Supplemental Figure S1: Abemaciclib and ribociclib radiosensitize TNBC with wild-type RB1

Cell viability was measured in each cell line 72 hours after treatment with either abemaciclib (green) or ribociclib (blue) in RB wild type MDA-MB-231 (A) and CAL-120 (B) cells to calculate IC$_{50}$ values. MCF10A cells (C) were treated with palbociclib alone (open circles) or palbociclib + RT (filled squares). Clonogenic survival assays were performed in MDA-MB-231 (D-E) and CAL-120 (F-G) cells with varying doses of either ribociclib or abemaciclib and a one-hour drug pretreatment. RB null BT-549 cells were treated with palbociclib + RT (H). MDA-MB-231 cells were treated with palbociclib 6 hours after RT, instead of a one hour drug pretreatment (I). SF 2 Gy graphs represent the mean of three independent experiments, and for each experiment a one-way ANOVA with Dunnett’s post hoc test was used to compare combination-treated groups to the control group treated with RT alone (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
Supplemental Figure S2: pRB levels decrease with CDK4/6 inhibition

Western blots were used to assess expression of pRB (S807), pRB (S780), and total RB in RB expressing breast cancer cell lines including MDA-MB-231 (A), CAL-120 (B), MCF-7 (C), and T47D (D) cells. Cells were pretreated with a CDK4/6 inhibitor one hour prior to radiation (4 Gy) and harvested 30 minutes after radiation treatment.
Supplemental Figure S3: CDK4/6 inhibition impairs HR in TNBC in vitro

Two stable BT-549 HR reporter clones were pretreated ± 500nM CDK4/6 inhibitor one hour before SceI-induction of dsDNA breaks (A,B). After a 1-hour pretreatment with ± 1μM palbociclib and ± 4 Gy radiation, coverslips were stained for RAD51 foci 6 hours and 16 hours after radiation in RB wild type SUM-159 cells (C). T-tests were performed between paired radiation and combination treated groups at each timepoint, correcting for multiple comparisons. Western blots were used to assess RAD51 protein expression (D). Representative images of RAD51 foci (red) 6 hours post radiation are shown in MDA-MB-231 cells (E) and CAL-851 cells (F).
Supplemental Figure S4: *RB1* knockout decreases CDK4/6 inhibitor potency in ER+ and TNBC cell lines

Cell viability was measured 72 hours after treatment with either palbociclib (grey), ribociclib (blue), or abemaciclib (green) in Cas9-expressing control cell lines (black circles) or *RB1* CRISPR knockout cells (colored squares). Dose-response curves were generated for each drug in CAL-120 (A-C), MDA-MB-231 (D-F), MCF-7 (G-H), and T47D (J-L) cell lines to calculate IC50 values after *RB1* knockout. IC50 experiments represent the aggregate of 3 independent replicates and data points display average with SEM.
Supplemental Figure S5: RB1 is essential for G1 cell-cycle arrest

Flow cytometry was used to quantify cell cycle distribution in G1 (black), G2 (white), and S (grey) phase after CDK4/6 inhibition using propidium iodine (PI) staining in CAL-120 cells (A, B), MDA-MB-231 cells (D,E) and MCF-7 cells (H) with intact RB1. Cell cycle progression was also quantified in RB null MDA-MB-468 cells (G) and in models of RB1 knockout (C,F,I). Cells were fixed at 24 hours and 48 hours post drug treatment with the IC50 concentration of palbociclib, abemaciclib, and ribociclib in all cell lines.
Clonogenic survival assays were performed in CAL-120 (A) and T47D (B) RB1 CRISPR cells along with MDA-MB-231 (C) and MCF-7 (D) Cas9 control cells to quantify radiosensitization and calculate radiation enhancement ratios (rER). Western blots were used to confirm successful knockout of RB in CAL-120 and T47D cells (E). rER were compared between parental CAL-120 cells and CAL-120 RB1 CRISPR knockout cells (F). Overexpression of RB was performed in MDA-MB-468 and BT-549 cells in order to assess radiosensitivity in clonogenic survival assays (G-I). All clonogenics represent the pooled results of 3 independent replicates. (*, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \); ****, \( P < 0.0001 \)).
Supplemental Figure S7: Palbociclib-resistant TNBC cells demonstrate loss of both RB protein and CDK4/6 inhibitor-mediated radiosensitization.

