

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

Corresponding Author: Dr Khandan Keyomarsi

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

● In this study, the authors evaluated the efficacy of combination therapy with CDK2 and CDK4/6 inhibitors using in vitro and in vivo models of HR+/HER2- and TNBC subtypes. The study is generally well executed, and results show significant negative correlation between CDK4/6is and the CDK2i (BLU-222) across different molecular subtypes of BC, including HR+/HER2-, HER2-enriched and TNBC, highlighting the therapeutic potential of drug combination in the different breast cancer molecular subtypes.

I have some reservations regarding the level of novelty and impact of the results presented in this study, in order to warrant publication in Nature communications. The study is mostly confirmatory in nature and provides limited advances in the field in terms of novel mechanisms. Indeed, previous studies have evaluated CDK2 inhibitor efficacy alone or in combination. In particular, BLU-222 has already been shown to demonstrate strong antitumor activity in preclinical models of CCNE1-amplified ovarian cancer and HR+/HER2- breast cancers that have progressed on CDK4/6i (refs 9,25 in the manuscript). These finding further led to an ongoing clinical trial (VELA trial (NCT05252416), to evaluate BLU-222 in combination with ribociclib and fulvestrant in CDK4/6i-resistant breast cancers (refs 26,27 in the text). Furthermore, as indicated by the authors, early results from a recent trial combining CDK2i with ribociclib showed reduced TK1 and Rb activities (ref 28 in manuscript).

● The Rb status should be considered in these studies, as the presence of the WT RB protein is an important determinant for the efficacy of CDK4/6 inhibitor monotherapy (Finn RS, et al. PD 0332991. Breast Cancer Res. 2009;11(5):R77.). Especially as some of the cell lines used in this study are Rb-deficient (i.e. MDA-MB-468)

● Luminal MCF7 and T47D PR cell lines are more resistant to palbociclib than parental cells but more sensitive to BLU-222 (Fig. 2B), highlighting an inverse relationship between sensitivity to palbociclib and BLU-222, and suggesting that BLU-222 can overcome palbociclib resistance. The authors should analyze the protein expression levels of all CDKs and cyclin partners in those resistant cell lines. Indeed, variable responses to the CDK2 inhibitors could be due to differential expression levels of the CDK2/cyclinE upon acquired palbociclib resistance. Similarly, protein expression levels of CDKs/cyclins should be analyzed in the TNBC MDA-MB-231 and BT20 palbociclib resistant cells compared to their parental counterpart.

● Fig.2 (J-P): To start evaluate the efficacy of BLU-222 in overcoming palbociclib resistance, the authors tested combination treatment in CDK4/6i intrinsic and acquired resistance models of TNBC. In the acquired resistance models, cells were treated with gradually increased palbociclib concentrations, and the combi-treatment shows strong synergistic effect. The inhibitors should also be tested, alone and in combination in the two parental cell lines to assess the relative contribution of the CDK4/6i resistance the drug synergism

● Fig.3A/B: total CDK2 protein levels should be assessed and included to compare with pCDK2 levels

● Decreased expression of the cell cycle inhibitor p21 in response to CDK4/6i is already well documented and should be properly referenced (DOI: 10.18632/oncotarget.27127; DOI: 10.1038/s41388-019-0850-2)

● the pivotal roles of p21 and p27 as CIP/KIP proteins in coordinating cross talks between CDK2 and CDK4/6 and CDK2

inhibition have been previously reported. CDK4/6 inhibitors, including palbociclib, ribociclib, or abemaciclib have been shown to dissociates p21 selectively from CDK4 complexes, to redistribute p21 to CDK2, thereby inhibiting CDK2 in breast cancer cells (Nature Com, 2021; <https://doi.org/10.1038/s41467-021-23612-z>). The PLA and CO-IP data generated in the MCF7 parental/PR cells should be compared to those described in the above-mentioned study. The palbociclib-induced decrease in p21 affinity for CDK4 and subsequent redistribution to other CDK complexes should also be investigated in TNBC models with intrinsic and acquired CDK4/6i resistance), alongside the changes in p21 expression. The above-mentioned study should also be properly referenced and discussed in the manuscript

- It has also been already previously shown that chronic exposure to palbociclib leads to decreased p21 expression levels in TNBC and that restoring p21 expression and that p21 plays a role sensitizing TNBC cells to palbociclib (Mol Cancer 23, 118 (2024). <https://doi.org/10.1186/s12943-024-02029-4>;

These studies should also be properly referenced and discussed in the manuscript

- The observed BLU-222 and palbociclib synergistic effects observed in vivo in the PDX TNBC models (without CCNE1 amplification) is interesting, as CDK4/6 inhibitor resistance is highly relevant to the TNBC molecular subtype, which exhibits no or poor initial response to palbociclib. TNBC standard of care contains a combination of chemotherapy drugs and in clinical trials, and treatments are typically added to standard of care. The potential for clinical translation would be increased by evaluating the synergy of proposed treatments alongside standard of care drugs.

- The authors do not interrogate the data of the RNA-Seq studies biologically in tumor suppression. How the interferon pathway causes tumor suppression? Also, no biological data were presented supporting the notion that the combination treatment causes senescence.

- The RNA-Seq data of tumors seems to lack biological replicates for Figure 8. Is the data generated using one or multiple tumors, or biological/technical replicates per model?

- Fig.8: In figures C, D, E and F, why are the numbers of samples (tumors?) in the vehicle groups varying from 4 to 6

- Fig.8i, 8j: The authors state....A heatmap revealed consistent upregulation of genes such as SAMD9, CD47, TRIM26, and several interferon-induced genes (IFI44L, IFI35,654 IFI27) across all models (Fig. 8i). Similarly, interferon-γ pathway genes, including OAS2, HLA-A, and PSMB8, were upregulated in the combination-treated group (Fig. 8j)..... However, in combination treatment groups, samples from the BCX070 model seem to show different signatures for interferon alpha and interferon gamma than the rest of the samples. The biology of this data has not been addressed.

Reviewer #2

(Remarks to the Author)

In this manuscript, Luo and colleagues present compelling data on the activity of a novel CDK2 inhibitor, BLU-222, in HR+ and TNBC models. The authors demonstrate that the CDK2 inhibitor synergizes with CDK4/6 inhibitors in resistant HR+ and TNBC models. Additionally, they describe how this combination profoundly affects apoptosis and cell cycle arrest through the upregulation of p21 and p27. Overall, this study provides evidence of the efficacy of a novel CDK2 inhibitor in breast cancer models resistant to CDK4/6 inhibitors. The manuscript also includes interesting molecular findings on p21 and p27 on CDK2-activity that have not been previously described. However, several key questions remain to be addressed:

1) The manuscript could be more concise while retaining the same level of information.

2) The references in the introduction should be reviewed. References 10–16 are too general and should be more CDK2-focused, particularly regarding the statements that ‘CDK2 inhibition induces cellular senescence’ and that ‘senescence involves the upregulation of p21 and p27, which inhibit CDK2 activity.’ Additionally, references 33–36, which include preclinical and clinical studies on cyclin E2 overexpression bypassing CDK4/6 inhibition, should be included in the introduction. Previous evidence on the activity of a selective CDK2 inhibitor (INX-315) in preclinical models of CCNE1-amplified ovarian cancer and HR+ breast cancer progressing on CDK4/6 inhibitors should be referenced. Please cite relevant publications by Shom Goel and Erik Knudsen in Cancer Discovery and Nature Communications, respectively.

3) The densitometry quantification of CDK4 in the Western blot from Figure S1 does not appear to correlate with the expression levels in Figure 1E. Specifically, in the Western blot, CDK4 levels seem to increase in shCDK2 cells with increasing doses of BLU-222, whereas the densitometry does not reflect this. Please review this discrepancy and revise the statement: “CDK4, CDK6, and cyclin D1 were upregulated in control and shCCNE1 cells but not in shCDK2 cells (Figure 1E, Supplemental Figures S1C–E).

4) In Figure 1H, the authors show that low-molecular-weight cyclin E (LMW-E) sensitizes T47D cells to BLU-222. Based on previous studies, cells overexpressing full-length cyclin E would be expected to be more sensitive to CDK2 inhibitors. However, the data indicate that T47D cells overexpressing full-length cyclin E are more resistant to BLU-222 than parental cells. How do the authors explain this effect? Additionally, please include parental and full-length cyclin E-overexpressing MDA-MB-231 cells in Figure S1L.

5) Acquired palbociclib-resistant MCF7 and T47D cells are valuable models for studying whether BLU-222 can overcome CDK4/6 inhibitor resistance. However, the manuscript does not provide the mechanism of acquired resistance for PR1.2 and PR4.8. Notably, Navarro-Yepes et al. (Cancer Research, 2023) Figure 3F shows that PR4.8 cells do not express RB1, which could explain their inherent resistance to CDK4/6 inhibitors and the absence of synergy with BLU-222. However, the profound sensitivity of PR4.8 cells to BLU-222 is unexpected and suggests CDK2 activity independent of RB1. The authors should:

1. Comment on the mechanisms of acquired resistance in PR1.2 (high cyclin E, as shown in Figure 3A) and PR4.8 cells (RB1 loss?).
2. Discuss why BLU-222, a CDK2 inhibitor, is effective as a single agent in PR4.8 cells, which lack RB1 expression. It would also be relevant to mention whether BLU-222 has antitumor activity in RB1-deficient tumors.
- 6) The authors generated palbociclib-resistant TNBC cell lines (MDA-MB-231 and BT20). However, in Figure 1N, the palbociclib sensitivity of the parental cells is not shown. According to Finn et al. (2009), MDA-MB-231 cells are relatively resistant to palbociclib. Including synergy plots for the combination treatment in parental MDA-MB-231 and BT20 cells would help clarify this point.
- 7) The combination of BLU-222 with CDK4/6 inhibitors in HR+/HER2- PDX models that progressed on palbociclib is highly consistent. However, it would be valuable to mention the resistance mechanisms of PR1, PR2, PR3, and PR4. Additionally, a comparison of the triplet therapy (BLU-222 + CDK4/6i + endocrine therapy) versus the standard of care (palbociclib + fulvestrant) would be beneficial. The authors should also consider:
 - Removing data on γH2AX as a marker of DNA damage in vivo, given that DNA damage has not been demonstrated in prior studies with the CDK2 inhibitor.
 - Instead, including apoptosis data based on Figure 2 and the role of p21-p53.
- 8) Regarding the GSEA results, the authors report that the G2M checkpoint signature is downregulated only in XC517. However, based on the findings from MCF7, T47D PR, and TNBC models, we would expect BLU-222 (alone or in combination with CDK4/6 inhibitors) to downregulate the G2M checkpoint in all models tested. Do the authors have any hypotheses for these differences?
 - If the RNA-seq timepoint corresponds to the endpoint of the in vivo experiments in Figure 7, it might be too late to capture cell cycle and apoptosis effects while instead reflecting a strong immune response.
 - The observed effects on the G2M checkpoint and E2F targets in XC517 may be influenced by treatment holidays and retreatment schedules.
- 9) In the discussion, please comment on Guiley et al 2021 related to p27 activating CDK4 and antagonizing palbociclib inhibition.

Minor comments

- 1) Include phosphorylation sites for CDK2 and RB in the Western blot figure legends (Figures 1A, 1E, and 1G).
- 2) In Figure 6, the combination of BLU-222 with ribociclib is shown. However, in panels 6D–I, the violet line is labeled as "Palbo + Fulv" instead of "Ribo + Fulv." According to the figure legend, it should be "Ribo + Fulv"—please correct this.
- 3) Consider reorganizing Figure 8 so that panels C–D, E–F, and K–L are aligned in parallel to improve readability.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Comments from the previous review have been addressed appropriately, the manuscript is now much improved

Reviewer #2

(Remarks to the Author)

The authors have addressed all the questions

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Dear Reviewers: We thank you for the thoughtful summary and the opportunity to revise our manuscript. Below, we first provide a detailed table summarizing every comment raised by both reviewers, outlining how each has been addressed — whether through expanded discussion, additional analyses, or the inclusion of new experiments — along with the corresponding updated figure and table numbers in the revised manuscript. Following the table, we present point-by-point responses to each reviewer. In these, we describe the rationale for our revisions, detail any newly generated data, summarize the new results, and indicate precisely where these changes have been incorporated into the revised text.

Reviewer 1 Comments	Author's responses
#1 In this study, the authors evaluated the efficacy of combination therapy with CDK2 and CDK4/6 inhibitors using in vitro and in vivo models of HR+/HER2- and TNBC subtypes. The study is generally well executed, and results show significant negative correlation between CDK4/6is and the CDK2i (BLU-222) across different molecular subtypes of BC, including HR+/HER2-, HER2-enriched and TNBC, highlighting the therapeutic potential of drug combination in the different breast cancer molecular subtypes. I have some reservations regarding the level of novelty and impact of the results presented in this study, in order to warrant publication in Nature communications. The study is mostly confirmatory in nature and provides limited advances in the field in terms of novel mechanisms. Indeed, previous studies have evaluated CDK2 inhibitor efficacy alone or in combination. In particular, BLU-222 has already been shown to demonstrate strong antitumor activity in preclinical models of CCNE1-amplified ovarian cancer and HR+/HER2- breast cancers that have progressed on CDK4/6i (refs 9,25 in the manuscript). These finding further led to an ongoing clinical trial (VELA trial (NCT05252416), to evaluate BLU-222 in combination with ribociclib and fulvestrant in CDK4/6i-resistant breast cancers (refs 26,27 in the text). Furthermore, as indicated by the authors, early results from a recent trial combining CDK2i with ribociclib showed reduced TK1 and Rb activities (ref 28 in manuscript).	Results and discussion highlighting the novel contributions of this study as follows. Novel Contributions include Pnew mechanistic insights, expands applicability to difficult-to-treat subtypes, and strengthens translational rationale for clinical development. Mechanism: First evidence that p21/p27 directly inhibit both CDK2 and CDK4, driving combination synergy; CRISPR loss abolishes effect. Drug sensitivity: Newly identified inverse CDK2i/CDK4/6i sensitivity correlation across BC subtypes. Therapeutic scope: Demonstrated efficacy in RB1-deficient, LMW-E, and CCNE1-amplified TNBC, plus HR+/HER2-models. In vivo impact: Durable regressions and survival benefit in 7 PDXs and an immune-competent model. Pathways & immunity: Senescence and interferon activation validated in vitro/in vivo; increased TILs suggest immunomodulatory potential.
#2 The Rb status should be considered in these studies, as the presence of the WT RB protein is an important determinant for the efficacy of CDK4/6 inhibitor monotherapy (Finn RS, et al. PD 0332991. Breast Cancer Res. 2009;11(5):R77.). Especially as some of the cell lines used in this study are Rb-deficient (i.e. MDA-MB-468)	Results: Supplementary Tables S1 and S2, Supplementary Figures S2D, S2H, S2M-O, and Main Figure 3A Discussion
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****REVIEWER REPORTS****

Reviewer #1

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We thank the reviewer for their positive assessment of the study's execution and their acknowledgment of the therapeutic potential of dual CDK2/CDK4/6 inhibition across multiple breast cancer subtypes. We appreciate the concern regarding novelty and agree that prior studies, including those cited in our manuscript, have evaluated CDK2 inhibition—sometimes in combination with CDK4/6 inhibitors—in certain contexts. However, our work extends this field in several important and novel directions, providing both new mechanistic insight and an expanded translational scope:

1. *Novel mechanistic insight into p21/p27-mediated synergy via direct interactions with CDK2 and CDK4:* We demonstrate that the synergistic effect of CDK2 inhibition (via BLU-222) in combination with CDK4/6i is mediated by the induction of the CDK inhibitors p21 and p27, which engage directly with both CDK2 and CDK4. Utilizing proximity ligation and co-immunoprecipitation assays in 4 matched sensitive and CDK4/6i resistant models, we show that BLU-222 enhances p21 binding to CDK2 and both p21 and p27 binding to CDK4—particularly in resistant models. This dual engagement represents a novel mechanistic insight not reported in previous studies involving CDK2 inhibitors, including those evaluating BLU-222.
2. *Functional validation using CRISPR knockouts of p21 and p27 as critical effectors of synergy:* We show that CRISPR-mediated knockout of either p21 or p27 eliminates the synergistic effect of the BLU-222 and CDK4/6i combination, shifting the drug interaction from synergistic to antagonistic. This directly demonstrates that p21 and p27 are not only biomarkers but essential mediators of therapeutic efficacy, offering novel mechanistic rationale for dual inhibition strategies.
3. *Systematic drug sensitivity profiling reveals a consistent inverse correlation between CDK2i and CDK4/6i response:* We demonstrate a significant inverse correlation in drug sensitivity to CDK2i and CDK4/6i across 15 breast cancer cell lines representing HR+/HER2-, HER2-enriched, and TNBC subtypes. This is the first study, to our knowledge, to quantitatively establish this relationship, providing a preclinical rationale for complementary targeting of these pathways and potential stratification of patients based on reciprocal sensitivities.
4. *First demonstration of strong synergy in triple-negative breast cancer (TNBC) models, including subtypes characterized by low-molecular-weight cyclin E (LMW-E) overexpression, RB1-deficient, CCNE1 amplification, and resistance to CDK4/6is:* A major novel contribution of our work is the demonstration that BLU-222 synergizes with CDK4/6 inhibitors in TNBC models, including those with RB1 deficiency, LMW-E overexpression, CCNE1 amplification, and acquired resistance, contexts where CDK4/6i monotherapy is typically ineffective. This underexplored therapeutic opportunity opens a potential avenue for expanding CDK2-targeted combination strategies beyond the HR+/HER2- population.
5. *Robust in vivo validation across multiple PDX models with both intrinsic and acquired resistance:* While previous reports highlighted the efficacy of BLU-222 in specific models, our study evaluates seven independent patient-derived xenograft (PDX) models, including four HR+/HER2- breast cancer that progressed on palbociclib, three TNBC models, and a transgenic model (T1p) overexpressing LMW-E to mimic the TNBC subtype. We show that BLU-222 in combination with

palbociclib or ribociclib ($\pm$ fulvestrant) induces durable tumor regression and significantly prolongs survival, in both acquired and intrinsically resistant settings. These findings add translational weight to our mechanistic observations and directly support the clinical development of this combination strategy.

6. *Transcriptomic and functional evidence of senescence and interferon pathway activation:*

RNA-seq analysis revealed a coordinated upregulation of senescence and interferon signaling pathways in PDX tumors treated with the combination of CDK2 and CDK4/6 inhibitors, suggesting a mechanistic basis for the observed therapeutic synergy. In addition to transcriptomic profiling, we validated the induction of cellular senescence across HR+/HER2- and TNBC cell lines, where β -galactosidase staining was significantly elevated in the combination treatment group. To functionally interrogate the role of the interferon pathway in mediating this antitumor response, we employed pharmacologic blockade using two independent JAK1/2 inhibitors. Inhibition of the pathway partially rescued tumor cell viability under combination treatment, supporting a critical role for interferon signaling in the efficacy of dual CDK inhibition.

