated with the combination of palbociclib, and BLU-222 compared to the vehicle treatment. FDR cutoff: 0.05; fold-change cutoff: 2. (N-R) Representative IHC images of cleaved caspase-3 staining in HR+/HER2- PDX models of (N) PR1 (vehicle, n = 5; BLU-222, n = 5; palbociclib, n = 4; BLU-222 + palbociclib, n = 4), (O) PR2 (vehicle, n = 4; palbociclib, n = 4; BLU-222, n = 5; BLU-222 + palbociclib, n = 5), (P) PR4 (vehicle, n = 4; BLU-222, n = 4; palbociclib, n = 6; BLU-222 + palbociclib, n = 6), and TNBC PDX models of (Q) XC517 (vehicle, n = 7; BLU-222, n = 5; palbociclib, n = 2; BLU-222 + palbociclib, n = 4), and (R) BCX070 tumors (vehicle, n = 5; BLU-222, n = 5; palbociclib, n = 3; BLU-222 + palbociclib, n = 4). Quantification of percentages of cleaved caspase-3-positive cells is shown below each image set as percentage of total cells. Scale bar = 50 μ m. Error bars represent the SEM calculated from n biological replicates using one-way ANOVA with Tukey's multiple comparisons test.

Table S1. List of IC₅₀ values of cell lines treated with three CDK4/6 inhibitors and BLU-222

Cell line	Rb status	TP53 status	Palbociclib	Abemaciclib	Ribociclib	BLU-222
HR+/HER2-						
MCF7 parental	WT	WT	0.60	0.077	0.13	2.45
MCF7 PR1.2	WT	WT	2.90	2.06 (Navarro-Yepes et al.)	0.91	0.55
MCF7 PR4.8	Deficient	Deletion	8.02	0.77 (Navarro-Yepes et al.)	8.24 (Kettner et al.)	0.32
MCF7 shRb#1	Negative	WT	5.5	NA	NA	0.20
MCF7 shRb#2	Negative	WT	6.3	NA	NA	0.60
MCL	Deficient	Deletion	7.79	NA	NA	0.36
T47D parental	WT	L194F Missense	1.01	0.12 (Kettner et al.)	0.42 (Kettner et al.)	2.98
T47D EL	WT	L194F Missense	6.50	NA	NA	5.21
T47D LMW-E	WT	L194F Missense		NA	NA	0.81
T47D PR1.2	WT	L194F Missense	4.43	NA	NA	1.78
T47D PR4.8	Deficient	Deletion	8.10	1.72 (Kettner et al.)	6.88 (Kettner et al.)	0.02
TCL	Deficient	Deletion	9.33	NA	NA	0.33
CAMA1	WT	WT	0.04	0.04	0.11	NA
HER2-enriched						
BT474 parental	WT	E285K Missense	0.60	0.20	NA	NA
BT474 PR	WT	E285K Missense	14.81	NA	NA	1.69
SKBR3 parental	WT	R175H Missense	1.02	0.22	1.76	NA
SKBR3 PR	WT	R175H Missense	8.10	4.21	94.06	3.21
MDA-MB-361	WT	Frame-shift	2.20	0.30	NA	NA
UACC812	WT	Splice mutation	1.92	0.27	8.40	NA
HCC1569 [†]	WT	R248Q Missense	4.35	1.50	7.90	NA
HCC1954 [†]	WT	R280T Missense	3.87	0.97	22.0	NA
TNBC						
MDA-MB-231 parental	WT	R280K Missense	0.17	0.23	4.17	0.46
MDA-MB-231 EV	WT	R280K Missense	NA	NA	NA	3.21
MDA-MB-231 EL	WT	R280K Missense	3.09	NA	NA	0.12
MDA-MB-231 LMW-E	WT	R280K Missense	NA	NA	NA	0.76
MDA-MB-231 PR	WT	R280K Missense	2.78	1.45	23.48	1.02
BT20 parental	Deficient	E294 Nonsense	2.6	0.14	83.8	1.20
BT20 PR	Deficient	E294 Nonsense	10.29	5.46	53.65	1.36
HCC38	WT	Splice site (c.672+1G>A)	0.09	0.07	0.4	1.18
MDA-MB-157 [†]	Deficient	R181C Missense	6.8	5.2	12.7	1.04
MDA-MB-468 [†] parental	Deficient	R273H Missense	5.05	0.62	11.6	0.47
scramble	Deficient	R273H Missense	5.04	NA	NA	0.48
shCDK2	Deficient	R273H Missense	1.61	NA	NA	1.48
HCC1806 [†] parental	Deficient	Y220C Missense	1.70	6.00	6.00	0.97
scramble	Deficient	Y220C Missense	1.80	NA	NA	0.41
shCDK2	Deficient	Y220C Missense	0.15	NA	NA	1.13
shCCNE1	Deficient	Y220C Missense	0.11	NA	NA	0.55
Non transformed BC cell line						
hMEC	WT	WT				
Empty vector	WT	WT	NA	NA	NA	15.1
FL-cycE	WT	WT	NA	NA	NA	9.60
LMW-E	WT	WT	NA	NA	NA	0.61

