Cancer Research

Kinome-Wide RNA Interference Screen Reveals a Role for PDK1 in Acquired Resistance to CDK4/6 Inhibition in ER-Positive Breast Cancer

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Abstract

Acquired resistance to cyclin-dependent kinases 4 and 6 (CDK4/6) small-molecule inhibitors in breast cancer arises through mechanisms that are yet uncharacterized. In this study, we used a kinome-wide siRNA screen to identify kinases that, when downregulated, yield sensitivity to the CDK4/6 inhibitor ribociclib. In this manner, we identified 3-phosphoinositide-dependent protein kinase 1 (PDK1) as a key modifier of ribociclib sensitivity in estrogen receptor–positive MCF-7 breast cancer cells. Pharmacologic inhibition of PDK1 with GSK2334470 in combination with ribociclib or palbociclib, another CDK4/6 inhibitor, synergistically inhibited proliferation and increased apoptosis in a panel of ER-positive breast cancer cell lines. Ribociclib-resistant breast cancer cells selected by chronic drug exposure displayed a relative increase in the levels of PDK1 and activation of the AKT pathway. Analysis of these cells revealed that CDK4/6 inhibition failed to induce cell-cycle arrest or senescence. Mechanistic investigations showed that resistant cells coordinately upregulated expression of cyclins A, E, and D1, activated phospho-CDK2, and phospho-S477/T479 AKT. Treatment with GSK2334470 or the CDK2 inhibitor dinaciclib was sufficient to reverse these events and to restore the sensitivity of ribociclib-resistant cells to CDK4/6 inhibitors. Ribociclib, in combination with GSK2334470 or the PI3Kα inhibitor alpelisib, decreased xenograft tumor growth more potently than each drug alone. Taken together, our results highlight a role for the PI3K–PDK1 signaling pathway in mediating acquired resistance to CDK4/6 inhibitors. *Cancer Res; 77(9); 2488–99. ©2017 AACR.*

Introduction

Small-molecule inhibitors of the cyclin-dependent kinases 4 and 6 (CDK4/6) demonstrated impressive activity in patients with

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ER-positive (ER[+]) HER2-negative (HER2^{−}) breast cancer when combined with antiestrogens (1–4). The CDK4/6 inhibitor palbociclib was approved by the FDA in 2015 for use in combination with letrozole for first-line treatment of postmenopausal women with ER[+] metastatic breast cancer. This was in part due to the outstanding results from the PALOMA-1 study, which demonstrated a marked improvement in progression-free survival (PFS) with the combination compared to letrozole alone (1). In the second-line setting, the PALOMA-3 study demonstrated a remarkable improvement in PFS for the combination of fulvestrant/palbociclib compared with fulvestrant alone (4). Currently, two other CDK4/6 inhibitors, abemaciclib (LY2835219; Lilly; ref. 5) and ribociclib (LEE011; Novartis; ref. 2) are under clinical investigation in patients with ER[+] breast cancer. Despite this positive clinical outcome, not all cancer patients benefit from CDK4/6 inhibition and a significant fraction of them eventually progress, underscoring the need to develop potent therapeutic combinations that circumvent drug resistance.

In seeking more effective rational drug combinations with CDK4/6 inhibitors, we utilized a high-throughput siRNA screen to identify kinases that, when inhibited, increased sensitivity to CDK4/6 inhibition. We identified upregulation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) as a mechanism of adaptation and eventual resistance to ribociclib. PDK1 has been implicated in important cellular processes including survival, metabolism, and tumorigenesis. PDK1 is highly expressed in

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many human cancer cell lines (6) and breast tumors (7), suggesting a role for PDK1 in cancer progression. In a cohort of patients with ER[+] breast cancer treated with endocrine therapy, high PDK1 expression was shown to predict for poor survival (8, 9). PDK1 functions downstream of PI3K and is required for the full activation of AKT (10) and other AGC kinases including serum glucocorticoid-dependent kinase (SGK), p90 ribosomal protein S6 kinase (RSK), p70 ribosomal protein S6 kinase (S6K), protein kinase C (PKC), and polo-like kinase 1 (PLK1; refs. 11–13). At the plasma membrane, PDK1 binds via its pleckstrin homology (PH) domain to phosphatidylinositol 3,4,5 trisphosphate (PIP3), the product of PI3K, where it phosphorylates and activates AKT at T308 (10, 11, 14). For substrates of PDK1 lacking a PH domain, such as S6K, RSK, and SGK, the interaction and subsequent activation depends on the hydrophobic motif of the target kinase binding to the PDK1-interacting fragment (PIF) rather than with PIP3 (15). Several PDK1-specific small molecules are in clinical development for the treatment of advanced cancers (16–18). On the basis of these data, we hypothesized that targeting the PI3K/PDK1 pathway in combination with CDK4/6 inhibitors will prolong the response to CDK4/6 inhibition and provide a novel treatment option for patients with ER[+] metastatic breast cancer.

