Early adaption and acquired resistance to CDK4/6 inhibition in ER-positive breast cancer

Maria Teresa Herrera-Abreu[1,#], Marta Palafox[2,#], Uzma Asghar[1], Martín A. Rivas[7], Rosalind J. Cutts[1], Isaac Garcia-Murillas[1], Alex Pearson[1], Marta Guzman[2], Olga Rodriguez[2], Judit Grueso[2], Meritxell Bellet[3], Javier Cortés[3], Richard Elliott[1], Sunil Pancholi[1], José Baselga[4], Mitch Dowsett[1,6], Lesley Ann Martin[1], Violeta Serra[2,*] and Nicholas C. Turner[1,5,*]

Supplementary figure legends:

Figure 1: Early adaptive resistance limits the efficacy of CDK4/6 inhibition. High-throughput drug screen identified PI3K-mTOR inhibitors as sensitizers to CDK4/6 inhibitors.

A. T47D and MCF-7 cells treated with palbociclib for 96 hours followed by 24 hours exposure to BrdU. Images show Brdu positive cells (green) and Brdu negative cells (red). Graphs show number of nuclei (DAPI staining), percentage of BrdU positive cells, and cell area (* $P<0.001$ vehicle vs. palbociclib, Student's T test). Magnification 100 x.

B. Western blot of cell lysates from T47D cells treated for the indicated time points with 500nM palbociclib.

C. MCF-7 cells treated with palbociclib for 72 hours followed by 0.5 hours exposure to EdU, then washed and exposed to Brdu for another 24 hours. Graph shows percentage of BrdU negative-EdU positive cells measured with Columbus software.

D and E. MCF-7 and T47D cells were exposed to a drug library along with SF80 dose of palbociclib, or vehicle for 72 hours. D. Bar charts showing hits with a significant effect on cell survival defined as a Z score <-2. PI3K-mTOR pathway inhibitors are shown in red. E. Bar charts showing hits that induce resistance to palbociclib with a significant effect on cell survival defined as a Z score >2. Hits include CHK1, NEDD and anti-mitotic inhibitors.

F. MCF-7 cells were exposed to the range of concentrations of palbociclib or GDC-0941 indicated, and the combination for 5 days (combination index 0.26 for 250nM GDC-0941/500nM Palbociclib).

G. MCF-7 cells exposed to palbociclib, MK2206, and the combination for 5 days (combination index 0.34 for 250nM Palbociclib and MK2206).

Figure 2: Determinants of sensitivity to the combination CDK4/6 and PI3K inhibition

A and B. Relative growth of a panel breast cancer cell lines treated for 6 days with control vehicle, 500nM palbociclib, 250nM GDC-0941, or the combination. A. Cell lines with increased sensitivity to the combination (***P<0.001, two-way ANOVA with Tukey's multiple comparison test, combination vs. each mono-therapy) are *RB1* proficient with activating mutations in *PI3KCA*. B. Cell lines were combination failed to increase sensitivity when compared to one of the mono-therapies (two-way ANOVA with Tukey's multiple comparison test), which include *PI3KCA* wild type or *RB1* null cell lines.

C and D. Clonogenic survival assays for the indicated cell lines exposed to 500nM palbociclib, 250nM GDC-0941, or combination for 14 days.

E. BrdU-ELISA cell proliferation assays for the indicated cell lines. Cells were treated as indicated for 72 hours (*P< 0.001 Student's T test palbociclib vs. combination, NS= not significant).

Figure 3: Early adaptation to CDK4/6 inhibition is mediated by non-canonical cyclin D1-CDK2 interaction. IGF1R inhibition increases sensitivity to CDK4/6 inhibition in MCF-7 cells.

A. Western blot of cell lysates from MCF-7 cells treated for 72 hours with vehicle, 500nM palbociclib, 250nM GDC-0941, or combination, and blotted with the indicated antibodies.

B. Relative growth of MCF-7 and T47D cells treated with vehicle, 500nM palbociclib, 250nM AEW541, or combination (***P<0.001, one-way ANOVA with Tukey's multiple comparisons test).

C. Phospho-Receptor Tyrosine Kinases (RTKs, R&D Systems) assays of lysates from MCF-7 cells treated with vehicle or 500nM palbociclib for 96 hours.

D. Western blot of cell lysates from MCF-7 cells treated with vehicle, 500nM palbociclib, 250nM AEW541, or combination and blotted with the indicated antibodies.

E, F, G and H. T47D and MCF-7 cells transfected for 4 days with control siCON2 or pool siRNA (E and F) and individual siRNA (G and H) targeting CDK2 or cyclin D1 and treated with vehicle or palbociclib for 72 or 96 hours as indicated. E, G and H. Brdu-ELISA proliferation assays. F. Number of nuclei, same experiment as in Figure 2B (*** P<0.001, palbociclib treated, siCON2 vs. other siRNAs, one-way ANOVA with Tukey's multiple comparisons test).

Supplementary Figure 4: Palbociclib induced senescence like morphology is reversible

A. Bright-field images of T47D cells treated with vehicle, 500nM palbociclib, 250nM GDC-0941, or combination for 72 hours with reduced senescence like morphology (cell flattening) with the combination. Bar chart scored for normal (black) or senescence like (grey) morphology of 300 cells counted in each condition.