† Intrinsic resistant to CDK4/6 inhibitors; NA, not available

Note: the IC₅₀ values (μM) represent the mean of at least three independent experiments.

Table S2. List of PDX and transgenic mouse models and characteristics.

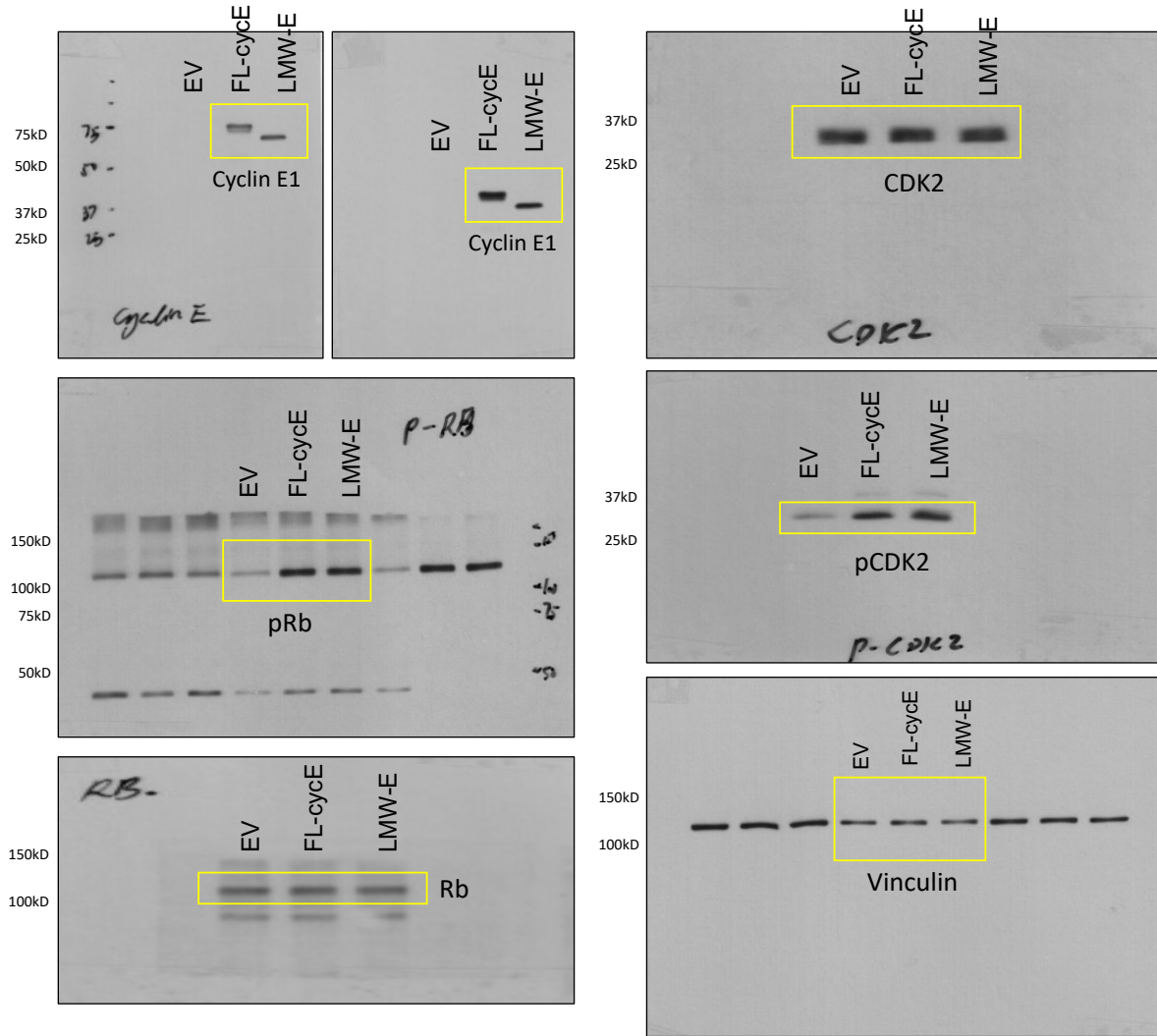
PDX Model	Receptor Status	Patient Treatment History	Genetic alterations
PR1	ER+/HER2-	Late progressor: disease progression after 16 months on Palbociclib + Letrozole	TP53, MYC, AURKB, RB1, PIK3CA, IGF1R, AKT3, CCNE2, MDM2, CHEK1, FGFR1, RAF1
PR2	ER+/HER2+	Late progressor: disease progression after 6 month on Trastuzumab + Palbociclib + Letrozole	TP53, MYC, AKT1, AURKB, RB1, NRAS, AKT3, AURKA, CCNE2, ERBB2, FGFR1, FGFR2
PR3	ER+/HER2-	Early progressor: disease progression after 3 months on Palbociclib + Letrozole	TP53, MYC, AKT1, PIK3CA, AKT3, AURKA, CCNE1, CCNE2, PKD1, HRAS, KRAS, CCND1, FGF3