Palbociclib-resistant MDA-MB-231 cells were generated through continuous culture in drug-containing media (A) and 72 hour cell viability was used to assess acquired resistance to palbociclib, ribociclib, or abemaciclib. Clonogenic survival assays were performed to quantify the rER for PalboR cells and western blots were used to assess RB expression compared to parental MDA-MB-231 cells (B). Radiosensitization was assessed after transient overexpression of GFP-RB and pretreatment ± palbociclib (C).
Supplemental Figure S8: Loss of p53 expression does not significantly impact radiosensitization

Knockout of p53 protein was assessed by western blot (A) and the viability of TP53 CRISPR cell lines was assessed using Alamar Blue 72 hours after treatment with varying concentrations of palbociclib (B). Clonogenic survival assays were used to assess palbociclib-mediated radiosensitization in Cas9 control cells (E) and TP53 knockout cell lines (C,D) and using a one hour pretreatment. A one-way ANOVA with Dunnett’s post hoc test was used to compare SF 2 Gy values for each cell line (*, P < 0.05).
Supplemental Figure S9: CDK4/6 inhibitor-mediated radiosensitization of TNBC in vivo

Time to tumor doubling is shown for MDA-MB-231 parental (RB wild type, A) xenografts treated with palbociclib, and a log-rank (Mantel-Cox) test was used to compare survival curves. Weights are shown for mice with parental (B), Cas9 control (E), and RB1 CRISPR (F) xenografts throughout the study. Ki67 staining (imaged at 40x) was used to assess proliferation of tumor cells in mice treated with short term palbociclib and/or RT (C,D). A one way ANOVA with Tukey’s post hoc test was used to compare Ki67 staining across treatment groups. (*, P < 0.05; **, P < 0.01; ***, P < 0.001, ****, P < 0.0001).
Supplemental Figure S10: RB1 loss does not result in increased micronuclei formation following ionizing RT.

Immunofluorescence was used to quantify micronuclei formation following 4 Gy RT in parental and RB1 CRISPR MCF-7 (A) and MDA-MB-231 cells (B) 72 hours after RT. Representative DAPI-stained slides are shown for all treatment groups (C,D).
Supplemental Figure S11: CDK4/6 inhibition suppresses RAD51 foci formation

Representative images of RAD51 foci at 6 hours post RT (4 Gy) are shown for parental RB wild type MCF-7 (A) and MDA-MB-21 (B) cells transfected with control or RB1-targeting siRNA. Images are also shown for MCF-7 Cas9 (C) and MDA-MB-231 Cas9 (D) and RB1 CRISPR (E) cells. GFP-RB and myc-MCL1 were transfected into HEK-293T cells and immunoprecipitation was performed 24 hours later (F).
### Supplemental Table S1: IC₅₀ values for parental and CRISPR TNBC and ER+ breast cancer cell lines

| Palbociclib | Ribociclib | Abemaciclib |
|-------------|------------|-------------|
| Cell Line   | IC₅₀       | CRISPR R Bí IC₅₀ | Cas9 Only IC₅₀ | IC₅₀ | CRISPR R Bí IC₅₀ | Cas9 Only IC₅₀ | IC₅₀ | CRISPR R Bí IC₅₀ | Cas9 Only IC₅₀ |
| MDA-MB-231  | 241.6nM    | >10 µM       | 222nM         | 1.99µM | >10 µM       | 1.95µM       | 1.07µM | 9.2 µM        | 568nM        |
| CAL-120     | 2.9µM      | >10 µM       | 1.3µM         | 1.77µM | >10 µM       | 1.25µM       | 2.61µM | 9.5 µM        | 2.54µM       |
| T47D        | 20nM       | > 5µM        | 17nM          | 40nM   | > 5 µM       | 33nM         | 10nM   | 2.6µM         | 21nM         |
| MCF-7       | 75nM       | 1.047 µM     | 184nM         | 200nM  | 741 nM       | 317.7nM      | 40nM   | 2.34µM        | 68nM         |