7. *Immune microenvironment remodeling and TIL enrichment in the T1p model: Furthermore, in an immune-competent transgenic mouse model (T1p) overexpressing LMW-E, we showed marked increase in tumor-infiltrating lymphocytes (TILs) following combination therapy, compared to both monotherapy and vehicle-treated groups. These findings implicate immunogenic activation within the tumor microenvironment as an additional contributor to therapeutic benefit and provide a strong preclinical rationale for integrating immunomodulatory strategies with CDK2 and CDK4/6 inhibition.*

Taken together, our study goes beyond previously published findings by elucidating a novel, mechanistically grounded rationale for dual CDK2/CDK4/6 inhibition, revealing previously unrecognized roles of p21 and p27 in driving this synergy, mediated by the coordinated activation of senescence and interferon-associated immune pathways. Furthermore, we expand the therapeutic scope, particularly in challenging contexts such as TNBC and RB1-deficient tumors. These contributions represent a meaningful advancement of both biological understanding and therapeutic strategy and provide a strong preclinical foundation to support ongoing and future clinical trials.

Revised “Discussion” in main manuscript (lines 1002-1018 of the revised manuscript)

In conclusion, our study demonstrates that BLU-222, when combined with CDK4/6 inhibitors, effectively overcomes resistance in both HR+ and TNBC models, including challenging contexts such as RB1-deficient and LMW-E-overexpressing tumors where CDK4/6i monotherapy is typically ineffective. We uncover a novel mechanistic framework in which upregulation of p21 and p27, coupled with their enhanced binding to both CDK2 and CDK4 complexes, drives therapeutic synergy. Functional CRISPR knockout experiments confirm these proteins as essential mediators rather than passive biomarkers. This effect is further reinforced by increased cellular senescence—validated in vitro and in vivo through biomarker analysis—activation of the p21–DREAM complex, and induction of type I and II interferon signaling, contributing to immune pathway engagement. We also show remodeling of the tumor immune microenvironment, including tumor-infiltrating lymphocyte enrichment in immune-competent models, underscoring a potential immunomodulatory component to the response. Importantly, systematic in vivo benchmarking against SOC regimens across multiple independent PDX and syngeneic models establishes the translational superiority of dual CDK2/CDK4/6 inhibition. Together, these findings advance our biological understanding of resistance mechanisms and provide a strong rationale for clinical testing of CDK2i combinations, as a promising therapeutic strategy for patients with CDK4/6i-resistant breast cancer across diverse molecular subtypes.

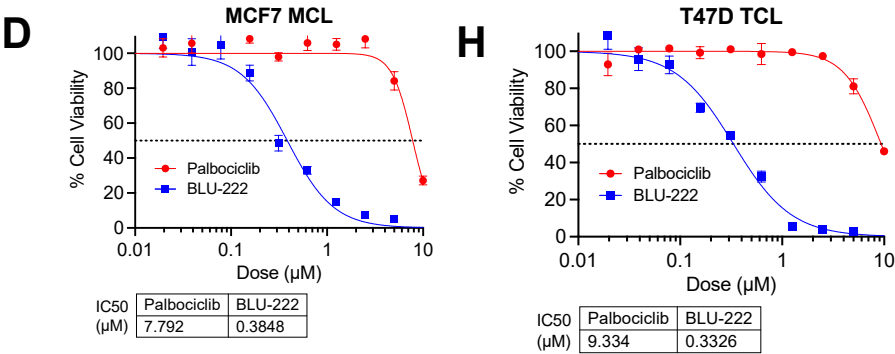
- The Rb status should be considered in these studies, as the presence of the WT RB protein is an important determinant for the efficacy of CDK4/6 inhibitor monotherapy (Finn RS, et al. PD 0332991. Breast Cancer Res. 2009;11(5):R77.). Especially as some of the cell lines used in this study are Rb-deficient (i.e. MDA-MB-468)

We thank the reviewer for highlighting the critical role of RB status in determining sensitivity to CDK4/6 inhibitor monotherapy, as established in the foundational study by Finn et al. (Breast Cancer Res. 2009). In the revised manuscript, we now explicitly report the RB status for all PDX models and cell lines used, including CDK4/6i-resistant clones (**Revised supplementary Tables S1 and S2**), and have integrated this information throughout the Results and Discussion sections.

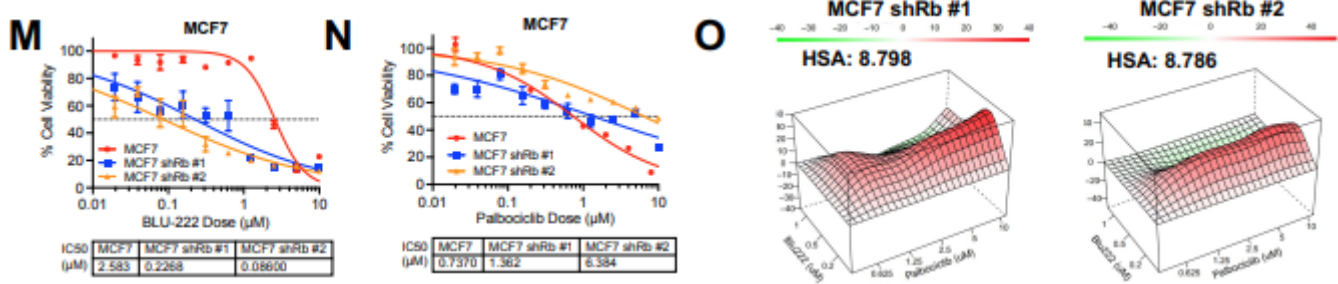
To address this point experimentally, we intentionally included both RB-proficient and RB-deficient models to reflect clinical heterogeneity and resistance patterns. RB-proficient models included HR+/HER2- cell lines MCF7 and T47D and TNBC cell lines MDA-MB-231 and BT20 cell lines; RB-deficient models included TNBC cell lines MDA-MB-468, HCC1806, and MDA-MB-157, as well as CDK4/6i-resistant HR+/HER2- clones derived in our lab—T47D TCL and MCF7 MCL—where RB loss emerged during long-term drug selection (**new supplemental Figure S2 D-H**). In addition, we generated a RB knockdown model of MCF7 (MCF7 shRB,) which is presented in the new **Figure 3A**, to further dissect the role of RB status in combination responses as presented in new **Figure S2M-O**

Below we provide the key experimental findings that we have included in the revised manuscript.

- 1) Response patterns of monotherapy in RB-deficient settings:** As expected, RB-deficient TNBC lines, CDK4/6i resistant pools (MCF-7 PR4.8 and T47D 4.8) and clones (TCL, MCL), and the MCF7 shRB model were refractory to CDK4/6 monotherapy, yet remained very sensitive to CDK2 inhibition with BLU-222 (Figures S2C, S2G, **new S2D,H and new Figure 3A**) This aligns with the known capacity of CDK2/cyclin E to drive S-phase progression independently of RB, and with literature showing RB1-deficient tumors' reliance on CDK2-mediated replication stress pathways.
- 2) Combination efficacy in RB-deficient and RB-proficient models:** BLU-222 plus palbociclib showed strong synergy (high HAS synergy scores) in RB-deficient TNBC cells [HCC1806 and MDA-MB-157 (Fig 2J, 2P)], RB-deficient resistant clones [TCL, MCL (**new Figure S2D-H**)], and the MCF7 shRB model (**new Figure S2M-O**), with marked increases in apoptosis and G2/M arrest (Figures 1D, 1E; Figures S2R,S). Moreover, in MCF7 and T47D cells, the combination was additive in both RB-intact and RB-knockout contexts **new Figure S2O**). These findings indicate that the combination therapy operates through mechanisms that do not strictly rely on Rb status. Instead, the synergy likely arises from coordinated suppression of CDK2/CDK4 via p21 and p27, along with activation of senescence and interferon-related pathways, as shown in our mechanistic experiments.



Supplemental Figure S2 (D, H)
Dose-response curves for palbociclib and BLU-222 monotherapy in **(D)** MCF7 MCL and **(H)** T47D TCL cells. Data are presented as mean ± SEM from three biological replicates.



Supplemental Figure S2 (M-O) (M-N) Dose-response curves of MCF7 parental and shRB clones (#1 and #2) treated with (**M**) Blu-222 and (**N**) Palbociclib monotherapy. (**O**) Synergy analysis of BLU-222 and palbociclib in MCF7 cells with Rb knockdown (shRb #1 and shRb #2), quantified using the HSA model.

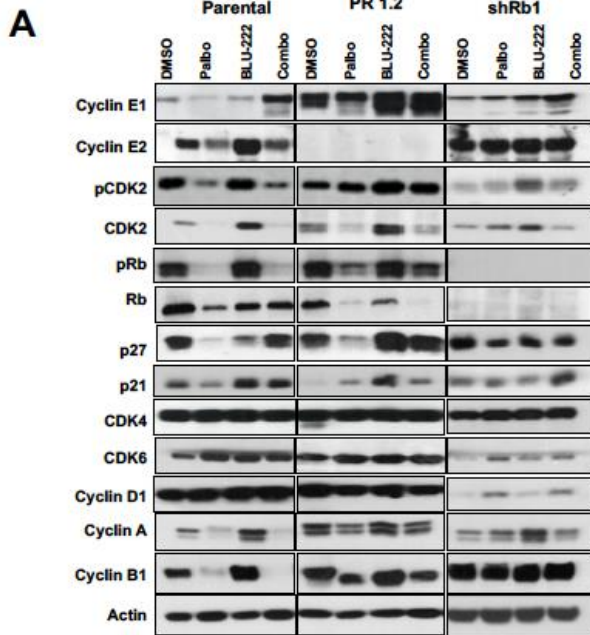


Figure 3A. Representative Western blots showing expression levels of pRb, Rb, Cyclin E1, cyclin E2, p27, p21, CDK2, p-CDK2, CDK4, CDK6, Cyclin D1, Cyclin A, Cyclin B1 and actin in p21, p27, cyclin D1, pCDK2, and actin in MCF7 parental and PR1.2 and Rb1 knockdown cell lines treated with 0.3 μM of single agents or a combination of palbociclib and BLU-222 for 3 days.

Revised Results in main manuscript (lines 276-295 of the revised manuscript)

Previous studies have established Rb as a critical determinant of sensitivity to CDK4/6 inhibitor (1). We assessed RB status across all cell lines and PDX models, including parental, resistant, and engineered lines (Supplemental Tables S1 and S2). RB-proficient models included HR+/HER2- MCF7 and T47D and TNBC MDA-MB-231; RB-deficient models included TNBC lines MDA-MB-468, HCC1806, and MDA-MB-157, as well as CDK4/6i-resistant clones T47D TCL and MCF7 MCL, where RB loss arose during long-term selection (Supplemental Fig. S2D and S2H). We also generated an RB knockdown MCF7 model (MCF7 shRB). Among the resistant pools, PR1.2 cells retained RB but exhibited high cyclin E expression, whereas PR4.8 cells showed complete RB loss, consistent with our prior reports (2). As expected, RB-deficient TNBC lines, resistant pools (MCF7 PR4.8, T47D 4.8), resistant clones (TCL, MCL), and MCF7 shRB were refractory to CDK4/6i monotherapy but remained highly sensitive to BLU-222 (Figs. S2C, S2G, S2D, S2H). Notably, BLU-222 monotherapy was particularly effective in RB-deficient PR4.8 cells, underscoring that CDK2 activity can drive proliferation independently of RB. BLU-222 plus palbociclib

also produced strong synergy in RB-deficient TNBC models, resistant clones, and MCF7 shRB (Figs. 2J, S2M-O), with marked apoptosis and G2/M arrest (Figs. 1C, 1D). In RB-proficient MCF7 and T47D parental cells, the combination was additive regardless of RB status. Collectively, these results establish that while RB loss confers resistance to CDK4/6i, it does not diminish sensitivity to CDK2 inhibition; rather, BLU-222 effectively exploits CDK2/cyclin E-driven replication stress vulnerabilities in both RB-intact (PR1.2) and RB-deficient (PR4.8) contexts, providing a strong rationale for therapeutic targeting of this subset.

Revised “Discussion” in main manuscript (lines 933-939 of the revised manuscript)

RB status, a known determinant of CDK4/6 inhibitor response [37], did not diminish BLU-222 activity. Resistant PR1.2 cells retained RB but displayed elevated cyclin E, while PR4.8 cells lost RB entirely. Both remained highly sensitive to BLU-222. Across RB-proficient and RB-deficient contexts—including TNBC lines, resistant clones, and MCF7 shRB—BLU-222 retained potency and synergized with CDK4/6 inhibition, producing apoptosis and G2/M arrest. These findings underscore that CDK2 dependence persists even in RB-null settings, consistent with reports that CDK2 inhibition is synthetically lethal in RB1-deficient cancers (3,4).

- Luminal MCF7 and T47D PR cell lines are more resistant to palbociclib than parental cells but more sensitive to BLU-222 (Fig. 2B), highlighting an inverse relationship between sensitivity to palbociclib and BLU-222, and suggesting that BLU-222 can overcome palbociclib resistance. The authors should analyze the protein expression levels of all CDKs and cyclin partners in those resistant cell lines. Indeed, variable responses to the CDK2 inhibitors could be due to differential expression levels of the CDK2/cyclinE upon acquired palbociclib resistance. Similarly, protein expression levels of CDKs/cyclins should be analyzed in the TNBC MDA-MB-231 and BT20 palbociclib resistant cells compared to their parental counterpart.

We thank the reviewer for this insightful comment. As recommended, we evaluated protein expression levels of CDK2, pCDK2, CDK4, CDK6, cyclins A, B, D1, E1, E2, Rb, pRb, and p21 and p27 in HR+ resistant models (MCF7-PR and T47D-PR; revised Figures 3A and 3B) as well as in TNBC palbociclib-resistant models (MDA-MB-231 -PR and BT20-PR; revised Figures S3A and S3B). We observed heterogeneous alterations in CDK/cyclin expression across these models. In HR+ resistant cells, cyclin E1 and CDK2 were consistently elevated compared with parental counterparts, while CDK4 levels remained stable or modestly increased, and cyclin D1 was downregulated specifically in T47D-PR. By contrast, TNBC resistant models did not exhibit upregulation of cyclin E1 or CDK2, and cyclin D1 and CDK4 levels varied between the two TNBC cell lines analyzed. Despite this variability, the most consistent finding across all HR+ and TNBC resistant models was the induction of p21 and p27 following BLU-222 treatment. This consistent response underscores p21 and p27 as central mediators of sensitivity to CDK2 inhibition, even as individual resistant lines maintain distinct CDK/cyclin alterations. Together, these findings suggest that while differential CDK/cyclin expression may influence the degree of sensitivity to BLU-222, the unifying mechanism across resistant settings is the induction of p21 and p27, providing a mechanistic basis for the observed therapeutic efficacy.

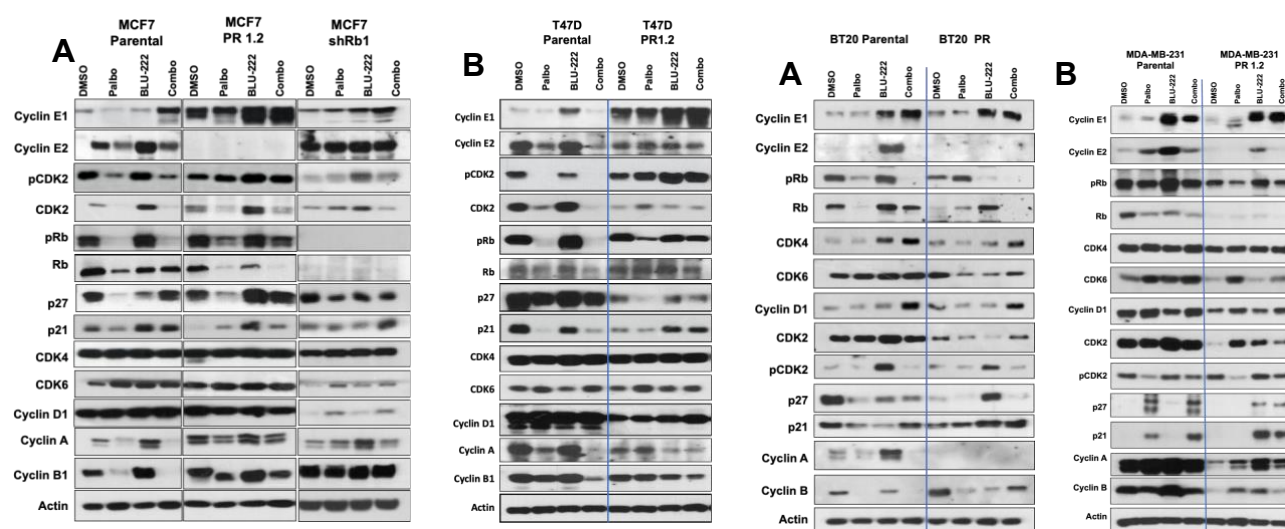


Figure 3A-B. (A-B) Representative Western blots showing expression levels of cyclin E1, E2, A, B1, D1, CDK2, CDK4, phosphorylated and total Rb, p21, p27 and actin in (A) MCF7 parental, PR1.2, and shRB1 knockdown cell lines and (B) T47D parental and PR1.2 cell lines treated with 0.3 μ M of single agents or a combination of palbociclib and BLU-222 for 3 days.

Supplementary Figure S3A-B. Representative Western blots showing expression levels of cyclin E1, E2, A, B1, D1, CDK2, CDK4, phosphorylated and total Rb, p21, p27 and actin in (A) BT20 parental and PR1.2 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.

Revised Results in main manuscript (lines 378-396 of the revised manuscript)

To elucidate the mechanisms underlying the observed synergy between BLU-222 and CDK4/6 inhibition, we examined cell cycle protein expression in MCF7 and T47D parental cells, their palbociclib-resistant (PR) derivatives, and RB-deficient variants following treatment with BLU-222 (0.3 μ M), palbociclib (0.3 μ M), or the combination for three days (**Figs. 3A–B**). Western blot analysis confirmed elevated cyclin E1 and phosphorylated CDK2 in HR+ PR cells, consistent with their CDK4/6i-resistant phenotype (2). Cyclin D1 was decreased in T47D but remained stable in MCF7, while CDK4 expression was stable or modestly increased in both lines. In contrast, TNBC PR models (MDA-MB-231-PR and BT20-PR; Supplemental Figs. S3A–B) showed no consistent elevation of cyclin E1 or CDK2, and cyclin D1/CDK4 levels varied, underscoring the heterogeneity of resistance mechanisms across subtypes. Despite these differences, a unifying feature across resistant models was the consistent elevated levels of p21 and/or p27 following BLU-222 treatment, either alone or in combination with palbociclib. In parental HR+ cells, p21 levels rose only with the combination, while p27 induction was diminished by palbociclib alone. Notably, in RB-deficient resistant cells, p21 was strongly induced by the combination, whereas p27 expression remained largely unchanged under all conditions. These findings indicate that RB is partially required for p27 induction in response to CDK4/6 blockade, while p21 induction can be triggered by CDK2 inhibition independently of RB. Thus, across HR+ and TNBC contexts—and irrespective of RB status—BLU-222 drives Cip/Kip upregulation, highlighting p21 and p27 as central mediators of the synergy between CDK2 and CDK4/6 inhibition.