PR4	ER+/HER2-	Early progressor: disease progress after 3 months on palbo+fulvestrant, skin biopsy	TP53, MYC, AKT1, AURKB, RB1, PIK3CA, IGF1R, NRAS, ESR1, CCNE1, HRAS, MDM2, ERBB2, CDH1, PTEN, KRAS, CCND1, FGF3, CHEK1, AKT2, AURKC, CDK2, CDK4, FAT1, NF1, CDK6
XC5172013 #	TNBC (Cyclin E low/negative)	Chemotherapy, radiation and endocrine therapy	STK11 HDEL, PIK3R1 HDEL, PTEN HDEL
BCX070	TNBC (Cyclin E high)	Chemotherapy	CCNE1 HAMP, AKT2 HAMP, NOTCH1 K2156, ATM DEL, BRCA2
BCX017	TNBC (Cyclin E high)	Chemotherapy	HDEL, STK11 HDEL, PIK3CA HAMP, CCNE1 HAMP, PIK3R1 HDEL
Transgenic Model			
T1p	TNBC	None	P53+/-, LMW-E

Notes: #: The model was generated from a local recurrence tumor. The patient was 49 years old at the time of the primary breast tumor diagnosis and had received chemotherapy, radiation therapy, and endocrine therapy for this HR+ cancer. After the local recurrence was surgically removed and found to be triple-negative, the patient subsequently developed chest wall metastasis.