### Supplemental Table S2: IC₅₀ values for additional TNBC cell lines

| Cell Line   | Concentration | IC₅₀ | rER     | RB       |
|-------------|---------------|------|---------|----------|
| SUM-159     | 5.5µM         | Wild Type |
| CAL-51      | 330nM         | Wild Type |
| MDA-MB-468  | >10µM         | Null  |
| CAL-851     | >10µM         | Null  |

### Supplemental Table S3: Radiation enhancement ratios for ER+ breast cancer cell lines treated with palbociclib

| Day | RT | Palbo | Expected | Observed | Ratio |
|-----|----|-------|----------|----------|-------|
| 7   | 0.790 | 0.762 | 0.602 | 0.651 | 0.925 |
| 16  | 0.455 | 0.572 | 0.260 | 0.325 | 0.801 |
| 18  | 0.381 | 0.490 | 0.187 | 0.257 | 0.725 |
| 21  | 0.348 | 0.476 | 0.165 | 0.215 | 0.768 |
| Final | 0.462 | 0.866 | 0.400 | 0.238 | 1.682 |

### Supplemental Table S4: Fractional Tumor Volume Calculations for MDA-MB-231 xenografts treated with palbociclib

| Day | RT | Abema | Expected | Observed | Ratio |
|-----|----|-------|----------|----------|-------|
| 7   | 0.738 | 0.678 | 0.500 | 0.624 | 0.801 |
| 11  | 0.625 | 0.608 | 0.380 | 0.446 | 0.852 |
| 16  | 0.482 | 0.703 | 0.338 | 0.329 | 1.030 |
| 18  | 0.448 | 0.712 | 0.319 | 0.299 | 1.067 |
| Final | 0.549 | 0.872 | 0.479 | 0.320 | 1.499 |

### Supplemental Table S5: Fractional Tumor Volume Calculations for MDA-MB-231 Cas9 Control and RB1 CRISP xenografts treated with abemaciclib

| Day | RT | Abema | Expected | Observed | Ratio |
|-----|----|-------|----------|----------|-------|
| 7   | 0.782 | 0.818 | 0.640 | 0.684 | 0.935 |
| 11  | 0.546 | 0.613 | 0.335 | 0.444 | 0.754 |
| 16  | 0.440 | 0.579 | 0.254 | 0.327 | 0.778 |
| 18  | 0.416 | 0.591 | 0.246 | 0.298 | 0.824 |
| Final | 0.427 | 0.721 | 0.308 | 0.327 | 0.939 |
| Timepoint | Treatment | Average Foci / Cell | Average Foci / Positive Cell |
|-----------|-----------|---------------------|-----------------------------|
| **CAL-120** |
| 6 hours  | DMSO      | 3.66 ± 0.74         |                             |
|          | Palbociclib | 2.82 ± 1.34         |                             |
|          | RT (4 Gy) | 11.28 ± 3.39        | 19.56 ± 6.37                |
|          | RT + Palbo | 7.38 ± 3.02         | 26.71 ± 10.37               |
| 16 hours | DMSO      | 0.91 ± 0.64         |                             |
|          | Palbociclib | 1.14 ± 0.67         |                             |
|          | RT (4 Gy) | 11.86 ± 2.01        | 21.51 ± 2.00                |
|          | RT + Palbo | 3.48 ± 0.92         | 17.46 ± 6.07                |
| **MDA-MB-231** |
| 6 hours  | DMSO      | 6.67 ± 4.77         |                             |
|          | Palbociclib | 2.84 ± 1.65         |                             |
|          | RT (4 Gy) | 12.81 ± 4.85        | 25.30 ± 8.16                |
|          | RT + Palbo | 4.12 ± 2.89         | 31.92 ± 15.83               |
| 16 hours | DMSO      | 1.54 ± 0.49         |                             |
|          | Palbociclib | 1.85 ± 0.34         |                             |
|          | RT (4 Gy) | 6.43 ± 0.55         | 12.07 ± 1.01                |
|          | RT + Palbo | 4.47 ± 3.02         | 18.11 ± 7.32                |
| **MDA-MB-468** |
| 6 hours  | DMSO      | 2.72 ± 1.13         |                             |
|          | Palbociclib | 2.18 ± 1.96         |                             |
|          | RT (4 Gy) | 15.57 ± 4.02        | 29.13 ± 8.61                |
|          | RT + Palbo | 11.39 ± 1.62        | 19.67 ± 3.47                |
| 16 hours | DMSO      | 1.92 ± 0.52         |                             |
|          | Palbociclib | 1.86 ± 0.84         |                             |
|          | RT (4 Gy) | 9.04 ± 0.51         | 18.18 ± 1.39                |
|          | RT + Palbo | 7.66 ± 0.32         | 15.51 ± 1.52                |
| **CAL-851** |
| 6 hours  | DMSO      | 1.07 ± 0.47         |                             |
|          | Palbociclib | 1.27 ± 0.80         |                             |
|          | RT (4 Gy) | 16.17 ± 2.71        | 44.24 ± 10.49               |
|          | RT + Palbo | 14.51 ± 3.80        | 34.48 ± 7.25                |
| 16 hours | DMSO      | 1.51 ± 0.60         |                             |
|          | Palbociclib | 2.46 ± 0.56         |                             |
|          | RT (4 Gy) | 7.97 ± 2.33         | 16.06 ± 4.38                |
|          | RT + Palbo | 9.32 ± 2.95         | 20.84 ± 6.95                |