- Fig.2 (J-P): To start evaluate the efficacy of BLU-222 in overcoming palbociclib resistance, the authors tested combination treatment in CDK4/6i intrinsic and acquired resistance models of TNBC. In the acquired resistance models, cells were treated with gradually increased palbociclib concentrations, and the combi-treatment shows strong synergistic effect. The inhibitors should also be tested, alone and in combination in the two parental cell lines to assess the relative contribution of the CDK4/6i resistance the drug synergism

We thank the reviewer for this insightful suggestion. In response, we have now evaluated the effects of BLU-222 and palbociclib, both as single agents and in combination, in the parental TNBC cell lines BT20 and MDA-MB-231. As shown in the newly included data (**Fig. 2J and 2P and Supplemental Figure S2L**) the combination of BLU-222 and palbociclib produced only minimal synergy in the parental lines, with HSA synergy scores of 2.48 (BT20) and 4.01 (MDA-MB-231). In sharp contrast, the corresponding palbociclib-resistant (PR) derivatives demonstrated a marked increase in synergy, with HSA synergy scores rising to 14.74 in BT20-PR and 13.37 in MDA-MB-231-PR (Fig. 2B, D). To extend this analysis, we compared across lineages using a synergy heatmap (**Fig. 2P**). These results demonstrate a consistent pattern: all parental cells, regardless of subtype (HR+ or TNBC), display additive responses to the combination, whereas all resistant cells exhibit robust synergy. This highlights that BLU-222/palbociclib synergy is a resistance-associated phenomenon, independent of lineage, and reinforces the conclusion that CDK2 inhibition preferentially benefits tumors with acquired CDK4/6i resistance.

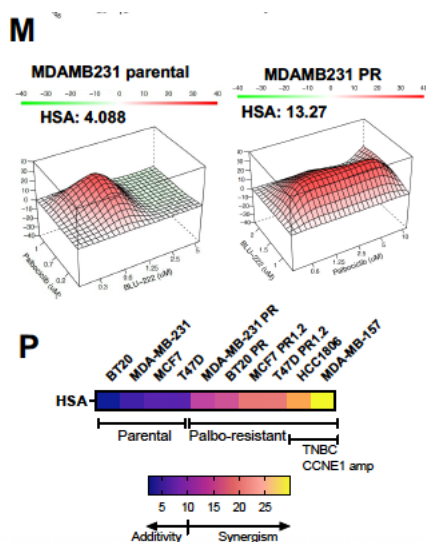
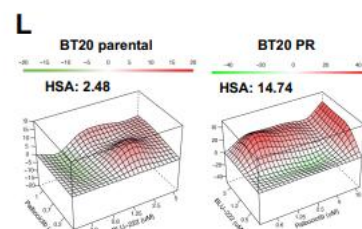


Fig. 2 (M) Representative 3D synergy plots illustrating the response of MDA-MB-231 parental and PR cells to combination treatment with different doses of palbociclib and BLU-222. **(P)** Heatmap of highest single agent (HSA) synergic scores across HR+, TNBC, and acquired palbo-resistant breast cancer cell lines. Error bars represent SEM in at least 3 independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



Revised Results in main manuscript (lines 317-319 of the revised manuscript)

Combination treatment of these PR cells revealed strong synergy (HSA scores 13.3–14.8) at low doses (0.3 μ M each), while parental counterparts displayed only minimal synergy (HSA scores 2.4–4.1) (**Figs. 2M and Supplemental Fig S2L**).

Revised Results in main manuscript (lines 324-332 of the revised manuscript)

To determine whether the observed synergy was subtype-specific, we compared responses across HR+ and TNBC parental and resistant models using HSA modeling (**Fig. 2P**). This analysis revealed a consistent trend: all parental cells, irrespective of subtype, exhibited additive effects, whereas all resistant cells displayed strong synergy, establishing BLU-222/palbociclib synergy as a hallmark of acquired resistance rather than lineage. In HR+ (MCF7 and T47D) and TNBC (MDA-MB-231 and BT20) parental cells, additive responses likely reflect their inherent sensitivity to CDK4/6 inhibition, while both HR+ (MCF7 PR1.2 and T47D PR1.2) and TNBC (MDA-MB-231 PR and BT20 PR) resistant cells showed enhanced synergy driven by compensatory CDK2 activation during CDK4/6i escape.

- Fig.3A/B: total CDK2 protein levels should be assessed and included to compare with pCDK2 levels

We thank the reviewer for this important suggestion. In response, we have now included total CDK2 protein levels alongside phospho-CDK2 in Figures 3A–B (see above western blots). These data demonstrate that in HR +ve parental cells, total and phosphorylated CDK2 levels are comparable, indicating that CDK2 activation largely mirrors CDK2 abundance. In contrast, in resistant models, total CDK2 levels do not parallel

phosphorylation, and pCDK2 is disproportionately elevated relative to total CDK2. This distinction suggests that CDK2 activation, rather than overall protein expression, is selectively enhanced in the resistant setting, reinforcing its role as a key driver of resistance and response to BLU-222.

- Decreased expression of the cell cycle inhibitor p21 in response to CDK4/6i is already well documented and should be properly referenced (DOI: 10.18632/oncotarget.27127; DOI: 10.1038/s41388-019-0850-2)
- the pivotal roles of p21 and p27 as CIP/KIP proteins in coordinating cross talks between CDK2 and CDK4/6 and CDK2 inhibition have been previously reported. CDK4/6 inhibitors, including palbociclib, ribociclib, or abemaciclib have been shown to dissociates p21 selectively from CDK4 complexes, to redistribute p21 to CDK2, thereby inhibiting CDK2 in breast cancer cells (Nature Com, 2021; <https://doi.org/10.1038/s41467-021-23612-z>). The PLA and CO-IP data generated in the MCF7 parental/PR cells should be compared to those described in the above-mentioned study. The palbociclib-induced decrease in p21 affinity for CDK4 and subsequent redistribution to other CDK complexes should also be investigated in TNBC models with intrinsic and acquired CDK4/6i resistance), alongside the changes in p21 expression. The above-mentioned study should also be properly referenced and discussed in the manuscript

We thank the reviewer for citing prior studies documenting decreased expression of p21 in CDK4/6i-resistant cells (5,6) and for highlighting the pivotal work of Pack et al. (7), which showed that CDK4/6 inhibitors promote selective dissociation of p21 from CDK4 complexes, redistributing it to CDK2 and indirectly suppressing CDK2 activity. These important studies are now cited and discussed in detail in the revised manuscript (Discussion, pp. 41–42).

While consistent with this prior literature, our findings reveal a distinct and novel mechanism. In agreement with earlier reports, we observed baseline downregulation of p21 (and p27) in CDK4/6i-resistant breast cancer cells compared to parental lines. However, we now show for the first time that direct CDK2 inhibition with BLU-222 induces robust de novo upregulation of both p21 and p27 across parental and resistant HR+ and TNBC models, in vitro and in vivo. This induction is not a redistribution of existing pools of Cip/Kip proteins but a true upregulation event initiated by CDK2 blockade itself.

Mechanistically, PLA and co-immunoprecipitation assays demonstrate that the induced p21 and p27 bind simultaneously to CDK2 and CDK4, enforcing dual inhibitory control over both G1 progression and S-phase entry. This dual engagement diverges from the redistribution model of Pack et al., where p21 movement indirectly restrained CDK2. Instead, in resistant tumor models, CDK2 inhibition is the initiating trigger that drives Cip/Kip induction and obligate engagement of both kinase complexes.

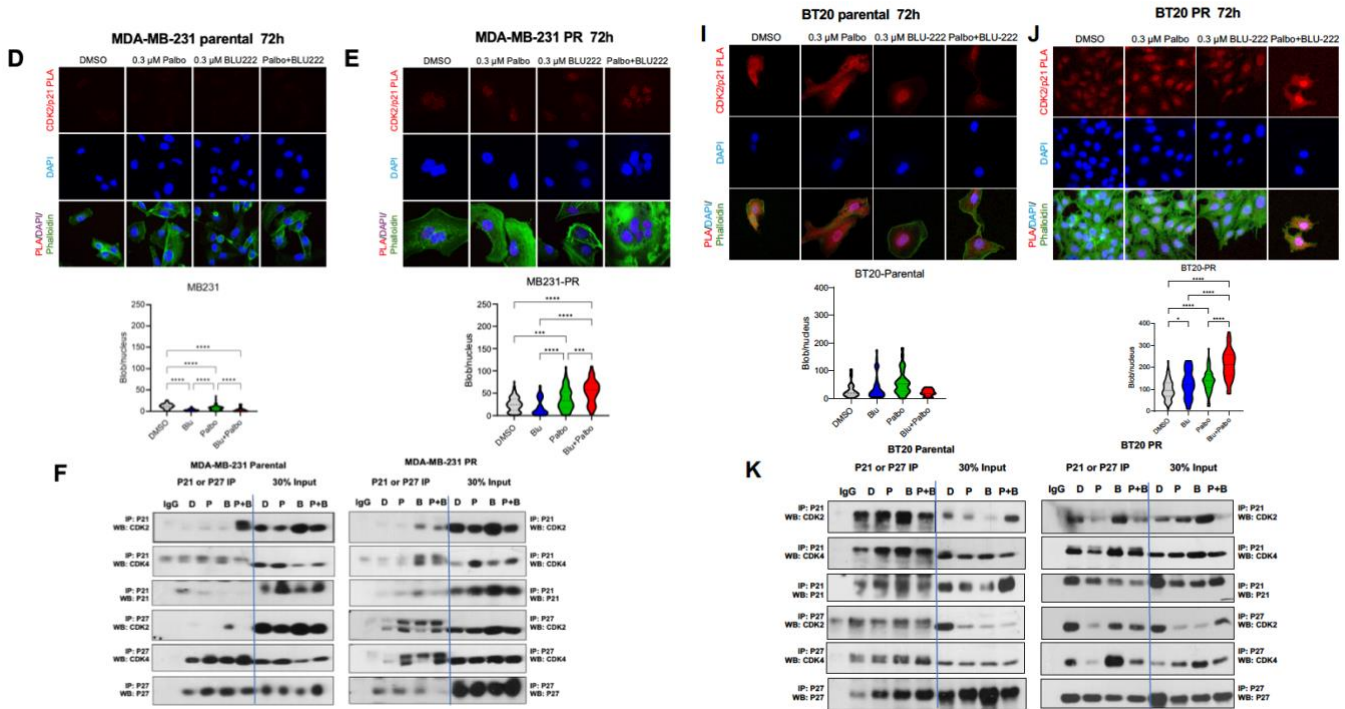
Functionally, this induction is essential. CRISPR knockout of either p21 or p27 completely abrogated the synergy between BLU-222 and palbociclib, proving that these proteins are not passive markers but active mediators of therapeutic efficacy. Thus, Cip/Kip induction by CDK2 inhibition defines a mechanism of dual-pathway blockade that directly restores CDK4/6i sensitivity.

To fully address the reviewer's request, we extended our analyses beyond HR+ models to TNBC. In both MDA-MB-231 and BT20 acquired CDK4/6i-resistant TNBC models (**New supplementary Figures S4D-F and S4I-K**), BLU-222 treatment similarly induced p21/p27 expression and promoted their binding to both CDK2 and CDK4, consistent with the mechanism observed in HR+ disease. By contrast, TNBC parental cells exhibited only modest additive effects with no significant synergy, further underscoring that synergistic induction and dual engagement of p21/p27 occur specifically in the resistant state, regardless of lineage.

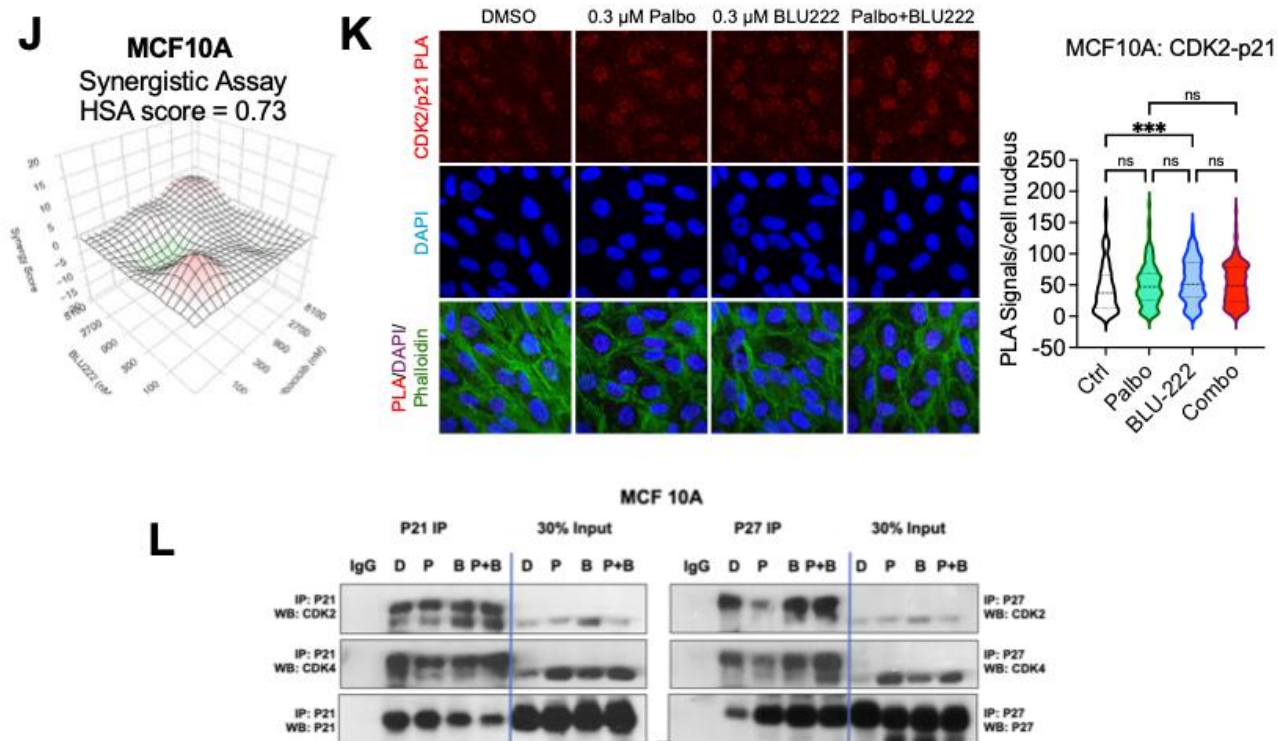
Finally, this phenomenon is not observed in non-tumorigenic cells. In MCF-10A mammary epithelial cells, palbociclib and BLU-222 each inhibited growth but only additively, not synergistically (**New supplementary Figures S4G-H**). Moreover, no change in p21 or p27 binding to CDK2 or CDK4 was detected following single-agent or combination treatment. These data reinforce that the Cip/Kip-driven dual suppression mechanism is specific to resistant tumor cells and not a general feature of cell cycle inhibition.

In summary, whereas prior studies described redistribution of existing Cip/Kip pools following CDK4/6 inhibition, our work establishes a novel therapeutic paradigm: CDK2 inhibition itself induces p21/p27, which are functionally required to simultaneously suppress CDK2 and CDK4, restoring sensitivity to CDK4/6 inhibitors in resistant breast cancers. To our knowledge, this is the first demonstration of Cip/Kip induction as an obligate driver of dual-pathway suppression, uniquely operative in resistant tumor contexts with direct translational significance.

To fully address the reviewer's comment and build upon our mechanistic findings, we have performed the following additional experiments: Proximity Ligation Assays and co-IP in TNBC Models (MDA-MB-231 and BT20 Parental and PR) Following single and combo treatment (Figure S4 D-F and S4I-K)



Supplementary Figure S4 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 μM), BLU-222 (0.3 μM), or the combination. Quantification of nuclear PLA signals per cell is shown below each panel. **(F)** 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)**. Immunoprecipitates (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-J)** PLA detecting CDK2-p21 interaction (red) in **(I)** BT20 parental and **(J)** PR cells following 72 h treatment. Quantification of PLA blob counts per nucleus is shown below each image set. **(K)** Co-IP analysis of p21 or p27 complexes in BT20 parental (left) and PR (right) cells after 72 h treatment. Immunoprecipitates were probed for CDK2, CDK4, p21, and p27, with IgG IP as negative control. 30% input lysates shown for comparison. Error bars represent SEM in at least 3 independent experiments, one-way ANOVA with Tukey's post-test, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.



Supplemental Figure S4 (J) Representative 3D synergy plots illustrating the response of MCF-10A (to combination treatment with different doses of palbociclib and BLU-222 (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. (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. Immunoprecipitates (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

Revised Results in main manuscript (lines 468-471 of the revised manuscript)

Consistently, BLU-222 monotherapy significantly enhanced p21-CDK2 interactions in the TNBC cell lines MDA-MB-231 and BT-20, which were significantly enhanced by the combination with palbociclib in both MDA-MB-231 PR and BT-20 PR cells (Supplemental Figures S4D-E and S4G-H).

Revised Results in main manuscript (lines 487-491 of the revised manuscript)

Our studies in TNBC also suggest a broader role of p27 in MDA-MB-231 PR and BT-20 PR TNBC cells, where treatment with palbociclib, BLU-222, or their combination markedly increased p27-CDK2 binding. In contrast, this effect was absent in the corresponding parental cells, in which p27–CDK2 interactions remained unchanged following either monotherapy or combination therapy. (Supplemental Figures S4F, S4I).

Revised Results in main manuscript (lines 492-500 of the revised manuscript)

Finally, to assess whether the p21/p27-mediated dual inhibition observed in resistant tumor cells is a general feature of cell cycle control, we tested the non-tumorigenic mammary epithelial line MCF-10A. Both palbociclib and BLU-222 reduced growth, but their effects were strictly additive, with no evidence of synergy by HSA analysis (Supplemental Figures S4J). Mechanistically, PLA and co-immunoprecipitation assays showed no changes in p21

or p27 binding to CDK2 or CDK4 following treatment with either agent or the combination (Supplemental Figures S4K-L). This implies that in normal epithelial cells, p21 and/or p27 may not be as dynamically regulated in response to CDK inhibitor treatment as observed in cancer cells, reflecting distinct mechanisms of cell cycle control between normal and transformed contexts.

Revised Discussion in main manuscript (lines 910-917 of the revised manuscript)

This mechanism is distinct from prior redistribution models. Previous studies have shown that CDK4/6 inhibitors can decrease p21 in resistant cells (5,6,8) and dissociate it from CDK4, leading to redistribution toward CDK2 (9). In contrast, our data show that BLU-222 leads to *de novo* induction of p21 and p27, whose dual engagement with CDK2 and CDK4 is essential for therapeutic synergy. These effects were consistent across HR+ and TNBC models, including both intrinsic and acquired resistance, highlighting their broad relevance. Notably, p21 and p27 induction coincided with senescence and interferon pathway activation, suggesting multilayered tumor-suppressive responses.