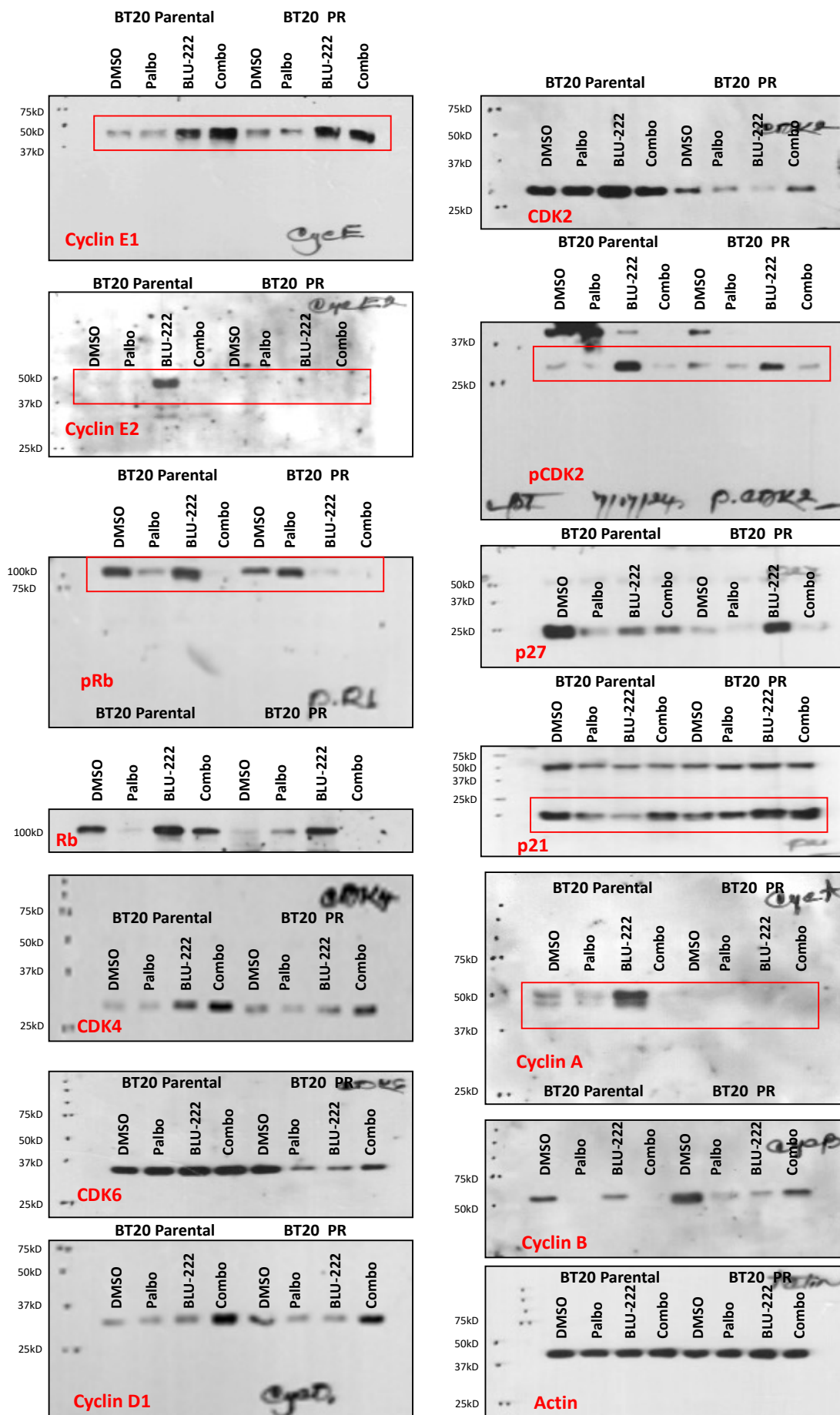
HAMP: > 4 gene copies; HDEL 1 gene copy.

Supplemental Table S3. Immunohistochemistry antibodies and conditions					
Marker	Primary antibody	Antigen retrieval	Primary antibody dilution	Secondary antibody source	Developing substrate
p21	Thermo Fisher Scientific #MA5-42680	Citrate-based antigen unmasking solution (#H-3300, Vector Labs)	1:100	VECTASTAIN Elite ABC kit (#PK6101, Vector Labs)	DAB substrate kit (#SK-4100, Vector Labs)
p27	Cell Signaling Technology #3698S	Citrate-based antigen unmasking solution (#H-3300, Vector Labs)	1:100	Horse Anti-Mouse IgG Antibody (H+L), Peroxidase (#PI-2000, Vector Labs)	ImmPACT DAB substrate (#SK-4105, Vector Labs)
γ H2AX (S139)	Cell Signaling Technology #2577S	Tris-based antigen unmasking solution (#H-3301, Vector Labs)	1:400	VECTASTAIN Elite ABC kit (#PK6101, Vector Labs)	DAB substrate kit (#SK-4100, Vector Labs)
Ki67	Abcam #ab16667	Citrate-based antigen unmasking solution (#H-3300, Vector Labs)	1:500	VECTASTAIN Elite ABC kit (#PK6101, Vector Labs)	DAB substrate kit (#SK-4100, Vector Labs)
Caspase3	BIOCARE Medical #CP229C	Citrate-based antigen unmasking solution (#H-3300, Vector Labs)	1:500	VECTASTAIN Elite ABC kit (#PK6101, Vector Labs)	DAB substrate kit (#SK-4100, Vector Labs)