**Supplemental Table 6:** Quantification of RAD51 foci in RB wild type and RB null TNBC cell lines.
| Timepoint | Treatment   | Average Foci / Cell | Average Foci / Positive Cell |
|-----------|-------------|---------------------|------------------------------|
| **CAL-120** |             |                     |                              |
| 30 min    | DMSO        | 2.29 ± 0.52         |                              |
|           | Palbociclib | 2.31 ± 0.30         |                              |
|           | RT (4 Gy)   | 20.40 ± 1.03        | 22.58 ± 0.64                 |
|           | RT + Palbo  | 21.16 ± 1.68        | 24.21 ± 0.89                 |
| 6 hours   | DMSO        | 2.73 ± 1.40         |                              |
|           | Palbociclib | 2.51 ± 0.47         |                              |
|           | RT (4 Gy)   | 17.05 ± 0.60        | 21.51 ± 0.35                 |
|           | RT + Palbo  | 14.51 ± 0.29        | 17.49 ± 0.54                 |
| 16 hours  | DMSO        | 2.77 ± 1.04         |                              |
|           | Palbociclib | 1.16 ± 0.65         |                              |
|           | RT (4 Gy)   | 13.40 ± 4.23        | 26.06 ± 0.34                 |
|           | RT + Palbo  | 10.85 ± 2.5         | 24.70 ± 1.42                 |
| 24 hours  | DMSO        | 1.55 ± 0.59         |                              |
|           | Palbociclib | 1.87 ± 1.02         |                              |
|           | RT (4 Gy)   | 7.32 ± 1.11         | 20.83 ± 4.66                 |
|           | RT + Palbo  | 8.27 ± 1.45         | 24.70 ± 1.42                 |
| **MDA-MB-231** |    |                     |                              |
| 30 min    | DMSO        | 1.24 ± 0.51         |                              |
|           | Palbociclib | 0.91 ± 0.34         |                              |
|           | RT (4 Gy)   | 12.84 ± 5.96        | 19.20 ± 0.90                 |
|           | RT + Palbo  | 18.94 ± 1.36        | 21.34 ± 0.80                 |
| 6 hours   | DMSO        | 2.28 ± 0.76         |                              |
|           | Palbociclib | 0.50 ± 0.02         |                              |
|           | RT (4 Gy)   | 11.63 ± 3.57        | 14.76 ± 3.05                 |
|           | RT + Palbo  | 12.07 ± 2.32        | 18.76 ± 2.71                 |
| 16 hours  | DMSO        | 1.02 ± 0.13         |                              |
|           | Palbociclib | 1.29 ± 0.49         |                              |
|           | RT (4 Gy)   | 10.08 ± 2.02        | 23.11 ± 1.52                 |
|           | RT + Palbo  | 7.00 ± 4.85         | 20.52 ± 1.01                 |
| 24 hours  | DMSO        | 0.69 ± 0.18         |                              |
|           | Palbociclib | 1.17 ± 0.54         |                              |
|           | RT (4 Gy)   | 3.78 ± 0.91         | 18.75 ± 3.49                 |
|           | RT + Palbo  | 2.52 ± 1.30         | 20.17 ± 2.21                 |