Revised Discussion in main manuscript (lines 926-932 of the revised manuscript)

Importantly, these mechanisms were restricted to tumor contexts. In the non-tumorigenic MCF-10A line, palbociclib and BLU-222 yielded only additive inhibition with no changes in p21/p27 binding, confirming that dual engagement is tumor-specific and not a universal feature of CDK inhibition. Moreover, BLU-222 activated the p21–DREAM pathway (10-12), providing transcriptional repression of cell cycle genes and reinforcing long-term arrest (13). The centrality of this axis highlights how replication stress, p21 induction, and DREAM-mediated repression converge to sustain durable responses.

● It has also been already previously shown that chronic exposure to palbociclib leads to decreased p21 expression levels in TNBC and that restoring p21 expression and that p21 plays a role sensitizing TNBC cells to palbociclib (Mol Cancer 23, 118 (2024). <https://doi.org/10.1186/s12943-024-02029-4>; These studies should also be properly referenced and discussed in the manuscript

We thank the reviewer for drawing our attention to the recent and highly relevant study by Poulet et al. (Molecular Cancer, 2024), which we have now cited and discussed in the revised manuscript. Poulet et al. demonstrated that chronic palbociclib exposure downregulates p21 in TNBC cells and that restoring p21 expression through modulation of TGFβ3 signaling can re-sensitize these cells to CDK4/6 inhibition in the monotherapy setting. Our work both builds upon and extends these findings. Whereas Poulet et al. focused on p21 restoration as a resistance-reversing mechanism in TNBC, our study identifies CDK2 inhibition as a mechanistically distinct approach that induces both p21 and p27 in CDK4/6i-resistant models. We further show that this induction is not merely correlative but functionally required for the therapeutic synergy between CDK2 and CDK4/6 inhibitors. Importantly, PLA and co-immunoprecipitation assays demonstrate that induced p21 and p27 concurrently engage CDK2 and CDK4 complexes, establishing dual inhibition as a mechanistic basis for synergy—a feature not addressed in prior studies.

Moreover, our data indicate that this Cip/Kip-driven mechanism is conserved across both luminal and TNBC models, and is accompanied by activation of senescence and interferon signaling pathways, thereby expanding the scope of the resistance-reversal strategy beyond the TNBC context described by Poulet et al. Taken together, our findings provide a broader mechanistic and translational framework for targeting CDK2 as a strategy to restore Cip/Kip function and overcome CDK4/6i resistance.

Revised Discussion in main manuscript (lines 910-917 of the revised manuscript)

This mechanism is distinct from prior redistribution models. Previous studies have shown that CDK4/6 inhibitors can decrease p21 in resistant cells (5,6,8) and dissociate it from CDK4, leading to redistribution toward CDK2 (9). In contrast, our data show that BLU-222 leads to *de novo* induction of p21 and p27, whose dual engagement with CDK2 and CDK4 is essential for therapeutic synergy. These effects were consistent

across HR+ and TNBC models, including both intrinsic and acquired resistance, highlighting their broad relevance. Notably, p21 and p27 induction coincided with senescence and interferon pathway activation, suggesting multilayered tumor-suppressive responses.

- The observed BLU-222 and palbociclib synergistic effects observed in vivo in the PDX TNBC models (without CCNE1 amplification) is interesting, as CDK4/6 inhibitor resistance is highly relevant to the TNBC molecular subtype, which exhibits no or poor initial response to palbociclib. TNBC standard of care contains a combination of chemotherapy drugs and in clinical trials, and treatments are typically added to standard of care. The potential for clinical translation would be increased by evaluating the synergy of proposed treatments alongside standard of care drugs.

We thank the reviewer for raising this important point regarding the clinical relevance of evaluating BLU-222 in the context of standard-of-care (SOC) chemotherapy for TNBC. We agree that translating our findings into a clinically meaningful framework requires testing the combination of CDK2 and CDK4/6 inhibition not only against resistant disease models but also alongside SOC therapies, which remain the backbone of TNBC treatment.

To address this, we performed additional studies testing BLU-222 in combination with carboplatin or paclitaxel in three different TNBC models: the BCX070 PDX (harboring CCNE1 amplification) and the HCC1569 CDX (also CCNE1-amplified) and XC517 PDX (lacking CCNE1 amplification). These new data, now included in Supplementary Figures S10A-F, demonstrate that the addition of BLU-222 to chemotherapy yields equal or slightly more durable responses than either monotherapy only in those TNBC PDX models that harbor CCNE1 amplification. These findings align with a recent Blueprint study showing that the activity of BLU-222 plus chemotherapy in ovarian models (OVCAR-3, ES-2) is dependent on CCNE1 amplification, reinforcing the mechanistic consistency across tumor types.

Equally compelling are our results in the XC517 TNBC PDX model, which lacks CCNE1 amplification and is resistant to palbociclib. In this model, BLU-222 combined with palbociclib achieved complete tumor eradication, whereas BLU-222 combined with SOC chemotherapy produced only modest responses, and palbociclib alone or SOC chemotherapy alone were ineffective. These findings are striking, as they establish that in the absence of CCNE1 amplification, BLU-222 synergizes most profoundly with CDK4/6 inhibition rather than with chemotherapy. Thus, while chemotherapy combinations may offer benefit in CCNE1-amplified tumors, dual CDK2 and CDK4/6 inhibition is uniquely effective in eradicating resistant TNBC lacking this amplification.

Taken together, these results provide a nuanced translational framework: BLU-222 combinations with chemotherapy hold promise in CCNE1-amplified TNBC, consistent with prior ovarian cancer studies, while the combination of BLU-222 and CDK4/6 inhibitors represents a highly effective therapeutic option in CCNE1 non-amplified TNBC, including settings where SOC chemotherapy and CDK4/6 inhibitors alone lack efficacy.

Figure S11

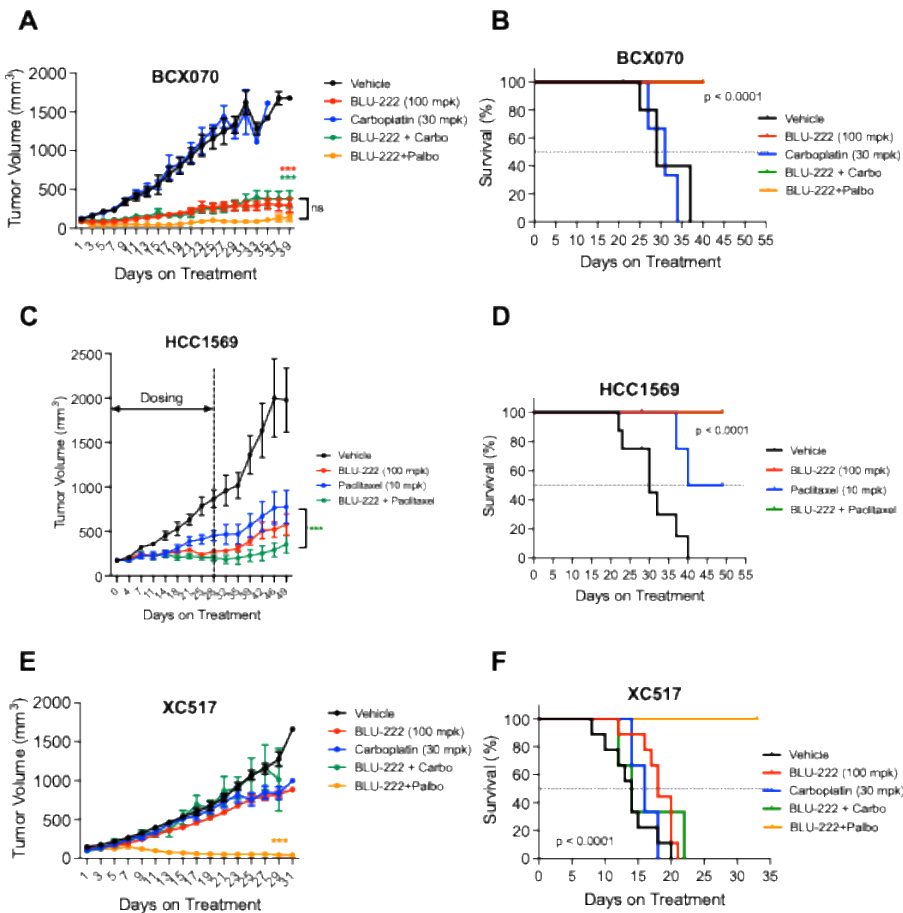


Fig. S11 | Efficacy of BLU-222 chemotherapy combination treatments in TNBC PDX xenografts models

(A-B) Tumor growth curves and Kaplan-Meier survival analysis of BCX070 PDX model treated with vehicle, BLU-222 (100 mpk), carboplatin (30 mpk), BLU-222 + carboplatin, or BLU-222 + palbociclib. 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, BLU-222 (100 mpk), paclitaxel (10 mpk), or BLU-222 + paclitaxel. Tumor volume data are mean $\pm$ SEM; significance determined as in (A-B). ***p < 0.001. (E-F) Tumor growth curves and Kaplan-Meier survival analysis of XC517 PDX model treated with vehicle, BLU-222 (100 mpk), carboplatin (30 mpk), BLU-222 + carboplatin, or BLU-222 + palbociclib. 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. ***p < 0.001.

Revised Results in main manuscript (lines 690-708 of the revised manuscript)

To evaluate the translational relevance of BLU-222 in TNBC, we tested its efficacy in combination with both SOC chemotherapies and CDK4/6 inhibition across multiple preclinical models (Supplemental Figures S11 A-F). In the TNBC PDX model BCX070 and the TNBC CDX model HCC1569, both harboring CCNE1 amplification, BLU-222 was combined with carboplatin (BCX-070) or paclitaxel (HCC1569). In both settings, the combination achieved equal or slightly more durable responses than either monotherapy (Supplemental Figures S11A-D). These findings parallel results from a recently published study demonstrating that BLU-222 synergizes with chemotherapy in CCNE1-abarrent ovarian cancer models (14), further reinforcing the dependence of this effect on CCNE1 amplification and its translational significance.

In contrast, in the XC5172013 TNBC PDX model lacking CCNE1 amplification, BLU-222 combined with carboplatin produced only modest improvements compared to monotherapies (Supplemental Figure S11 E-F). By comparison, BLU-222 combined with palbociclib resulted in complete tumor eradication, whereas neither palbociclib nor BLU-222 monotherapy had therapeutic impact. Importantly, none of the treatment regimens, including the BLU-222 plus carboplatin combination, caused measurable body weight loss, indicating that the therapies were well tolerated (Supplemental Figs S7I-J). These results highlight a critical distinction: while BLU-222 synergizes with chemotherapy in CCNE1-amplified tumors, its most profound therapeutic effect in non-amplified TNBC emerges from dual CDK2 and CDK4/6 inhibition, which can overcome resistance where both SOC chemotherapy and CDK4/6 inhibitors fail.

Revised Discussion in main manuscript (lines 958-964 of the revised manuscript)

The translational impact of these findings is highlighted by direct comparisons against SOC regimens. Benchmarking showed that the triplet therapy (BLU-222 + CDK4/6 inhibitor + endocrine therapy) consistently outperformed palbociclib + fulvestrant, producing more durable tumor regressions and survival benefit in resistant PDX models. In TNBC, therapeutic partners differed by genomic context: BLU-222 synergized with chemotherapy in CCNE1-amplified tumors, but in non-amplified models, only BLU-222 plus CDK4/6 inhibition achieved durable control. These results argue for tailoring CDK2-based combinations to molecular subsets of breast cancer.

- The authors do not interrogate the data of the RNA-Seq studies biologically in tumor suppression. How the interferon pathway causes tumor suppression? Also, no biological data were presented supporting the notion that the combination treatment causes senescence.

We thank the reviewer for raising this important point. In the revised manuscript, we have expanded both the Results and Discussion to provide experimental biological context for interferon pathway activation and direct experimental evidence supporting senescence induction.

First, to address senescence, we now include in vitro β -galactosidase staining data from HR+ (MCF7, T47D) and TNBC (MDA-MB-231) models treated with BLU-222 and palbociclib. These results show that BLU-222 enhances therapy-induced senescence in both sensitive and resistant settings. Collectively, these findings provide functional validation of our RNA-seq results and highlight senescence as a biologically relevant mechanism contributing to the antitumor effects of CDK2 and CDK4/6 co-inhibition.

To extend these findings in vivo, we assessed senescence by Sudan Black B (SBB) staining for lipofuscin an established marker of senescence, in two HR+/HER2- PDX models (PR1 and PR4) and two TNBC PDX models (BCX070 and XC517). All four models exhibited increased senescence following BLU-222 treatment, with the most pronounced effects observed in the combination group. These findings offer functional in vivo validation of senescence as a therapeutic mechanism, extending our earlier in vitro observations based on β -galactosidase staining. Therefore, the consistency of results across multiple independent models underscores the robustness and translational potential of this therapeutic approach.

Second, we also determine experimentally whether interferon signaling mediates tumor suppressive activity in CDK inhibitor-treated models. To this end, we treated CDK4/6 inhibitor-resistant TNBC and HR+ breast cancer cell lines with BLU-222 and palbociclib in combination with small-molecule inhibitors targeting the JAK pathway, a key downstream effector of IFNAR (type I interferons) and IFNGR (type II interferons). Notably, the addition of a JAK inhibitor (Upadacitinib or Tofacitinib) to BLU-222 and palbociclib rescued cell viability in resistant PR1.2 cells across multiple lines, underscoring the essential role of JAK/STAT signaling in mediating the synergistic cytotoxicity observed with CDK2 and CDK4/6 co-inhibition. In contrast, parental cells exhibited no significant difference in viability between dual and triple combinations. These findings suggest that interferon signaling is necessary for the full cytotoxic effect of CDK2/4/6 inhibition in resistant cells. The ability of JAK inhibition to reverse or attenuate growth suppression induced by BLU-222 and palbociclib provides functional evidence that activation of the interferon pathway contributes to therapeutic vulnerability in this context.

Taken together, these new findings strengthen the biological relevance of our RNA-seq results by showing that senescence and interferon signaling are not correlative, but mechanistically contribute to tumor suppression in this therapeutic context.

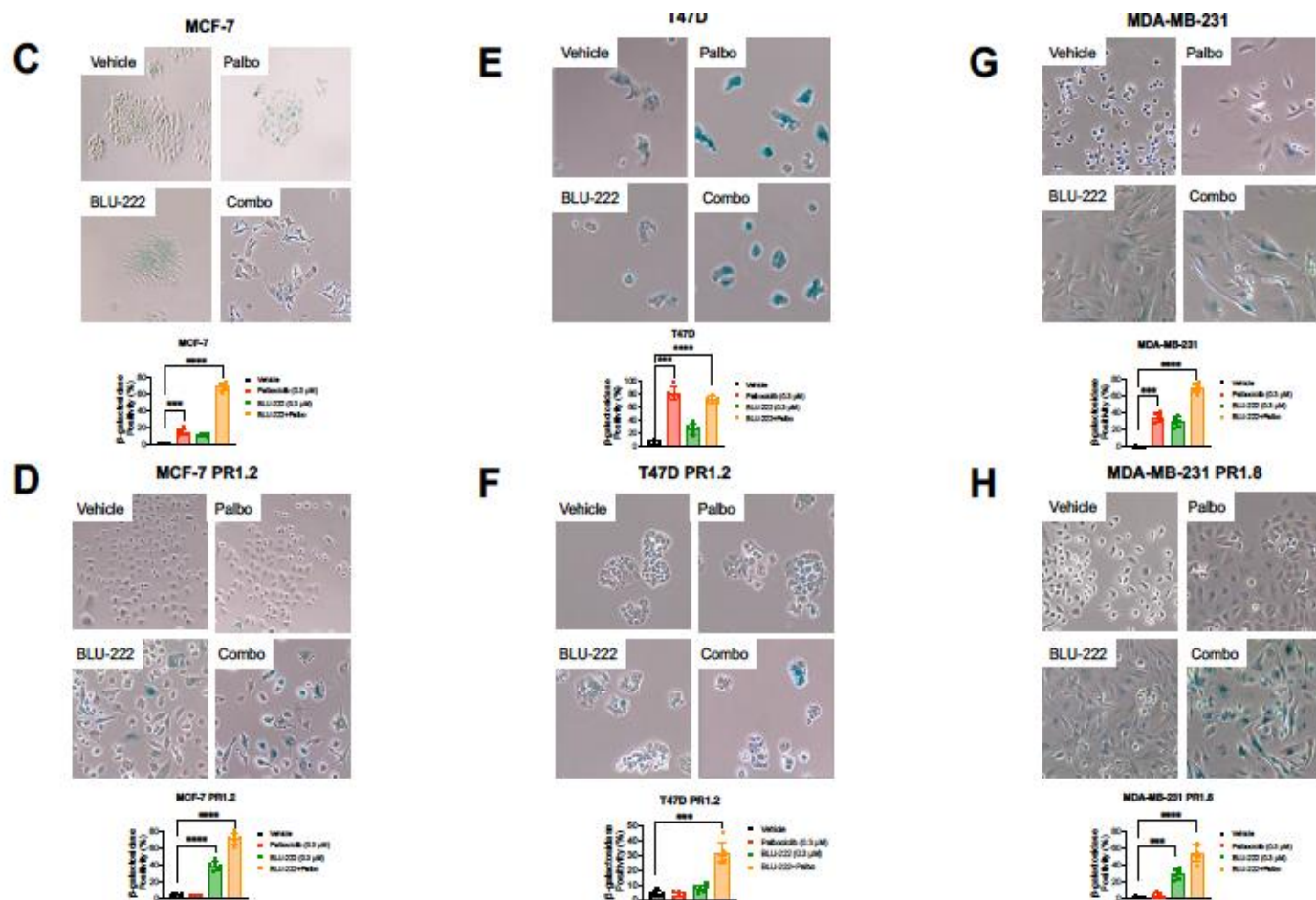


Fig. S13 | Senescence induction in breast cancer models following BLU-222 and palbociclib combination treatment

(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 PR cells following 5 days of treatment with vehicle, palbociclib (0.3 mM), BLU-222 (0.3 mM), or combination. Representative images are shown (top), and quantification of SA-β-gal–positive cells (% total) is presented below. Data are mean ± SEM; one-way ANOVA with Tukey's multiple comparisons test. **p < 0.01, ***p < 0.001, ****p < 0.0001.