B. Relative caspase 3/7 activation in MCF-7 cells treated with vehicle or palbociclib for 72 hours, with the addition of vehicle or GDC-0941 for a further 6, 18, or 24 hours (* P<0.05, ***P<0.001, two-way ANOVA with Tukey's multiple comparisons test).

C. Clonogenic survival in MCF7 cells treated with vehicle, palbociclib, GDC-0941 or combination for 16 days, or first with palbocicilib for 10 days with addition of GDC-0941 or combination for another 6 days.

D. T47D cells treated with vehicle or 500nM palbociclib for 72 hours. Subsequently palbociclib continued for another 96 hours or washed cells grown in the presence of

vehicle. Bright-field images show senescence like morphology (cell flattening and increased cell area) after 72 hours of palbociclib treatment that is reversed by palbociclib withdrawal. Bar chart shows scores for normal (black) or senescence like (grey) morphology of 300 cells counted in each condition.

Supplementary Figure 5: Acquired palbociclib resistance through *RB1* loss or *CCNE1* amplification/expression.

A. MCF-7 palbociclib resistant (pR) cells treated with palbociclib for 72 hours followed by 2 hours exposure to BrdU. Images show Brdu positive cells (green). Graphs show number of nuclei (DAPI staining), percentage of BrdU positive cells, and cell area (* $P<0.001$ vehicle vs. palbociclib, Student's T test).

B. Comparison of read depth for the *CCNE1* (cyclin E1) gene in the parental and resistant cell lines. A sashimi plot of coverage in the parental (wt) and resistant (pR) MCF-7 cell lines at the *CCNE1* gene plotted on the same scale using the Integrated Genome Browser (IGV) shows the increased read count of cyclin E1 in the resistant cell line.

C. Comparison of variant allele frequency of mutations found in the parental MCF7 and resistant MCF7pR cell lines. There is good agreement in variant allele frequency for both mutant and resistant cell lines (r=0.87) including the previously reported *PIK3CA* missense mutation (highlighted). Mutations acquired in the resistant cell line are coloured in blue and an additional *PIK3CA* mutation present only in the resistant cell line is highlighted. Both samples had a minimum sequencing depth of 50x for all mutations.

D. Digital PCR (ddPCR) plots for *CCNE1* Copy Number Variation assessment on MCF-7 and MCF-7pR. In each ddPCR plot, green dots represent *TERT* DNA (VIC labeled), blue dots represent *CCNE1* DNA (FAM - 6-fluorescein amidite labeled), brown dots represent droplets containing both, *TERT* and *CCNE1*, DNA, and black dots are droplets with no DNA incorporated.

E. Cell growth in MCF-7pR and T47DpR cells, or corresponding parental cell line transfected 4 days earlier with control siCON2 or SMARTpool siRNA targeting CDK4 or cyclin D1 or UBB (ubiquitin B) as a positive control.

F and G. MCF-7 palbociclib resistant (pR) cells were transfected 4 days earlier with control siCON2 or indicated siRNA and treated with vehicle or palbociclib for 72 hours. F. BrdU incorporation measured by ELISA and corrected for viable cell number (***P<0.001, two-way ANOVA with Tukey's multiple comparisons test). G. Percentage of BrdU positive cells (BrdU staining vs. number of nuclei) and cell area measured in more than 1000 cells using Columbus software (*** P<0.001, two-way ANOVA with Tukey's multiple comparisons test).

Supplementary Figure 6: Body weight of mice during vehicle, BYL719, LEE011 or combination treatment.

A. Body weight (g) of PDX191 following vehicle, BYL719 (35mg/kg, QD, 6IW), LEE011 (75mg/kg, QD, 6IW) and BYL719 plus LEE011 treatment (n.s., not significant, two-way ANOVA). The total number of mice in each arm (n) is indicated.

B. Body weight (g) of PDX244 following vehicle, BYL719 (35mg/kg, QD, 6IW), LEE011 (75mg/kg, QD, 6IW) and BYL719 plus LEE011 treatment. The total number of mice in each arm (n) is indicated.

Supplementary Figure 7: The combination LEE011 and BYL719 is more effective in ER positive PDX than single agent treatments

A. Relative tumor growth (right) of PDX244 following vehicle, BYL719 (35mg/kg, QD, 6IW), LEE011 (75mg/kg, QD, 6IW) and BYL719 plus LEE011 treatment. The total number of tumors in each arm (n) and SEM of each point are indicated.

Western blot (left) showing phospho-pRb Ser807/811, pRb, cyclin E2, cyclin D1 and Tubulin expression in PDX244 after 14 days of vehicle, BYL719, LEE011 and BYL719 plus LEE011 treatment. Each lane belongs to one individual tumor.

1. Wang, K., M. Li, and H. Hakonarson, *ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data.* Nucleic Acids Res, 2010. **38**(16): p. e164.
2. Forbes, S.A., et al., *COSMIC: exploring the world's knowledge of somatic mutations in human cancer.* Nucleic Acids Res, 2015. **43**(Database issue): p. D805-11.