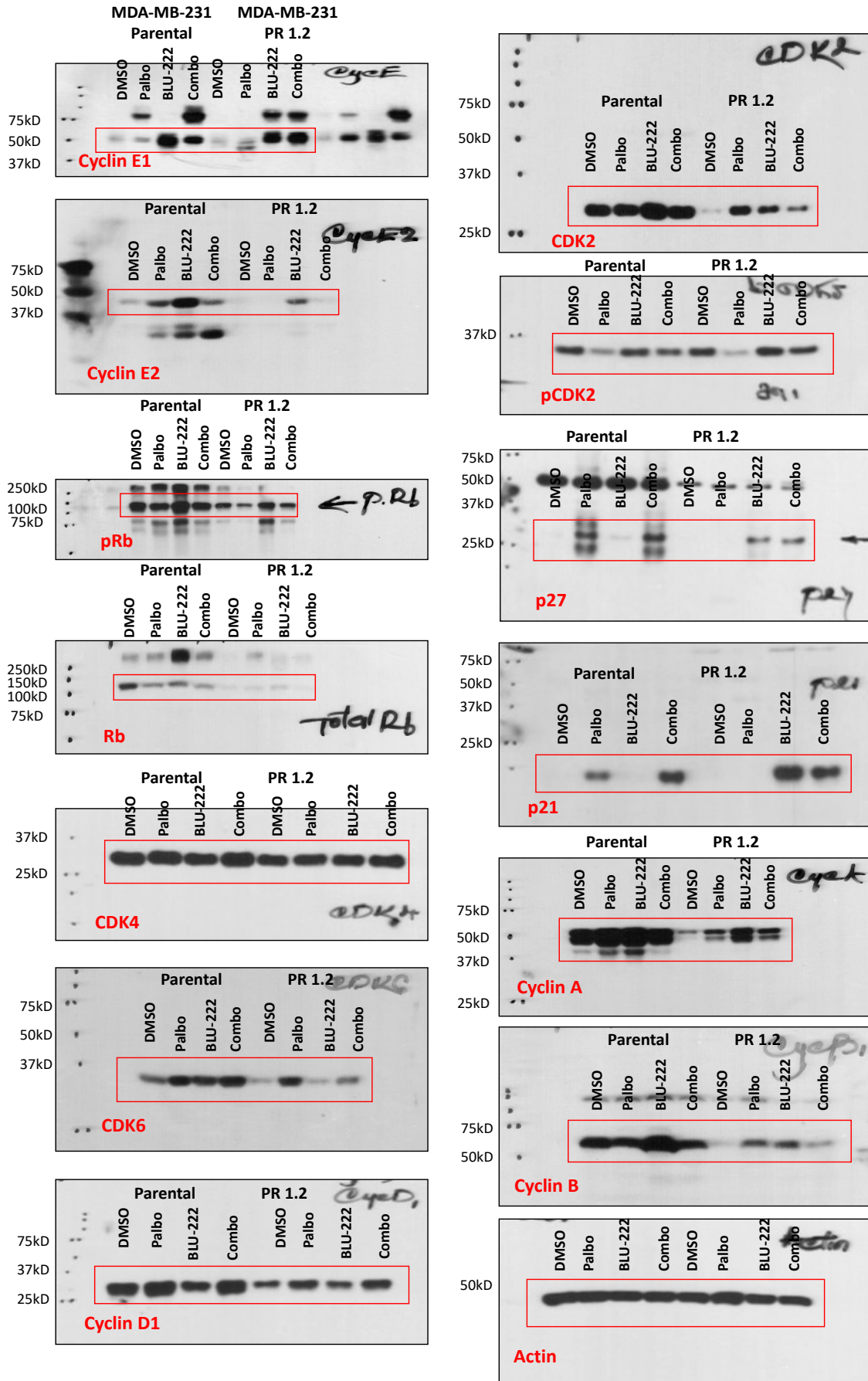
Unprocessed scans of Figure S10



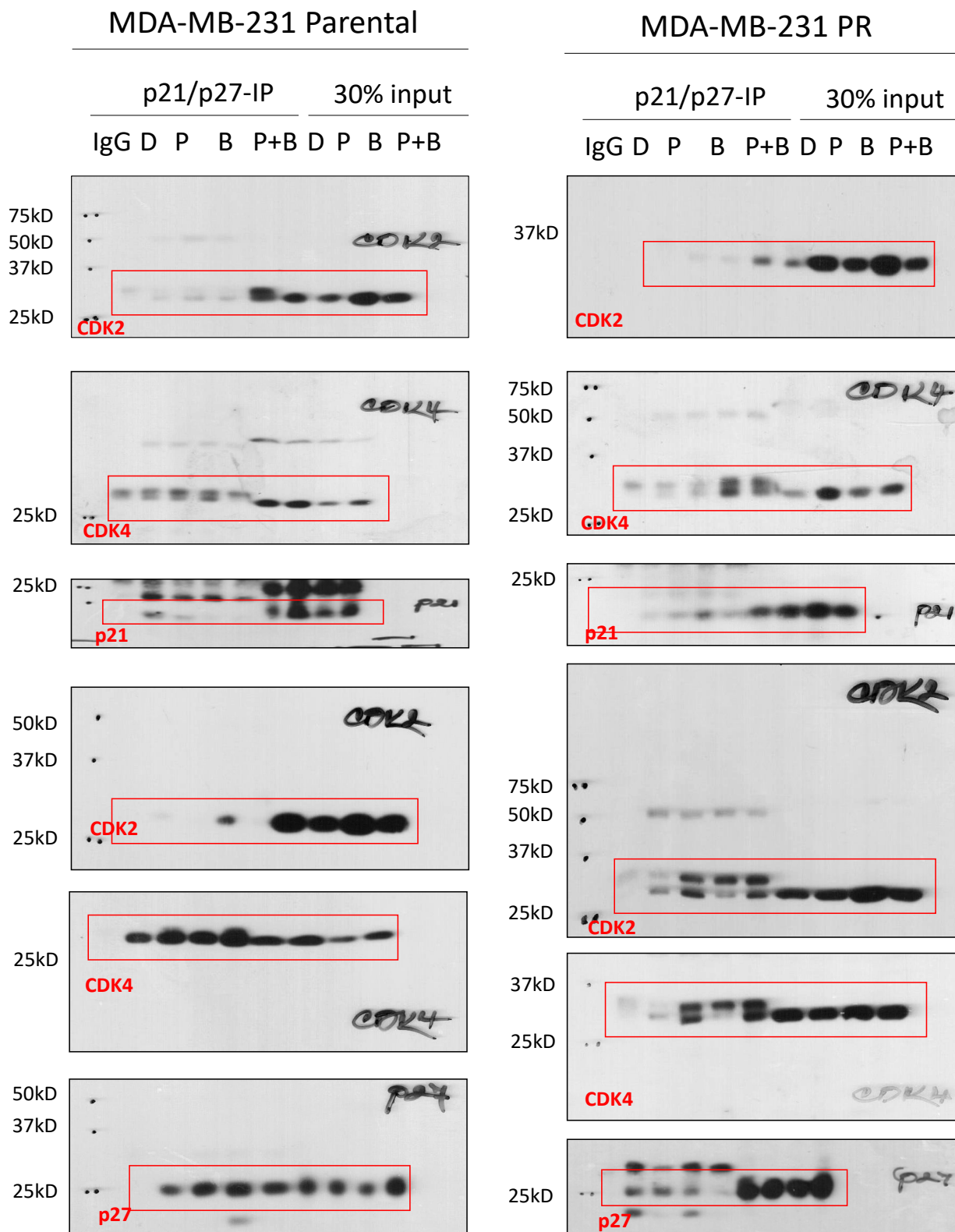