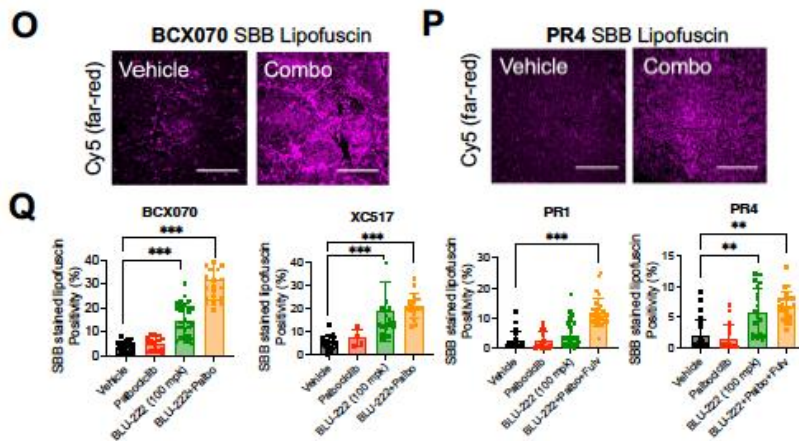


Figure 8 (O-P) Representative images of Sudan Black B (SBB)-stained lipofuscin (far-red, Cy5), indicative of therapy-induced senescence, in (O) BCX070 and (P) PR4 tumors from vehicle-treated and combination-treated (BLU-222+palbociclib) mice. Scale bars, 100 μ m. (Q) Quantification of SBB-positive lipofuscin area (%) in BCX070, XC517, PR1, and PR4 tumors across indicated treatment groups. Data are shown as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

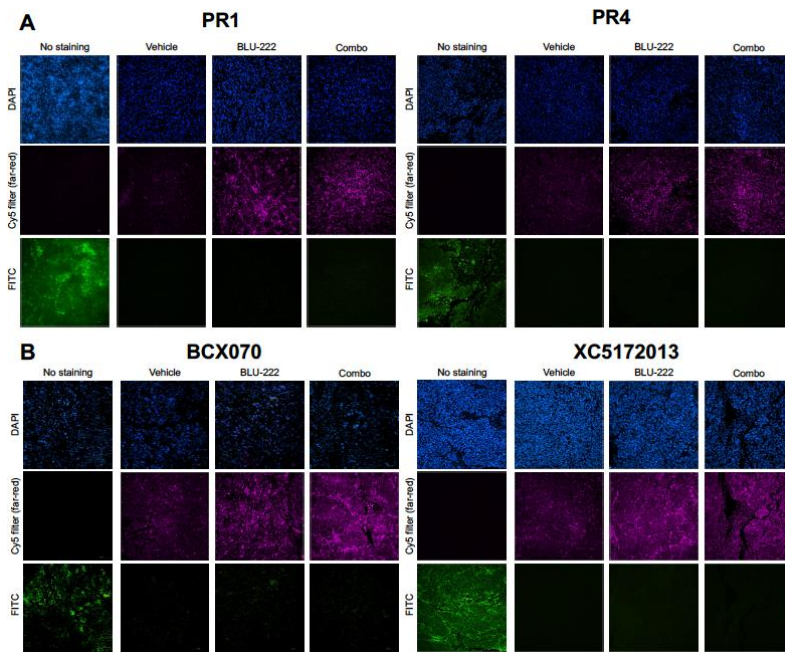
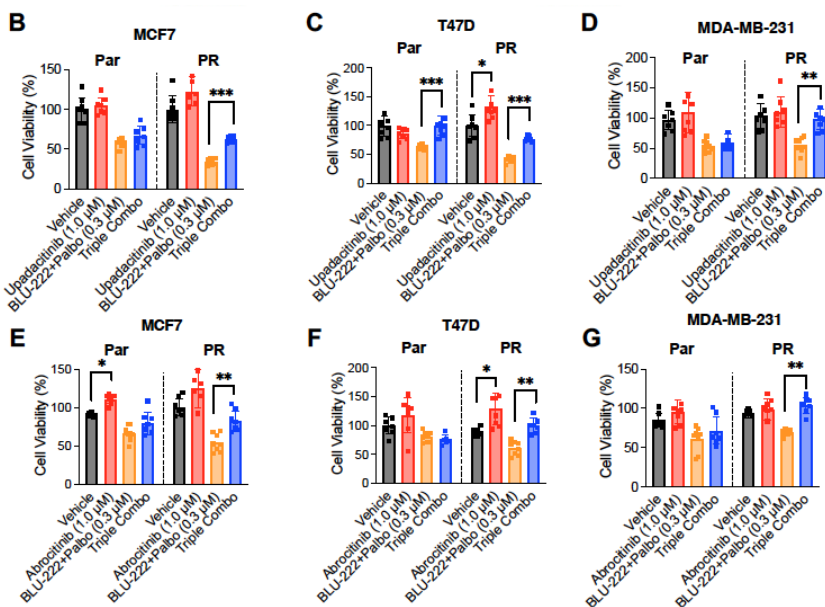


Fig. S13 | Senescence induction in breast cancer models following BLU-222 and palbociclib combination treatment

(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.



Supplementary Figure S12 (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, upadacitinib (UPA; 1 μ M), BLU-222 + palbociclib (Combo), or UPA + Combo (Triple Combo) 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, abrocitinib (ABR; 1 μ M), Combo, or ABR + Combo (Triple Combo) for 5 days.

Revised Results (in vitro senescence) in main manuscript (lines 813-821 of the revised manuscript)

To functionally validate transcriptomic findings, we assessed senescence induction following single agents and combination treatment with BLU-222 and palbociclib *in vitro*. β -galactosidase staining revealed robust induction of cellular senescence following BLU-222 and CDK4/6 inhibitor combination treatment across multiple models, including Palbociclib-resistant MCF7, T47D, and MDA-MB-231 derivatives (Supplemental Figs S13 C–H). These results further support senescence as a key mechanism contributing to the therapeutic efficacy of CDK2 and CDK4/6 co-inhibition. These results, observed consistently across HR+ and TNBC in both *in vitro* and *in vivo* models, highlight senescence as a conserved and biologically relevant mechanism that contributes to the therapeutic efficacy of CDK2 and CDK4/6 co-inhibition in resistant tumors.

Revised Results (in vivo senescence) in main manuscript (lines 804-812 of the revised manuscript)

To further investigate the cellular consequences of combined CDK2 and CDK4/6 inhibition, we analyzed senescence induction using Sudan Black B (SBB) staining for lipofuscin accumulation, a hallmark of senescent cells (15). SBB staining revealed a significant increase in lipofuscin-positive cells in tumors treated with the combination of BLU-222 and CDK4/6 inhibitors across multiple models, including two HR+ (PR1, and PR4) and two TNBC (BCX070, XC5172013) PDX models (Figures 8 P–R, Supplemental Figs S13 A–B). These results indicate robust induction of cellular senescence, suggesting that combination of BLU-222 and CDK4/6 inhibition contributes to therapeutic efficacy not only through cell cycle suppression but also by promoting a senescent phenotype in tumor cells.

Revised Results (interferon signaling) in main manuscript (lines 771-783 of the revised manuscript)

To investigate whether interferon signaling contributes to the cytotoxic effects of CDK2 and CDK4/6 co-inhibition, we treated HR+ and TNBC parental and PR cells with BLU-222 and palbociclib in the presence or absence of JAK inhibitors (Upadacitinib or Tofacitinib), which block downstream signaling from both IFNAR (type I) and IFNGR (type II) interferon receptors. In resistant lines, including HR+ MCF7 and T47D cells and TNBC MDA-MB-231 cells, JAK inhibition with Upadacitinib markedly rescued cell viability (Supplemental Figs. S12B–D), indicating that JAK/STAT signaling is required for the full cytotoxic effect of combined CDK2 and CDK4/6 inhibition. Similar results were obtained with the JAK inhibitor Abrocitinib (Supplemental Figs. S12E–G), further confirming that interferon signaling is necessary for the observed therapeutic activity. Collectively, these findings support a model in which activation of interferon signaling via JAK/STAT contributes to the anti-tumor effects of CDK2/4/6 co-inhibition specifically in resistant cells and provide functional validation of the interferon pathway enrichment identified in our RNA-seq analyses.

- The RNA-Seq data of tumors seems to lack biological replicates for Figure 8. Is the data generated using one or multiple tumors, or biological/technical replicates per model?
- Fig.8: In figures C, D, E and F, why are the numbers of samples (tumors?) in the vehicle groups varying from 4 to 6

We now provide the number of biological replicates per condition in the Figure 8 legend and Methods section. Variation in sample numbers was due to tumor dropout during collection, which we have clarified in the revised figure caption

Revised Results (Figure 8 legends) in main manuscript (line 871 of the revised manuscript)

Dot plot showing normalized enrichment scores (NES) of Hallmark gene sets from gene set enrichment analysis (GSEA) for the combination BLU-222 and palbociclib treatment arm across HR+/HER2– (PR1, n = 3 and PR4, n = 4) and TNBC (BCX070, n = 5 and XC5172013, n = 4) PDX models.

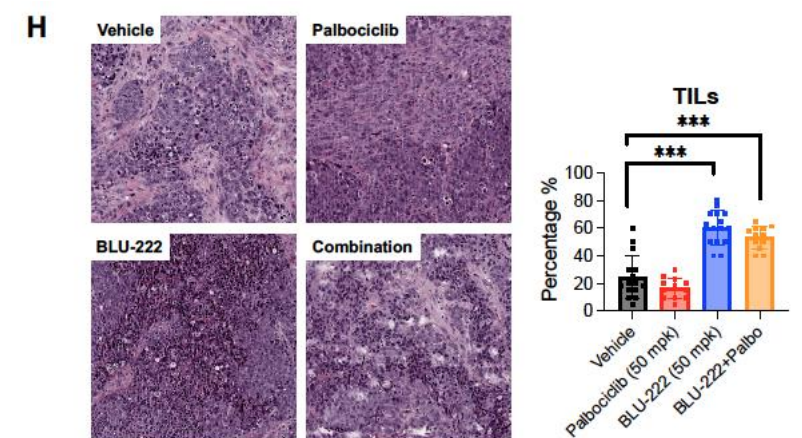
- Fig.8i, 8j: The authors state....A heatmap revealed consistent upregulation of genes such as SAMD9, CD47, TRIM26, and several interferon-induced genes (IFI44L, IFI35,654 IFI27) across all models (Fig. 8i). Similarly, interferon- γ pathway genes, including OAS2, HLA-A, and PSMB8, were upregulated in the combination-treated group (Fig. 8j)..... However, in combination treatment groups, samples from the BCX070 model seem to show

different signatures for interferon alpha and interferon gamma than the rest of the samples. The biology of this data has not been addressed.

We appreciate the reviewer's careful observation regarding the distinct interferon signature in the BCX070 model following combination treatment. We now acknowledge this heterogeneity in the Results and Discussion sections. Notably, BCX070 was the only model to respond significantly to BLU-222 monotherapy, and correspondingly, BLU-222 alone produced a strong induction of interferon signaling in this model. BCX070 harbors CCNE1 amplification, and our prior clinical studies demonstrated that overexpression of cyclin E, particularly the low-molecular-weight isoforms, is frequently associated with increased tumor-infiltrating lymphocytes (TILs) and heightened immune activation in TNBC (16) (These findings suggest that intrinsic cyclin E-driven biology may sensitize tumors to immune modulation, providing a plausible explanation for the distinct interferon response observed in BCX070.

To further test this hypothesis, we analyzed TIL dynamics in the immune-competent T1P mouse model treated with either BLU-222 alone or in combination with palbociclib (Figure 7D). Both regimens significantly increased TIL infiltration (Supplementary Figure S12H), supporting the notion that CDK2 inhibition, either alone or in combination with CDK4/6 inhibition, can promote antitumor immune activation within the tumor microenvironment.

In addition, RNA-seq analysis of a new CCNE1-overexpressing PDX model, BCX017, revealed a similar induction of interferon signatures in response to BLU-222 monotherapy, consistent with the findings in BCX070. These results reinforce the idea that cyclin E status may influence intrinsic immune responsiveness to CDK2 inhibition. We have now included these new data in revised Figure 8A, D, I and J and expanded the Discussion to address the biological basis for these findings. Collectively, these results provide a clearer mechanistic rationale linking cyclin E amplification, interferon pathway activation, and enhanced immunogenicity, thereby strengthening the translational significance of our study.



Supplementary Figure S12 (H) Representative H&E-stained tumor sections from PR1 xenografts treated with vehicle, palbociclib, BLU-222, or combination for the indicated duration. Quantification of tumor-infiltrating lymphocytes (TILs) is shown at right. Data are mean $\pm$ SEM; one-way ANOVA with Tukey's multiple comparisons test. *** $p < 0.001$.

Revised Results in main manuscript (line 784-797 of the revised manuscript)

Interestingly, in the CCNE1-amplified BCX070 and BCX017 models, which displayed unique sensitivity to BLU-222 monotherapy, we observed a marked induction of interferon signaling following CDK2 inhibition (Fig 8A, 8J and 8K). To further evaluate the immunomodulatory consequences of this treatment, we assessed tumor-infiltrating lymphocytes (TILs) in the immune-competent T1P model, which overexpresses low-molecular-weight cyclin E. Histological analysis and quantitative assessment of TILs revealed that BLU-222, administered either as a single agent or in combination with palbociclib, significantly increased TIL density compared to vehicle or palbociclib alone ($p < 0.001$; Supplemental Figure S12H). These findings suggest that CDK2 inhibition, in addition to disrupting tumor cell cycle progression, promotes remodeling of the tumor immune microenvironment.

Importantly, this effect appears particularly pronounced in cyclin E-driven tumors, highlighting a potential link between CDK2 inhibition, interferon pathway activation, and enhanced antitumor immunity. Together, these results raise the possibility that CDK2 inhibition could potentiate immune responsiveness and improve outcomes when integrated with immunotherapeutic strategies.

Revised Discussion in main manuscript (line 965-980 of the revised manuscript)

Beyond cell cycle arrest, BLU-222 induced senescence and immune remodeling. Upregulation of interferon pathways suggested immune-mediated elimination of senescent cells, consistent with prior reports linking CDK4/6 inhibition to cGAS-STING activation (17). Importantly, these effects were not uniform across all tumors but appeared to be shaped by cyclin E status. In CCNE1-amplified BCX070, BLU-222 monotherapy induced a particularly strong interferon response, highlighting that cyclin E amplification confers an immune-primed phenotype. This aligns with prior clinical evidence linking low-molecular-weight cyclin E to heightened tumor-infiltrating lymphocyte (TIL) presence and immune activity in TNBC (18). Supporting this concept, in the immune-competent T1P model engineered to overexpress LMW-E, CDK2 inhibition significantly increased TIL density, with even greater effects when combined with palbociclib. Together, these findings support a dual role for CDK2 inhibition: directly suppressing proliferation while indirectly remodeling the tumor immune microenvironment. Cyclin E-driven tumors may therefore be uniquely poised to couple CDK2 blockade with immune remodeling. More broadly, the convergence of interferon signaling and enhanced immune infiltration positions CDK2 inhibition as a potential strategy to augment antitumor immunity and strengthens the rationale for its integration with immunotherapies in defined breast cancer subsets.

Reviewer #2

In this manuscript, Luo and colleagues present compelling data on the activity of a novel CDK2 inhibitor, BLU-222, in HR+ and TNBC models. The authors demonstrate that the CDK2 inhibitor synergizes with CDK4/6 inhibitors in resistant HR+ and TNBC models. Additionally, they describe how this combination profoundly affects apoptosis and cell cycle arrest through the upregulation of p21 and p27. Overall, this study provides evidence of the efficacy of a novel CDK2 inhibitor in breast cancer models resistant to CDK4/6 inhibitors. The manuscript also includes interesting molecular findings on p21 and p27 on CDK2-activity that have not been previously described. However, several key questions remain to be addressed:

1) The manuscript could be more concise while retaining the same level of information.

We appreciate the reviewer's comments. We have carefully revised the text to improve clarity and flow, reducing redundancy while retaining all essential content. We also incorporated additional results and discussion that were requested by both reviewers.

2) The references in the introduction should be reviewed. References 10–16 are too general and should be more CDK2-focused, particularly regarding the statements that 'CDK2 inhibition induces cellular senescence' and that 'senescence involves the upregulation of p21 and p27, which inhibit CDK2 activity.' Additionally, references 33–36, which include preclinical and clinical studies on cyclin E2 overexpression bypassing CDK4/6 inhibition, should be included in the introduction. Previous evidence on the activity of a selective CDK2 inhibitor (INX-315) in preclinical models of CCNE1-amplified ovarian cancer and HR+ breast cancer progressing on CDK4/6 inhibitors should be referenced. Please cite relevant publications by Shom Goel and Erik Knudsen in Cancer Discovery and Nature Communications, respectively.

We appreciate the reviewer's comments. In response, we have revised the Introduction by replacing several generalized references with more specific, mechanistically relevant citations. These include studies highlighting CDK2-mediated senescence, cyclin E2-driven resistance mechanisms, and prior investigations of CDK2 inhibition in HR+ breast cancer models (e.g., Cancer Discovery, Nature Communications). Please refer to the page 4 of the revised manuscript lines 90-96.

3) The densitometry quantification of CDK4 in the Western blot from Figure S1 does not appear to correlate with the expression levels in Figure 1E. Specifically, in the Western blot, CDK4 levels seem to increase in shCDK2 cells with increasing doses of BLU-222, whereas the densitometry does not reflect this. Please review this discrepancy and revise the statement: "CDK4, CDK6, and cyclin D1 were upregulated in control and shCCNE1 cells but not in shCDK2 cells (Figure 1E, Supplemental Figures S1C–E).

We thank the reviewer for carefully noting this discrepancy. Upon revisiting the Western blot and densitometry, we agree that CDK4 levels appear to increase in shCDK2 cells with BLU-222 treatment, whereas our original quantification and text implied otherwise. We have corrected this inconsistency by re-analyzing the blot and adjusting the densitometry presentation in Supplemental Figure S1C. Accordingly, the Results section text has been revised to state that while CDK4, CDK6, and cyclin D1 were upregulated in control and shCCNE1 cells, CDK4 also showed an increase in shCDK2 cells following BLU-222 treatment. This revision ensures consistency between the visual Western blot data and the quantitative analysis.

Revised Results in main manuscript (line 138-141 of the revised manuscript)

Additionally, CDK4, CDK6, and cyclin D1 were upregulated in control and shCCNE1 cells, and while CDK6 and cyclin D1 induction was not observed in shCDK2 cells, CDK4 levels did increase following BLU-222 treatment (Fig. 1E, Supplemental Figs. S1C–E).

4) In Figure 1H, the authors show that low-molecular-weight cyclin E (LMW-E) sensitizes T47D cells to BLU-222. Based on previous studies, cells overexpressing full-length cyclin E would be expected to be more sensitive to CDK2 inhibitors. However, the data indicate that T47D cells overexpressing full-length cyclin E are more resistant to BLU-222 than parental cells. How do the authors explain this effect? Additionally, please include parental and full-length cyclin E-overexpressing MDA-MB-231 cells in Figure S1L.