Supplemental Table S7: Quantification of γH2AX foci after RT and CDK4/6 inhibition.
| Timepoint | Treatment | MCF-7 | Average Foci / Cell | Average Foci / Positive Cell |
|-----------|-----------|-------|---------------------|-----------------------------|
| 6 hours   | RT (siNT) | 17.07 ± 0.17 | 33.04 ± 0.66 |
|           | RT + Palbo (siNT) | 4.71 ± 1.07 | 20.71 ± 5.63 |
|           | RT (siRB1) | 6.5 ± 0.82 | 17.40 ± 2.98 |
|           | RT + Palbo (siRB1) | 10.13 ± 1.72 | 23.74 ± 2.73 |
|           | RT (siNT) | 11.57 ± 3.75 | 28.58 ± 3.25 |
|           | RT + Palbo (siNT) | 5.65 ± 0.37 | 18.68 ± 3.01 |
|           | RT (siRB1) | 5.18 ± 0.38 | 12.56 ± 0.88 |
|           | RT + Palbo (siRB1) | 7.99 ± 1.10 | 21.18 ± 3.10 |

**Supplemental Table S8:** Quantification of RAD51 foci in MCF-7 and MDA-MB-231 cells after RT and CDK4/6 inhibition ± siRB1.

| Timepoint | Treatment | MCF-7 Cas9 | Average Foci / Cell | Average Foci / Positive Cell |
|-----------|-----------|------------|---------------------|-----------------------------|
| 6 hours   | NT        | 2.51 ± 0.68 |                     |
|           | Palbociclib | 1.31 ± 0.51 |                     |
|           | RT (4 Gy) | 22.96 ± 4.26 | 35.79 ± 3.30 |
|           | RT + Palbociclib | 10.65 ± 1.59 | 37.61 ± 6.77 |
|           | NT        | 2.02 ± 0.70 |                     |
|           | Palbociclib | 0.71 ± 0.29 |                     |
|           | RT (4 Gy) | 15.24 ± 2.83 | 35.38 ± 5.82 |
|           | RT + Palbociclib | 14.55 ± 3.19 | 34.82 ± 5.40 |
|           | NT        | 2.11 ± 1.29 |                     |
|           | Palbociclib | 2.42 ± 0.75 |                     |
|           | RT (4 Gy) | 19.98 ± 3.42 | 40.57 ± 7.33 |
|           | RT + Palbociclib | 6.68 ± 2.87 | 50.70 ± 12.39 |
|           | NT        | 2.60 ± 1.12 |                     |
|           | Palbociclib | 3.61 ± 0.49 |                     |
|           | RT (4 Gy) | 24.69 ± 5.25 | 33.14 ± 5.86 |
|           | RT + Palbociclib | 8.36 ± 3.24 | 22.32 ± 9.56 |
|           | NT        | 4.36 ± 1.35 |                     |
|           | Palbociclib | 3.29 ± 0.39 |                     |
|           | RT (4 Gy) | 12.52 ± 0.76 | 23.60 ± 0.96 |
|           | RT + Palbociclib | 9.01 ± 0.23 | 33.14 ± 2.29 |
|           | NT        | 1.09 ± 0.73 |                     |
|           | Palbociclib | 1.51 ± 0.79 |                     |
|           | RT (4 Gy) | 20.42 ± 4.99 | 45.81 ± 6.48 |
|           | RT + Palbociclib | 14.97 ± 2.51 | 38.74 ± 10.26 |

**Supplemental Table S9:** Quantification of RAD51 foci in MCF-7 and MDA-MB-231 Cas9 control and RB1 CRISPR cells ± transient GFP-RB overexpression.