Unprocessed scans of Figure S3A



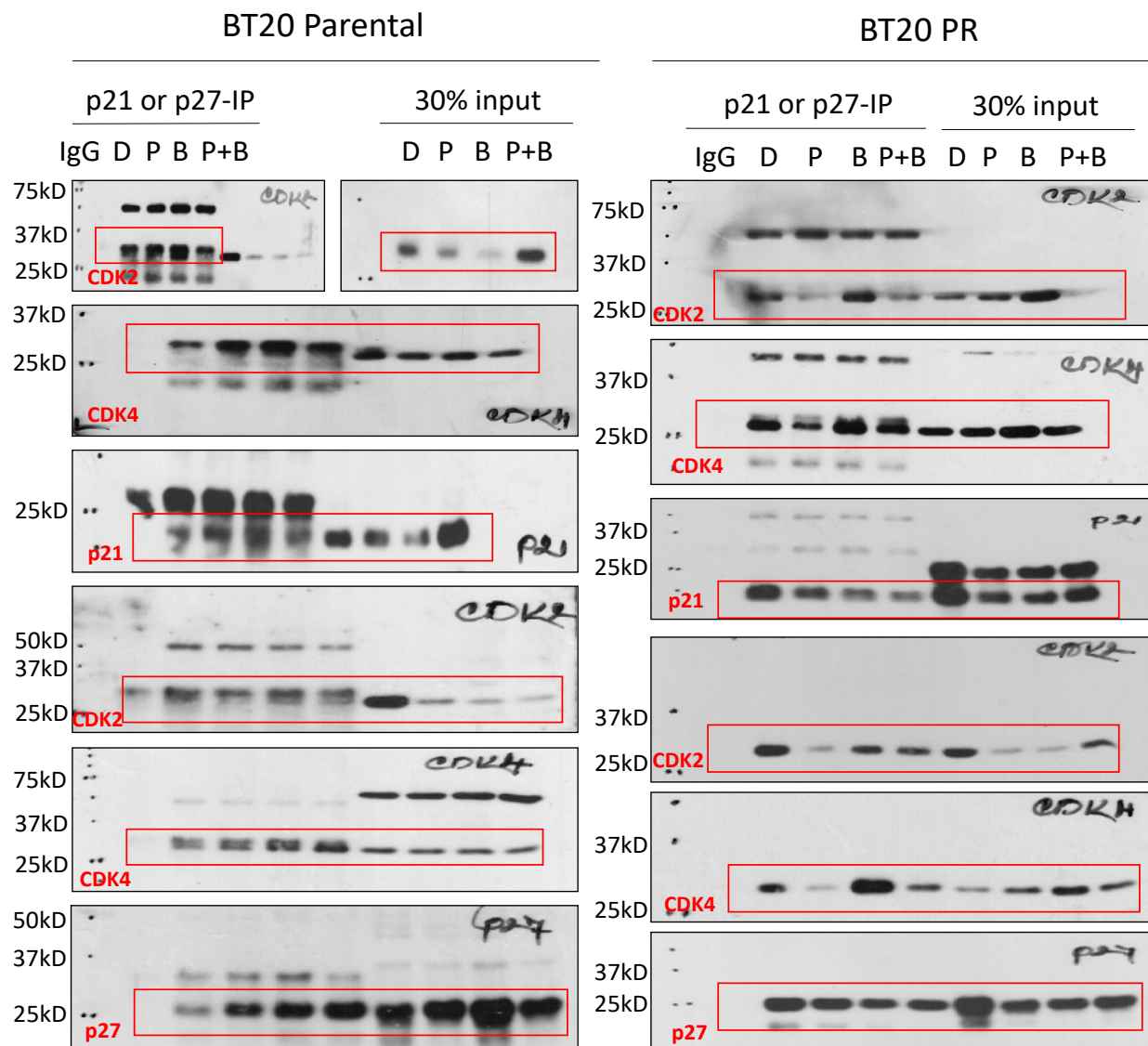
Unprocessed scans of Figure S3B



Unprocessed scans of Figure S4F



Unprocessed scans of Figure S4I



Unprocessed scans of Figure S4L

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