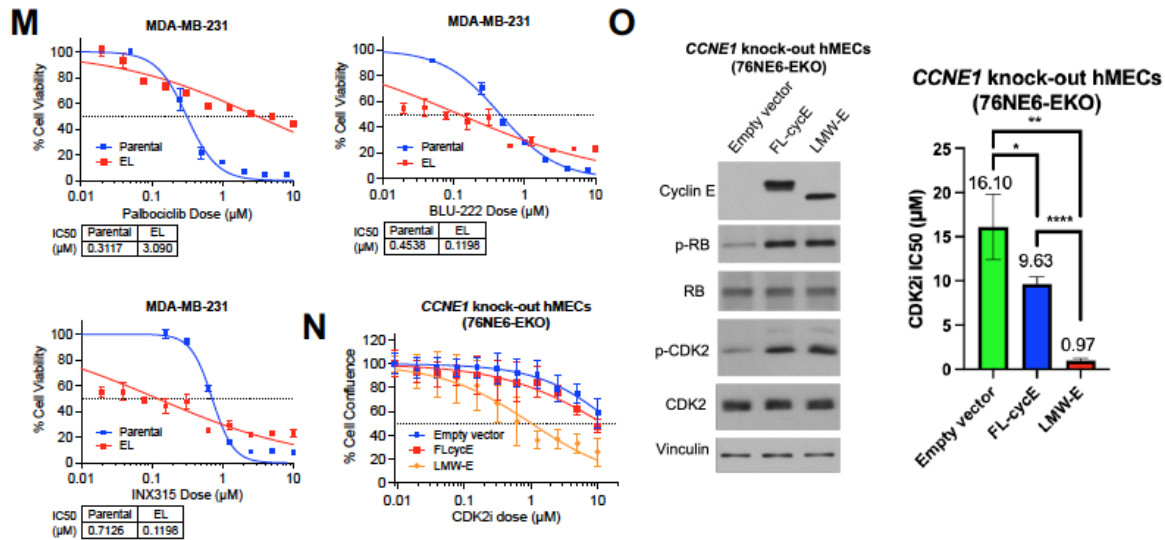
We thank the reviewer for this thoughtful observation. As the reviewer notes, in Figure 1H, T47D cells overexpressing low-molecular-weight cyclin E (LMW-E) were markedly sensitized to BLU-222, whereas cells overexpressing full-length cyclin E (EL) appeared more resistant than their parental counterparts. This observation is consistent with our prior work showing that full-length cyclin E can, under certain conditions, delay cell cycle progression rather than accelerate it. Specifically, in our 2010 study (19) we demonstrated that EL overexpression activates checkpoints and induces G1 delay, thereby dampening the cytotoxic effects of CDK2 inhibition. In contrast, the hyperactive, oncogenic LMW-E isoforms drive unchecked CDK2 activity, resulting in heightened reliance on CDK2 and greater sensitivity to its inhibition. These distinct biological properties likely explain why T47D EL cells show relative resistance to BLU-222 compared with parental cells, while T47D LMW-E cells display profound sensitivity.

However, in TNBC MDA-MB-231 cells, we observed that EL overexpression actually increased sensitivity to BLU-222 relative to parental controls (new data in revised Fig. S1M). We believe this context-dependent difference reflects intrinsic lineage variation in how cyclin E isoforms interact with checkpoint and replication stress pathways. Unlike luminal T47D cells, MDA-MB-231 cells, a basal cell line, are genomically unstable and harbor higher baseline replication stress; in this setting, EL and LMW-E overexpression both likely exacerbates CDK2-driven origin firing and replication stress, thereby creating increased vulnerability to CDK2 inhibition. This contrasts with the checkpoint-proficient T47D background, where EL overexpression induces G1 delay and reduces CDK2 dependence.

To further dissect the isoform-specific effects, we leveraged our doxycycline-inducible 76NE6 model system expressing either EL or LMW-E (20). In this controlled genetic background, inducible LMW-E robustly sensitized cells to BLU-222, producing a significant leftward shift in the dose–response curve, consistent with strong CDK2 addiction (Supplementary Figure S1N–O). By contrast, inducible EL did not enhance such sensitivity, with cells showing attenuated growth inhibition, compared to LMW-E overexpressing cells, following BLU-222 exposure.

These results validate that LMW-E promotes a dependency on CDK2 activity, whereas EL overexpression alone does not consistently augment sensitivity to CDK2 inhibition and may instead blunt it through checkpoint-mediated slowing of proliferation.

Taken together, these findings suggest that LMW-E universally drives strong sensitivity to CDK2 inhibition due to its hyperactive CDK2 activation, while EL's effects are more context-dependent: protective in luminal backgrounds such as T47D, but sensitizing in TNBC backgrounds such as MDA-MB-231. This reinforces the conclusion that the oncogenic properties of cyclin E isoforms—and the lineage-specific cellular context—critically shape the therapeutic response to CDK2 inhibition. We have incorporated these results and their interpretation into the revised manuscript



Supplementary Figure S1 (M) Dose–response curves showing the effect and IC₅₀ values of palbociclib, BLU-222, and INX-315 on cell viability in MDA-MB-231 parental versus full-length cyclin E overexpression (EL) cells. Data are presented as mean ± SEM from three biological replicates. **(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). Data are mean ± SEM. **(O)** Immunoblot analysis in CCNE1 knock-out hMECs expressing empty vector, FL-cycE, or LMW-E. Right: quantification of CDK2 inhibitor IC₅₀ values from panel N. Bars represent mean ± SEM; statistical significance determined by one-way ANOVA with Tukey's post-test. *p < 0.05, **p < 0.01, ****p < 0.0001.

Revised Results in main manuscript (line 168-189 of the revised manuscript)

Interestingly, the impact of EL expression differed by breast cancer subtype. In T47D cells, a luminal cell line, EL overexpression conferred relative resistance to BLU-222 compared with parental cells, consistent with prior evidence that EL can delay cell cycle progression and checkpoint activation (19). In contrast, EL-overexpressing MDA-MB-231 cells, a basal cell line, were ~10-fold more resistant to palbociclib but showed increased sensitivity to CDK2 inhibition, with a 3.8-fold reduction in BLU-222 IC₅₀ and a 6-fold reduction with INX-315 compared to parental cells (Supplemental Fig. S1M). To directly validate these isoform-specific effects in a controlled background, we used CCNE1-knockout 76NE6 human immortalized mammary epithelial cells reconstituted with doxycycline-inducible EL or LMW-E (76NE6-EKO)(20). Both isoforms increased CDK2 activation and RB phosphorylation, but LMW-E induction produced robust sensitization to CDK2 inhibition, with an IC₅₀ of 0.97 μM—approximately 16.6-fold lower than empty vector controls and 10-fold lower than EL-expressing cells (Supplemental Fig. S1N-O). In contrast, EL induction yielded only modest sensitization or, in some contexts,

relative protection against BLU-222. Together, these findings demonstrate that LMW-E consistently drives CDK2 hyperactivation and therapeutic vulnerability across subtypes, whereas the effects of EL are context dependent—attenuating sensitivity in luminal T47D cells but enhancing it in basal MDA-MB-231 cells. Collectively, these results highlight the oncogenic role of LMW-E as a biomarker of CDK2 inhibitor sensitivity and underscore the importance of tumor subtype and isoform context in determining therapeutic response. Importantly, they also suggest an inverse relationship between palbociclib and BLU-222 sensitivity, where LMW-E expression confers resistance to CDK4/6 inhibition but enhances susceptibility to CDK2 inhibition, providing a mechanistic rationale for dual targeting in cyclin E-driven tumors.

Revised Discussion in main manuscript (line 940-949 of the revised manuscript)

Cyclin E isoforms further shaped therapeutic response. Full-length cyclin E (EL) delayed cell cycle progression in luminal T47D cells, attenuating BLU-222 sensitivity, whereas LMW-E (21) consistently drove hyperactive CDK2 activity and hypersensitivity to BLU-222 (21). This dichotomy, validated in inducible 76NE6 models (22), established an inverse relationship between CDK4/6 and CDK2 inhibitor sensitivity: LMW-E drove palbociclib resistance but enhanced BLU-222 efficacy. Mechanistically, LMW-E upregulation allows cells to bypass the G1 checkpoint by hyperactivating CDK2, sustaining proliferation despite CDK4/6 inhibition. Although prior studies suggest that CDK2 inhibition can initially suppress this activity before adaptive entry into S phase via E2F and cyclin A2 (23) our findings demonstrate that dual inhibition is required to sustain durable arrest.

5) Acquired palbociclib-resistant MCF7 and T47D cells are valuable models for studying whether BLU-222 can overcome CDK4/6 inhibitor resistance. However, the manuscript does not provide the mechanism of acquired resistance for PR1.2 and PR4.8. Notably, Navarro-Yepes et al. (Cancer Research, 2023) Figure 3F shows that PR4.8 cells do not express RB1, which could explain their inherent resistance to CDK4/6 inhibitors and the absence of synergy with BLU-222. However, the profound sensitivity of PR4.8 cells to BLU-222 is unexpected and suggests CDK2 activity independent of RB1. The authors should:

1. Comment on the mechanisms of acquired resistance in PR1.2 (high cyclin E, as shown in Figure 3A) and PR4.8 cells (RB1 loss?).
2. Discuss why BLU-222, a CDK2 inhibitor, is effective as a single agent in PR4.8 cells, which lack RB1 expression. It would also be relevant to mention whether BLU-222 has antitumor activity in RB1-deficient tumors.

We thank the reviewer for emphasizing the importance of delineating the mechanisms of acquired resistance in the PR1.2 and PR4.8 models and for highlighting their relevance to understanding CDK2 inhibitor activity.

Mechanisms of resistance in PR1.2 and PR4.8: In line with our observations, PR1.2 cells display elevated cyclin E expression (Fig. 3A), a well-established mechanism that drives resistance to CDK4/6 inhibition by activating compensatory CDK2 signaling. In contrast, PR4.8 cells exhibit complete loss of RB1 expression, as we reported previously Figure 1D in (2). Since functional RB is required for CDK4/6 inhibitors to induce G1 arrest, RB1 loss represents a canonical mechanism of resistance.

BLU-222 activity in RB-deficient cells: Despite RB1 loss, PR4.8 cells remain profoundly sensitive to BLU-222, a CDK2 inhibitor. This finding is not unique to PR4.8, as BLU-222 also demonstrated potent activity in multiple RB1-deficient TNBC models (MDA-MB-468, HCC1806, **see revised Table S1**), in resistant clones derived from PR4.8 (T47D TCL and MCF7 MCL; **new Fig. S2D-H**), and the newly generated MCF7 shRB model which were refractory to CDK4/6 monotherapy, yet remained very sensitive to CDK2 inhibition with BLU-222 (Figures S2C, S2G, **new S2D,H and new Figure 3A**) Together, these data indicate that the efficacy of CDK2 inhibition is independent of RB status. Mechanistically, this likely reflects the continued reliance of RB-deficient cells on CDK2/cyclin E to sustain S-phase entry and genome replication in the absence of G1/S checkpoint control. Moreover, RB1-deficient cancers are often characterized by heightened genomic instability and replication stress. By targeting CDK2, BLU-222 may exploit these vulnerabilities, consistent with emerging literature

demonstrating synthetic lethality between CDK2 inhibition and RB1 deficiency due to replication stress overload and impaired origin firing.

Clinical significance: These findings highlight that CDK2 inhibitors such as BLU-222 may have therapeutic value in RB1-deficient tumors—subsets of breast cancers where CDK4/6 inhibitors are ineffective and clinical options are limited. Importantly, this includes both de novo RB1-deficient TNBC and acquired RB1 loss in luminal breast cancers progressing on CDK4/6 inhibitors. Our results therefore extend the translational potential of CDK2 inhibition and suggest that RB1 loss, historically considered a barrier to CDK4/6 therapy, may instead identify tumors vulnerable to CDK2 blockade.

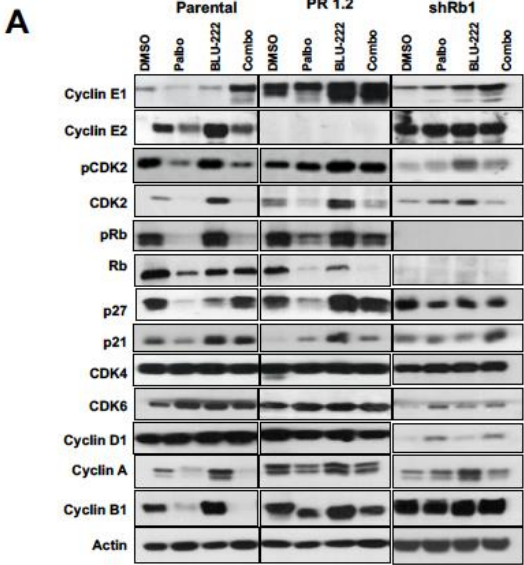
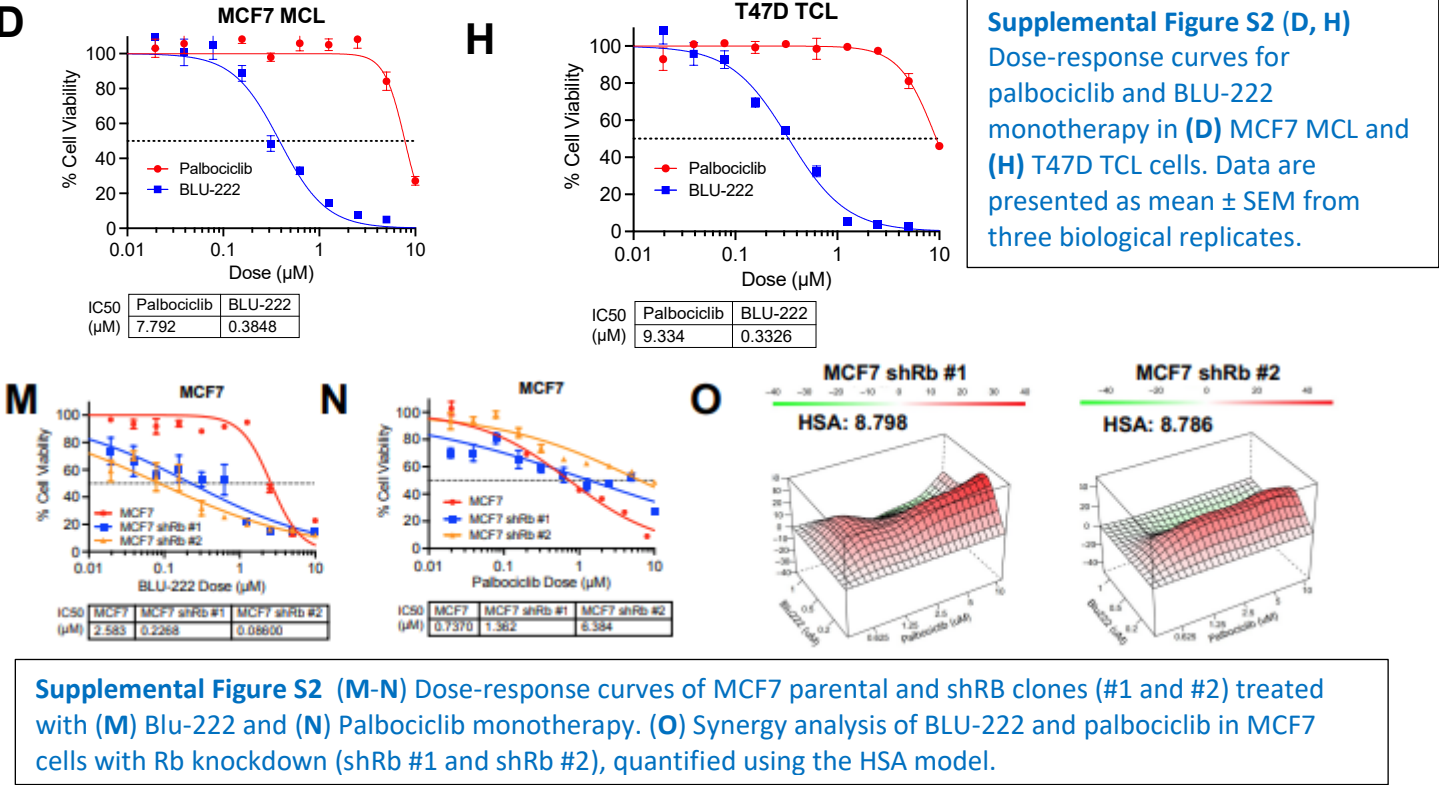


Figure 3A. Representative Western blots showing expression levels of pRb, Rb, Cyclin E1, cyclin E2, p27, p21, CDK2, p-CDK2, CDK4, CDK6, Cyclin D1, Cyclin A, Cyclin B1 and actin in p21, p27, cyclin D1, pCDK2, and actin in MCF7 parental and PR1.2 and Rb1 knockdown cell lines treated with 0.3 μM of single agents or a combination of palbociclib and BLU-222 for 3 days.

Revised Results in main manuscript (lines 276-295 of the revised manuscript)

Previous studies have established Rb as a critical determinant of sensitivity to CDK4/6 inhibitor (1). We assessed RB status across all cell lines and PDX models, including parental, resistant, and engineered lines (Supplemental Tables S1 and S2). RB-proficient models included HR+/HER2- MCF7 and T47D and TNBC MDA-MB-231; RB-deficient models included TNBC lines MDA-MB-468, HCC1806, and MDA-MB-157, as well as CDK4/6i-resistant clones T47D TCL and MCF7 MCL, where RB loss arose during long-term selection (Supplemental Fig. S2D and S2H). We also generated an RB knockdown MCF7 model (MCF7 shRB). Among the resistant pools, PR1.2 cells retained RB but exhibited high cyclin E expression, whereas PR4.8 cells showed complete RB loss, consistent with our prior reports (2). As expected, RB-deficient TNBC lines, resistant pools (MCF7 PR4.8, T47D 4.8), resistant clones (TCL, MCL), and MCF7 shRB were refractory to CDK4/6i monotherapy but remained highly sensitive to BLU-222 (Figs. S2C, S2G, S2D, S2H). Notably, BLU-222 monotherapy was particularly effective in RB-deficient PR4.8 cells, underscoring that CDK2 activity can drive proliferation independently of RB. BLU-222 plus palbociclib also produced strong synergy in RB-deficient TNBC models, resistant clones, and MCF7 shRB (Figs. 2J, S2M-O), with marked apoptosis and G2/M arrest (Figs. 1C, 1D). In RB-proficient MCF7 and T47D parental cells, the combination was additive regardless of RB status. Collectively, these results establish that while RB loss confers resistance to CDK4/6i, it does not diminish sensitivity to CDK2 inhibition; rather, BLU-222 effectively exploits CDK2/cyclin E-driven replication stress vulnerabilities in both RB-intact (PR1.2) and RB-deficient (PR4.8) contexts, providing a strong rationale for therapeutic targeting of this subset.

Revised “Discussion” in main manuscript (lines 933-939 of the revised manuscript)

RB status, a known determinant of CDK4/6 inhibitor response [37], did not diminish BLU-222 activity. Resistant PR1.2 cells retained RB but displayed elevated cyclin E, while PR4.8 cells lost RB entirely. Both remained highly sensitive to BLU-222. Across RB-proficient and RB-deficient contexts—including TNBC lines, resistant clones, and MCF7 shRB—BLU-222 retained potency and synergized with CDK4/6 inhibition, producing apoptosis and G2/M arrest. These findings underscore that CDK2 dependence persists even in RB-null settings, consistent with reports that CDK2 inhibition is synthetically lethal in RB1-deficient cancers (3,4).

6) The authors generated palbociclib-resistant TNBC cell lines (MDA-MB-231 and BT20). However, in Figure 1N, the palbociclib sensitivity of the parental cells is not shown. According to Finn et al. (2009), MDA-MB-231 cells are relatively resistant to palbociclib. Including synergy plots for the combination treatment in parental MDA-MB-231 and BT20 cells would help clarify this point.

We thank the reviewer for this insightful suggestion. In response, we have now evaluated the effects of BLU-222 and palbociclib, both as single agents and in combination, in the parental TNBC cell lines BT20 and MDA-MB-231. As shown in the newly included data (Fig. 2J and 2P and Supplemental Figure S2L) the combination of BLU-222 and palbociclib produced only minimal synergy in the parental lines, with HSA synergy scores of 2.48 (BT20) and 4.01 (MDA-MB-231). In sharp contrast, the corresponding palbociclib-resistant (PR) derivatives demonstrated a marked increase in synergy, with HSA synergy scores rising to 14.74 in BT20-PR and 13.37 in MDA-MB-231-PR (Fig. 2B, D). To extend this analysis, we compared across lineages using a synergy heatmap (Fig. 2P). These results demonstrate a consistent pattern: all parental cells, regardless of subtype (HR+ or TNBC), display additive responses to the combination, whereas all resistant cells exhibit robust synergy. This highlights that BLU-222/palbociclib synergy is a resistance-associated phenomenon, independent of lineage, and reinforces the conclusion that CDK2 inhibition preferentially benefits tumors with acquired CDK4/6i resistance.

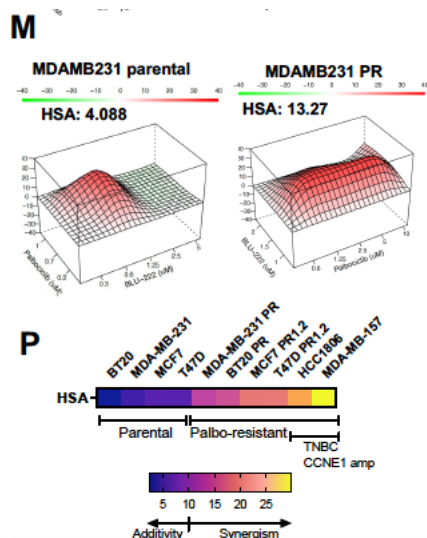
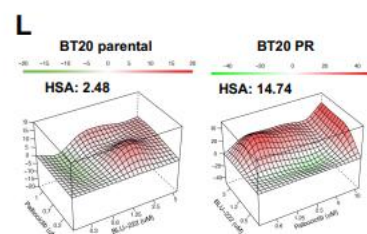


Fig. 2 (M) Representative 3D synergy plots illustrating the response of MDA-MB-231 parental and PR cells to combination treatment with different doses of palbociclib and BLU-222. (P) Heatmap of highest single agent (HSA) synergic scores across HR+, TNBC, and acquired palbo-resistant breast cancer cell lines. Error bars represent SEM in at least 3 independent experiments (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$).



Supplementary Fig. S2 L Representative 3D synergy plots illustrating the response of BT20 parental and PR cells to combination treatment with different doses of palbociclib and BLU-222.

Revised Results in main manuscript (lines 317-319 of the revised manuscript)

Combination treatment of these PR cells revealed strong synergy (HSA scores 13.3–14.8) at low doses ($0.3 \mu\text{M}$ each), while parental counterparts displayed only minimal synergy (HSA scores 2.4–4.1) (Figs. 2M and Supplemental Fig S2L).

Revised Results in main manuscript (lines 324-332 of the revised manuscript)

To determine whether the observed synergy was subtype-specific, we compared responses across HR+ and TNBC parental and resistant models using HSA modeling (Fig. 2P). This analysis revealed a consistent trend: all parental cells, irrespective of subtype, exhibited additive effects, whereas all resistant cells displayed strong synergy, establishing BLU-222/palbociclib synergy as a hallmark of acquired resistance rather than lineage. In HR+ (MCF7 and T47D) and TNBC (MDA-MB-231 and BT20) parental cells, additive responses likely reflect their inherent sensitivity to CDK4/6 inhibition, while both HR+ (MCF7 PR1.2 and T47D PR1.2) and TNBC (MDA-MB-231 PR and BT20 PR) resistant cells showed enhanced synergy driven by compensatory CDK2 activation during CDK4/6i escape.

7) The combination of BLU-222 with CDK4/6 inhibitors in HR+/HER2- PDX models that progressed on palbociclib is highly consistent. However, it would be valuable to mention the resistance mechanisms of PR1, PR2, PR3, and PR4. Additionally, a comparison of the triplet therapy (BLU-222 + CDK4/6i + endocrine therapy) versus the standard of care (palbociclib + fulvestrant) would be beneficial. The authors should also consider:

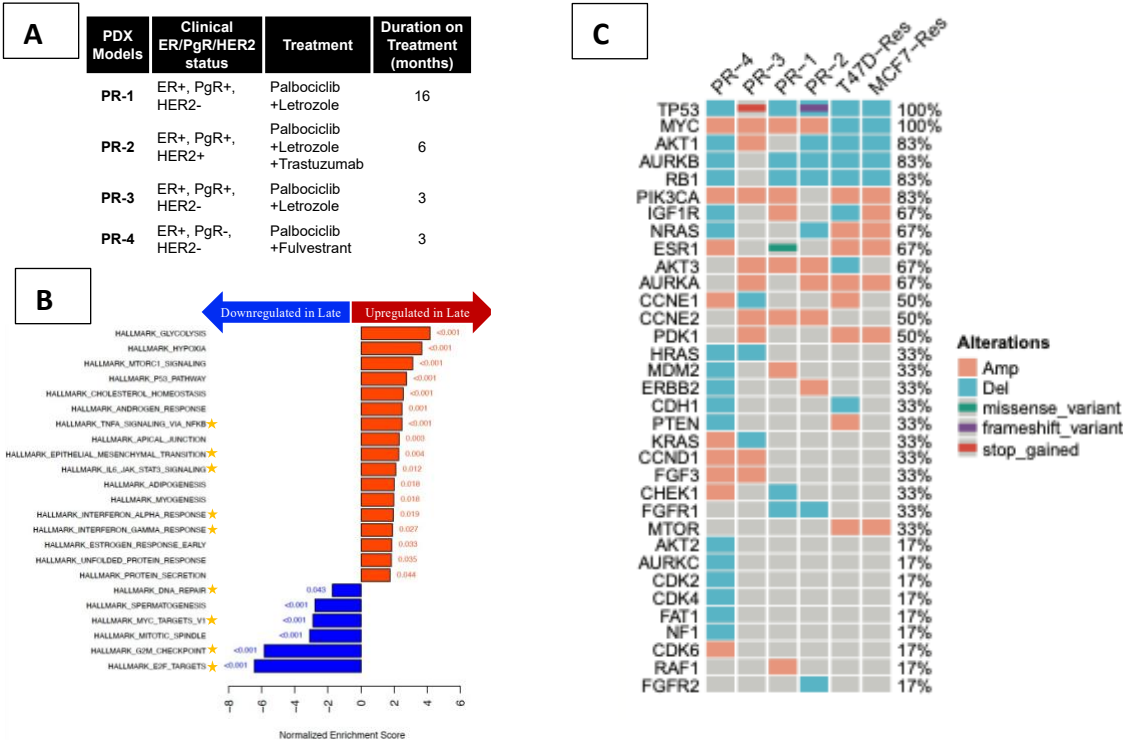
- Removing data on γH2AX as a marker of DNA damage in vivo, given that DNA damage has not been demonstrated in prior studies with the CDK2 inhibitor.
- Instead, including apoptosis data based on Figure 2 and the role of p21-p53.

We thank the reviewer for this important comment and agree that further contextualization of the resistance biology and refinement of our mechanistic presentation would strengthen the manuscript.

Resistance mechanisms in PR1–PR4 models: We have recently published (2,24) on the characterization of our 4 PDX models and provide the pertinent figures, here in the rebuttal letter for your perusal). The rebuttal letter Figure 1 below and our 2 publications provide detailed genomic and transcriptomic analyses of resistance mechanisms. Specifically:

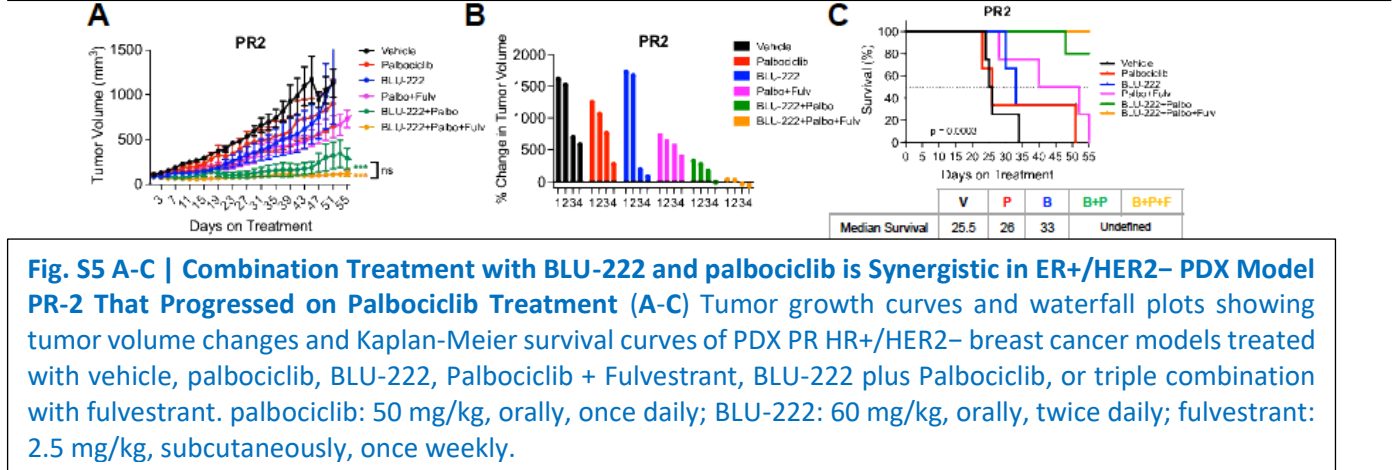
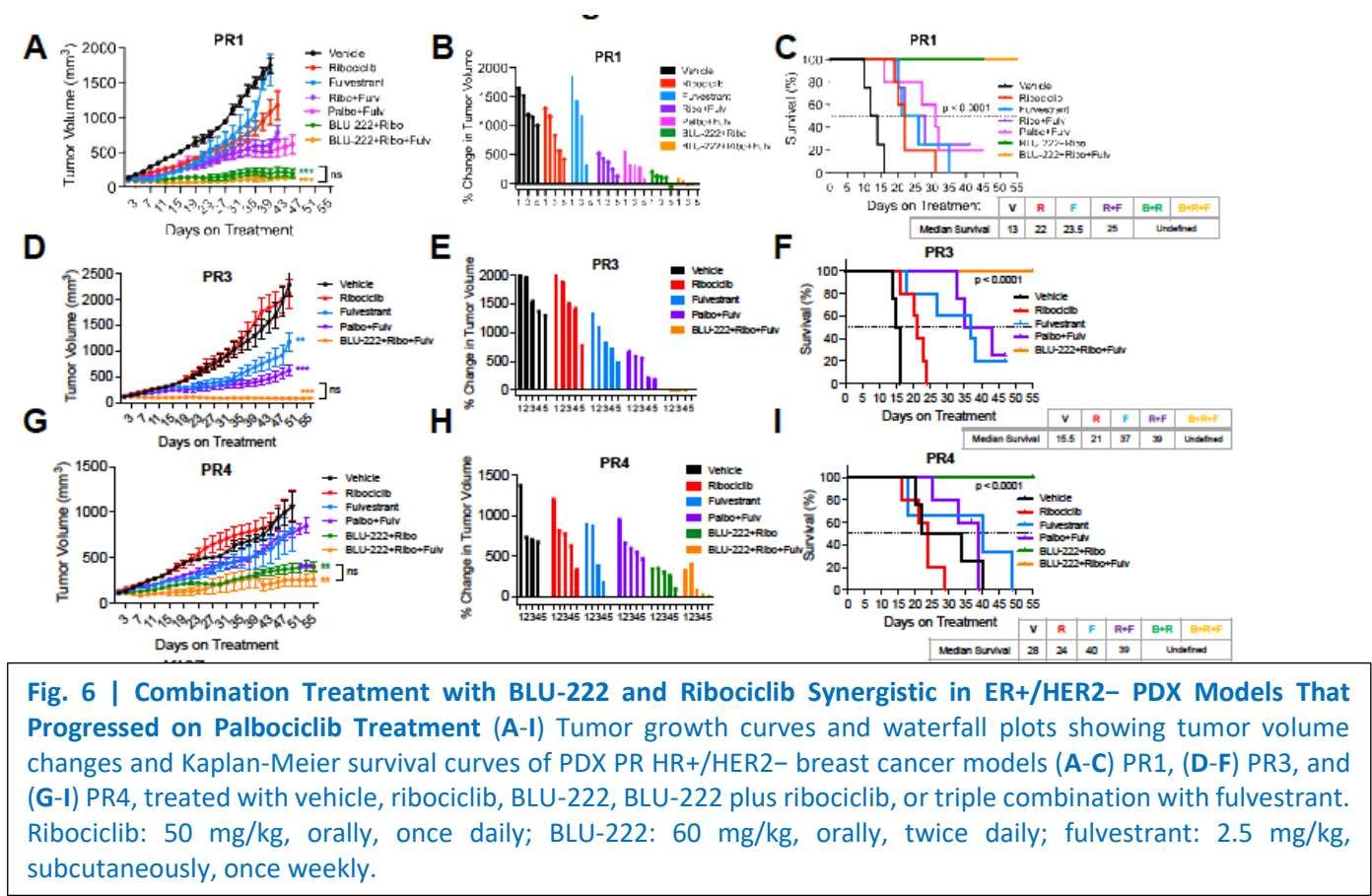
- PR-1 and PR-2 (late progressors, ≥ 6 months on palbociclib, panels A and B below): RNA-seq revealed enrichment of IL-6/STAT3, EMT, and inflammatory pathways, with slower tumor growth and lower Ki67 indices.

- PR-3 and PR-4 (early progressors, <3 months on palbociclib, panels A and B below): RNA-seq demonstrated enrichment of cell-cycle/DNA damage programs (E2F, G2/M checkpoint, AURKA), consistent with more rapid growth and higher proliferative indices.
- Additionally, all PR 1-4 models as well as our MCL and TCL models are either deleted for P53 or harbor mutations as in indicated in the Oncoprint panel below (Rebuttal Letter Figure 1C). PR-1, PR-4, PR-2, MCL and TCL also harbor Rb deletions and PR-1 harbors ESR mutations. These mutations further highlighting heterogeneity across these models.



Rebuttal Letter Figure 1: Related to characterization of palbociclib-resistant metastatic breast cancer patient-derived xenograft (PDX) models. **A.** Patient characteristics of four different palbociclib-resistant (PR) metastatic breast cancer PDX models, derived from patients who developed disease progression between 6 and 16 months (late resistance, PR-1 and PR-2) while receiving treatment with palbociclib plus ET or from patients who developed disease progression after 3 months while receiving treatment with palbociclib plus ET (early resistance, PR-3 and PR-4). **B** Gene set enrichment analysis comparing PDX models from patients who progressed after ≥ 6 months vs. those who progressed within 3 months. Starred pathways overlap with those found in our previously published PR cell line models (**C**) Oncoprint for PDX models (PR1-4) and MCF7 and T47D cell lines resistant to palbociclib (MCL and TCL) (n=6) on the 34 genes curated from literature associated with potential drivers of resistance to CDK4/6 inhibitors based on the somatic mutation and CNV data. For cell line somatic mutation calling, parental cells were used as a control. Genes are sorted by mutation frequency from high to low. The percentage of samples with any gene alteration is indicated on the right side of the plot. Each column represents a sample. Colors and shapes refer to different variant types.

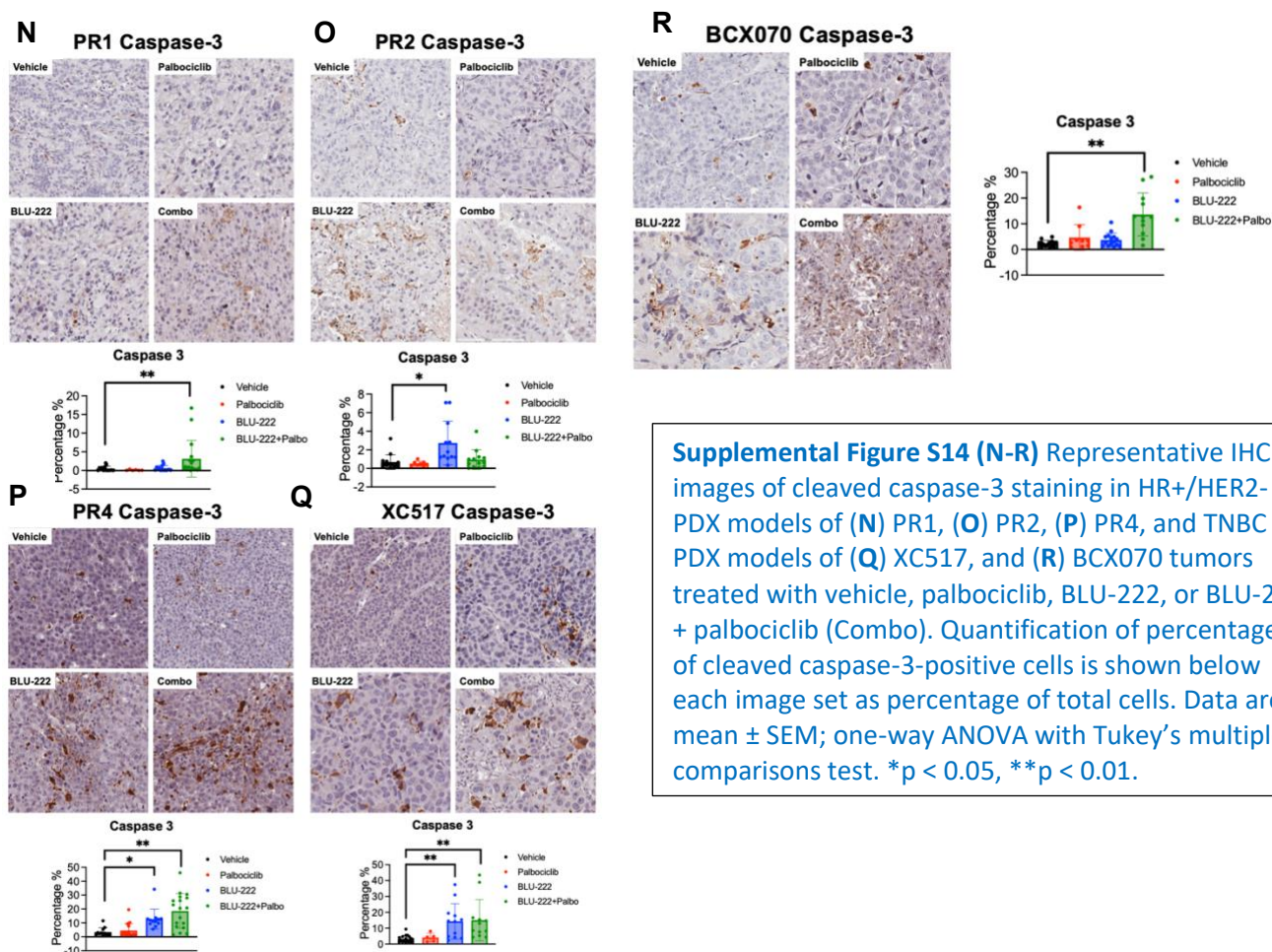
Triplet therapy vs. standard of care-We agree that evaluating the triplet combination (BLU-222 + CDK4/6 inhibitor + endocrine therapy) against the current standard of care (palbociclib + fulvestrant) provides important translational relevance. In response, we performed new in vivo experiments directly comparing these regimens, which are now presented in revised **Figures 6 and Supplementary Figure S5**. The triplet therapy consistently demonstrated superior tumor growth inhibition and more durable regressions compared to palbociclib plus fulvestrant, particularly in models with high cyclin E expression. These findings indicate that CDK2 inhibition not only complements CDK4/6 inhibition but also helps overcome endocrine resistance, likely through suppression of CDK2/cyclin E–driven proliferation that persists despite ER targeting. By benchmarking the triplet regimen against the clinical standard-of-care, our results highlight the potential of CDK2 inhibition to extend therapeutic benefit in resistant HR+/HER2– disease and provide strong preclinical justification for clinical trials testing this strategy.



γ H2AX, Caspase 3, and Senescence: We thank the reviewer for this insightful comment. While we acknowledge that the role of CDK2 in inducing DNA damage has not been universally demonstrated across all tumor models, prior studies have shown that CDK2 hyperactivation—particularly in the context of cyclin E overexpression—can drive replication stress and DNA damage through deregulated origin firing and impaired fork stability (20,25,26). In our study, we observed increased γ H2AX levels *in vivo* following BLU-222 treatment, which we interpret as a marker of replication stress and DNA damage response specifically in resistant tumors with elevated cyclin E. Thus, we believe γ H2AX is a mechanistically relevant readout of CDK2 inhibition in this context, consistent with the established biology of CDK2-driven replication stress.

We agree, however, that γ H2AX should not be interpreted in isolation. To strengthen our conclusions, we now present γ H2AX alongside apoptosis markers (Caspase 3 staining) (**new Supplemental Figure S14N-R**) and Sudan Black B staining (senescence) (**new Figure 8O-P**) in matched tumor sections. These analyses show that while apoptosis occurs in certain models, the predominant biological outcome of CDK2/CDK4/6 co-inhibition is not widespread apoptotic cell death, but rather a durable cytostatic response marked by increased γ H2AX and induction of senescence. This interpretation is further supported by our *in vitro* and *in vivo* β -galactosidase and SBB staining results, which show that BLU-222 induces robust senescence across HR+ and TNBC resistant models.

Taken together, these findings suggest that γ H2AX and senescence are mechanistically linked outcomes of CDK2 inhibition in resistant, cyclin E–driven tumors. While apoptosis may contribute to the overall response in selected contexts, our data indicate that the central therapeutic mechanism of BLU-222 is the induction of replication stress that culminates in senescence and durable tumor suppression. We have revised the manuscript to clarify this point and included apoptosis data, as requested, alongside γ H2AX and senescence markers.



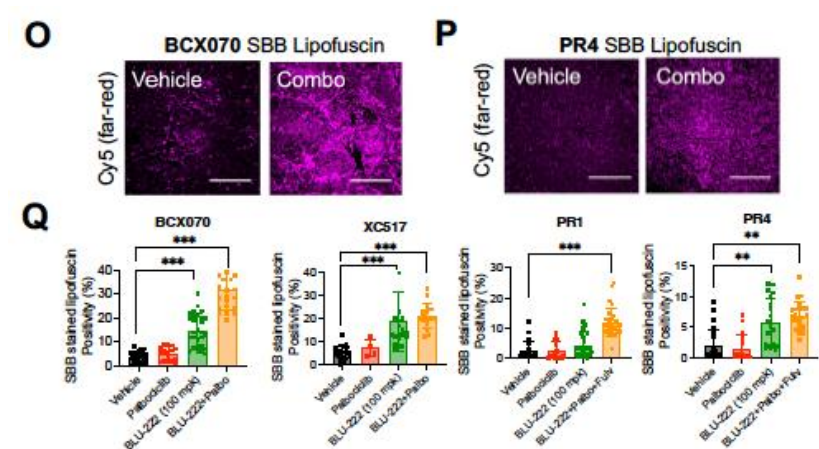


Figure 8 (O-P) Representative images of Sudan Black B (SBB)-stained lipofuscin (far-red, Cy5), indicative of therapy-induced senescence, in (O) BCX070 and (P) PR4 tumors from vehicle-treated and combination-treated (BLU-222+palbociclib) mice. Scale bars, 100 μ m. (Q) Quantification of SBB-positive lipofuscin area (%) in BCX070, XCS17, PR1, and PR4 tumors across indicated treatment groups. Data are shown as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

p21–p53 signaling axis: We thank the reviewer for raising this important point. While p21 is classically known as a transcriptional target of p53, our data indicate that the induction of p21 by CDK2 inhibition occurs independently of p53 status. Specifically, all four HR+/HER2– PDX models (PR1–PR4) and the highly resistant PR4.8 clone are p53-mutated (see **Rebuttal Letter Figure Panel C**), as are the majority of TNBC models examined. We have now included p53 mutational status for all models in **Supplementary Tables S1 and S2**. Importantly, despite lacking functional p53, these models consistently showed robust induction of p21 following BLU-222 treatment, both in vitro and in vivo. This observation suggests that BLU-222-mediated p21 upregulation is not reliant on canonical p53 transcriptional control. Instead, it is likely mediated through alternative regulatory pathways, such as those activated by CDK inhibition or replication stress. This interpretation is consistent with prior reports that p21 can be induced through p53-independent mechanisms, including via Sp1, STAT, or E2F family members under conditions of oncogenic stress (need reference).

Revised Results (mechanism of resistance of PR1-4 PDX models) in main manuscript (lines 533-545 of the revised manuscript)

To evaluate the in vivo efficacy of BLU-222 in combination with CDK4/6i, we used four previously established ER+/HER2– PDX models (PR1–PR4), derived from patients whose tumors had progressed on palbociclib and endocrine therapy (2,24). PR1 and PR2 represent acquired resistance, with progression after 16 and 6 months of treatment, respectively, whereas PR3 and PR4 represent intrinsic resistance, with progression within 3 months (Supplemental Table S2). Mechanistically, RNA-seq profiling revealed enrichment of IL-6/STAT3, EMT, and inflammatory signaling in the late progressors (PR1 and PR2), while the early progressors (PR3 and PR4) exhibited enrichment of cell cycle and DNA damage–associated programs, including E2F, G2/M checkpoint, and AURKA(2,24). Genomic profiling further showed that all PR models harbor TP53 alterations, with additional RB1 loss in PR1, PR2, and PR4, and an ESR1 mutation in PR1 (Supplemental Tables S2). Together, these models capture clinically relevant mechanisms of acquired and intrinsic CDK4/6 inhibitor resistance and provide a robust platform to test whether CDK2 inhibition can overcome diverse resistance contexts.

Revised Results (triple therapy vs standard of care) in main manuscript (lines 588-595 of the revised manuscript)

Compared to the SOC doublet of CDK4/6i plus fulvestrant, which produced only tumor growth delay without durable regression, addition of the CDK2 inhibitor BLU-222 significantly enhanced therapeutic efficacy across multiple resistant HR+/HER2– PDX models. The triplet regimen (BLU-222 + CDK4/6i + fulvestrant) induced robust, often complete or near-complete regressions, extended survival, and delayed recurrence (Figs. 6A–I; Supplemental Figs. S5A–C). These results highlight the potential of the triplet combination to overcome resistance to standard therapy and provide strong preclinical rationale for its clinical evaluation in HR+/HER2– breast cancer.

Revised Results (triple therapy vs standard of care) in main manuscript (lines 838-849 of the revised manuscript)

To functionally validate apoptotic induction, we assessed caspase-3 activation following single-agent and combination treatment with BLU-222 and CDK4/6i across five PDX models. IHC staining revealed modest caspase-3 positivity with BLU-222 monotherapy, minimal staining with CDK4/6i, and robust induction of apoptosis with the combination in PR1, PR2, PR4, XC5172013, and BCX070 (Supplemental Figs. S14N–R). While these findings establish apoptosis as an important contributor to therapeutic efficacy, they complement rather than replace the broader biological outcomes observed. In particular, earlier analyses demonstrated that CDK2/CDK4/6 co-inhibition triggers replication stress marked by γ H2AX and drives durable growth arrest through senescence, as evidenced by β -galactosidase and Sudan Black B staining (Fig. 8O–P). Together, these data suggest that apoptosis occurs in selected contexts, but that DNA damage–associated senescence represents the predominant and durable therapeutic mechanism, with apoptosis acting in parallel to enhance overall tumor suppression.

Revised Results (p21-p53 axis) in main manuscript (lines 604-611 of the revised manuscript)

Importantly, BLU-222 retained efficacy across all PR models despite diverse genomic alterations, including RB1 loss, ESR1 mutations, and PIK3CA mutations, demonstrating that its therapeutic benefit is not limited by these resistance-associated lesions (Supplemental Table S2). Notably, strong p21 induction was detected even in the TP53-deficient or mutated PR1-4 models, indicating that CDK2 inhibition activates p21 expression through p53-independent mechanisms. Together, these findings establish that PR1–4 not only recapitulate clinically relevant resistance mechanisms but also provide a robust platform showing that dual CDK2/CDK4 inhibition can overcome diverse contexts of resistance.

8) Regarding the GSEA results, the authors report that the G2M checkpoint signature is downregulated only in XC517. However, based on the findings from MCF7, T47D PR, and TNBC models, we would expect BLU-222 (alone or in combination with CDK4/6 inhibitors) to downregulate the G2M checkpoint in all models tested. Do the authors have any hypotheses for these differences?

- If the RNA-seq timepoint corresponds to the endpoint of the in vivo experiments in Figure 7, it might be too late to capture cell cycle and apoptosis effects while instead reflecting a strong immune response.
- The observed effects on the G2M checkpoint and E2F targets in XC517 may be influenced by treatment holidays and retreatment schedules.

We thank the reviewer for this insightful comment and appreciate the opportunity to further clarify the model-specific transcriptional responses. The observation that G2M checkpoint downregulation is most prominent in the XC517 model, despite consistent in vitro findings, highlights important differences in tumor context and treatment timing that merit deeper discussion.

As noted, the RNA-seq analyses were performed at the study endpoint, corresponding to the final tumor collection timepoint in Figure 7. At this stage, many tumors had undergone substantial regression, and our transcriptional data were dominated by interferon signaling, TNF- α , and inflammatory response gene sets, especially in combination-treated tumors. These immune and stress-related pathways likely represent a later-stage adaptive response, which may obscure the more transient cell cycle-specific effects seen earlier in treatment. This is a likely explanation for why G2M checkpoint and E2F targets were only significantly downregulated in the XC517 model.

In addition, we agree with the reviewer that the retreatment schedule used in the XC517 study (due to initial regression followed by re-dosing) may have allowed us to capture an intermediate timepoint where cell cycle suppression was still active, in contrast to other models where tumors were already senescent, necrotic, or undergoing immune clearance. The influence of treatment holidays or variable tumor regression kinetics likely

contributes to model-specific transcriptional profiles at the endpoint. We have included these explanations in the discussion section.

Revised Discussion in main manuscript (lines 918-925 of the revised manuscript)

Transcriptomic analyses provided further mechanistic clarity. Downregulation of the G2/M checkpoint and E2F targets was most evident in XC5172013, reflecting treatment timing and model-specific kinetics. Because RNA-seq was performed at study endpoints—after regression in most tumors—the dominant transcriptional signatures reflected interferon signaling, TNF- α , and inflammatory pathways consistent with senescence and immune activation. The retreatment schedule in XC5172013 likely captured an intermediate stage where cell cycle suppression was still detectable. Together, these findings suggest that suppression of G2/M and E2F programs are early events, whereas senescence and immune remodeling dominate at later stages.

9) In the discussion, please comment on Guiley et al 2021 related to p27 activating CDK4 and antagonizing palbociclib inhibition.

We appreciate the reviewer's suggestion. We have incorporated this important reference and expanded our discussion of p27 as a modulator of CDK4 activity and potential resistance to CDK4/6 inhibitors.

Revised Discussion in main manuscript (lines 904-909 of the revised manuscript)

This reprogramming contrasts with structural studies showing that p27 can assemble CDK4/6–cyclin D complexes and antagonize palbociclib inhibition (27) emphasizing that BLU-222 fundamentally shifts CIP/KIP function from potential activators to obligate inhibitors in resistant tumor contexts. By restoring this inhibitory control, BLU-222 not only enhances antitumor efficacy but also introduces a mechanistic framework for overcoming drug resistance.

Minor comments

1) Include phosphorylation sites for CDK2 and RB in the Western blot figure legends (Figures 1A, 1E, and 1G). Phosphorylation sites are now included in all relevant figure legends.

2) In Figure 6, the combination of BLU-222 with ribociclib is shown. However, in panels 6D–I, the violet line is labeled as "Palbo + Fulv" instead of "Ribo + Fulv." According to the figure legend, it should be "Ribo + Fulv"—please correct this.

Labelling error in Figure 6D–I has been corrected to "Ribo + Fulv."

3) Consider reorganizing Figure 8 so that panels C–D, E–F, and K–L are aligned in parallel to improve readability.

Figure 8 layout has been restructured and has been restructured for improved clarity and visual alignment.

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In this manuscript, Luo and colleagues present compelling data on the activity of a novel CDK2 inhibitor, BLU-222, in HR+ and TNBC models. The authors demonstrate that the CDK2 inhibitor synergizes with CDK4/6 inhibitors in resistant HR+ and TNBC models. Additionally, they describe how this combination profoundly affects apoptosis and cell cycle arrest through the upregulation of p21 and p27. Overall, this study provides evidence of the efficacy of a novel CDK2 inhibitor in breast cancer models resistant to CDK4/6 inhibitors. The manuscript also includes interesting molecular findings on p21 and p27 on CDK2-activity that have not been previously described. However, several key questions remain to be addressed:

- 1) The manuscript could be more concise while retaining the same level of information.
- 2) The references in the introduction should be reviewed. References 10–16 are too general and should be more CDK2-focused, particularly regarding the statements that ‘CDK2 inhibition induces cellular senescence’ and that ‘senescence involves the upregulation of p21 and p27, which inhibit CDK2 activity.’ Additionally, references 33–36, which include preclinical and clinical studies on cyclin E2 overexpression bypassing CDK4/6 inhibition, should be included in the introduction. Previous evidence on the activity of a selective CDK2 inhibitor (INX-315) in preclinical models of CCNE1-amplified ovarian cancer and HR+ breast cancer progressing on CDK4/6 inhibitors should be referenced. Please cite relevant publications by Shom Goel and Erik Knudsen in *Cancer Discovery* and *Nature Communications*, respectively.
- 3) The densitometry quantification of CDK4 in the Western blot from Figure S1 does not appear to correlate with the expression levels in Figure 1E. Specifically, in the Western blot, CDK4 levels seem to increase in shCDK2 cells with increasing doses of BLU-222, whereas the densitometry does not reflect this. Please review this discrepancy and revise the statement: “CDK4, CDK6, and cyclin D1 were upregulated in control and shCCNE1 cells but not in shCDK2 cells (Figure 1E, Supplemental Figures S1C–E).
- 4) In Figure 1H, the authors show that low-molecular-weight cyclin E (LMW-E) sensitizes T47D cells to BLU-222. Based on previous studies, cells overexpressing full-length cyclin E would be expected to be more sensitive to CDK2 inhibitors. However, the data indicate that T47D cells overexpressing full-length cyclin E are more resistant to BLU-222 than parental cells. How do the authors explain this effect? Additionally, please include parental and full-length cyclin E-overexpressing MDA-MB-231 cells in Figure S1L.
- 5) Acquired palbociclib-resistant MCF7 and T47D cells are valuable models for studying whether BLU-222 can overcome CDK4/6 inhibitor resistance. However, the manuscript does not provide the mechanism of acquired resistance for PR1.2 and PR4.8. Notably, Navarro-Yepes *et al.* (*Cancer Research*, 2023) Figure 3F shows that PR4.8 cells do not express RB1, which could explain their inherent resistance to CDK4/6 inhibitors and the absence of synergy with BLU-222. However, the profound sensitivity of PR4.8 cells to BLU-222 is unexpected and suggests CDK2 activity independent of RB1. The authors should:
 1. Comment on the mechanisms of acquired resistance in PR1.2 (high cyclin E, as shown in Figure 3A) and PR4.8 cells (RB1 loss?).
 2. Discuss why BLU-222, a CDK2 inhibitor, is effective as a single agent in PR4.8 cells, which lack RB1 expression. It would also be relevant to mention whether BLU-222 has antitumor activity in RB1-deficient tumors.

- 6) The authors generated palbociclib-resistant TNBC cell lines (MDA-MB-231 and BT20). However, in Figure 1N, the palbociclib sensitivity of the parental cells is not shown. According to Finn et al. (2009), MDA-MB-231 cells are relatively resistant to palbociclib. Including synergy plots for the combination treatment in parental MDA-MB-231 and BT20 cells would help clarify this point.
- 7) The combination of BLU-222 with CDK4/6 inhibitors in HR+/HER2- PDX models that progressed on palbociclib is highly consistent. However, it would be valuable to mention the resistance mechanisms of PR1, PR2, PR3, and PR4. Additionally, a comparison of the triplet therapy (BLU-222 + CDK4/6i + endocrine therapy) versus the standard of care (palbociclib + fulvestrant) would be beneficial. The authors should also consider:
 - Removing data on γ H2AX as a marker of DNA damage in vivo, given that DNA damage has not been demonstrated in prior studies with the CDK2 inhibitor.
 - Instead, including apoptosis data based on Figure 2 and the role of p21-p53.
- 8) Regarding the GSEA results, the authors report that the G2M checkpoint signature is downregulated only in XC517. However, based on the findings from MCF7, T47D PR, and TNBC models, we would expect BLU-222 (alone or in combination with CDK4/6 inhibitors) to downregulate the G2M checkpoint in all models tested. Do the authors have any hypotheses for these differences?
 - If the RNA-seq timepoint corresponds to the endpoint of the in vivo experiments in Figure 7, it might be too late to capture cell cycle and apoptosis effects while instead reflecting a strong immune response.
 - The observed effects on the G2M checkpoint and E2F targets in XC517 may be influenced by treatment holidays and retreatment schedules.
- 9) In the discussion, please comment on Guiley et al 2021 related to p27 activating CDK4 and antagonizing palbociclib inhibition.

Minor comments

- 1) Include phosphorylation sites for CDK2 and RB in the Western blot figure legends (Figures 1A, 1E, and 1G).
- 2) In Figure 6, the combination of BLU-222 with ribociclib is shown. However, in panels 6D–I, the violet line is labeled as "Palbo + Fulv" instead of "Ribo + Fulv." According to the figure legend, it should be "Ribo + Fulv"—please correct this.
- 3) Consider reorganizing Figure 8 so that panels C–D, E–F, and K–L are aligned in parallel to improve readability.