Herein, we demonstrate that genetic and pharmacologic inhibition of PI3K/PDK1 in combination with CDK4/6 inhibition synergistically blocked cell proliferation and increased apoptosis of ER[+] breast cancer cells *in vitro* and *in vivo*. In ribociclib-resistant cell lines, we observed that the PI3K/PDK1 pathway mediates cell survival and proliferation through upregulation of AKT and non-AKT targets of PDK1, all of which culminates in aberrant cell-cycle progression in the presence of CDK4/6 inhibition. Inhibition of PDK1 with the small-molecule GSK2334470 resensitized ribociclib-resistant cells to CDK4/6 inhibitors. These results provide a rationale for cotargeting of the PI3K/PDK1 and CDK4/6 pathways in patients with ER[+] metastatic breast cancer.

Materials and Methods

Cell lines and reagents

Parental lines were obtained from ATCC within the past 10 years (2006–2014) and maintained in 10% FBS (Gibco). Cell lines were authenticated by ATCC prior to purchase by the short tandem repeat method. Cell lines were not authenticated after purchase. Mycoplasma testing was conducted for each cell line before use. All experiments were performed less than 2 months after thawing early passage cells. All experiments were performed in Iscove's modified Dulbecco's medium (IMEM)/10% FBS/0.002% phenol red under estrogen-containing conditions unless otherwise noted. To generate ribociclib-resistant ER[+] cell lines, MCF-7, T47D, HCC1428, and HCC1500 cells were cultured in the presence of progressively increasing concentrations of ribociclib starting at 10 nmol/L. Cells were deemed resistant when they grew at the same rate as parental cells in 1,000 nmol/L of ribociclib. For the experiments outlined herein, resistant cells were removed from drug for at least 24 to 48 hours prior to retreatment. Ribociclib and alpelisib were obtained through a materials transfer agreement (MTA) with Novartis. Fulvestrant was obtained from the Vanderbilt Chemotherapeutic Pharmacy. GSK2334470 and palbociclib were obtained from Selleckchem. Abemaciclib (LY2835219) was obtained from MedChemExpress. Primary antibodies for immunoblot analyses were from Cell Signaling Technology

except for cyclin D1 (Santa Cruz Biotechnology, M-20; sc-718). The S477/T479 P-AKT antibody was a kind gift from Dr. Wenyi Wei (Harvard Medical School, Boston, MA; ref. 19).

IHC

Primary antibodies for IHC were from Cell Signaling Technology. See Supplementary Materials for assay details.

RNAi screen

MCF-7 cells were screened using the Dharmacon Human siGENOME Protein Kinase siRNA Library (GU-003505) available through the Vanderbilt high-throughput screening (HTS) facility as described in Supplementary Methods. Secondary validation was performed with two independent siRNAs against PDK1. siPDK1.1: GUGAGGAAAUGGAAGGAUAUU; siPDK1.2: CAAGAGACCUCGUGGAGAAUU.

Cell proliferation assays

Cells were seeded in IMEM/10% FBS for proliferation in two-dimensional (2D) growth assays and counted or fixed/stained with crystal violet as described previously (20). Media and inhibitors were replenished every 2 to 3 days; after 10 to 21 days, adherent cells were trypsinized and counted using a Coulter Counter or fixed/stained with crystal violet followed by image analysis of the plates using an Odyssey Infrared Imaging System (LI-COR Biosciences). For siRNA experiments, cells were transfected with siRNAs targeting PDK1.1, PDK1.2, CDK4 (Santa Cruz Biotechnology), or a nonsilencing control (Santa Cruz Biotechnology) using Lipofectamine RNAiMax Transfection Reagent (Invitrogen) according to the manufacturer's protocol. The next day, cells were reseeded for proliferation assays and/or immunoblot analyses as described previously (20). Three-dimensional (3D) growth assays were conducted in growth factor–reduced Matrigel (BD Biosciences) as described previously (21). Phase-contrast pictures were taken using an Olympus CK40 microscope and colonies were counted using the GelCount scanning software.

β-Galactosidase staining

Cells (2×10^5) were plated in triplicate in 6-well plates and treated with DMSO, 1 μmol/L ribociclib, 1 μmol/L GSK2334470, or the combination for 72 hours. Cells were stained with β-galactosidase at pH 6.0 following the manufacturer's protocol (Cell Signaling Technology, #9860). Cells were photographed and β-galactosidase–positive cells were counted using a light-field microscope.

Flow cytometry

Cells (1×10^6) were plated in serum-free media and treated 24 hours later with inhibitors. For cell-cycle analyses, cells were treated for 24 hours, then washed with PBS and fixed in 99% methanol for 3 hours at $-20°C$. Cells were incubated with 0.1 mg/mL RNase A (Qiagen) and 40 μg/mL propidium iodide (PI; Sigma-Aldrich) for 10 minutes at room temperature. For apoptosis assays, cells were treated for 72 hours and then washed and stained using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit according to the manufacturer's protocol (Thermo Scientific). FACS analysis was performed on the LSRFortessa X-20 Cell Analyzer (BD Biosciences) and the data were analyzed with FlowJo software.

Immunoblot analysis

Cells were lysed with RIPA buffer [150 mmol/L NaCl, 1.0% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris, pH 8.0 (Sigma), and 1× protease inhibitor cocktail (Roche)]. Lysates (20 µg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes; these were first incubated with primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Thermo Scientific).

Xenograft studies

Mouse experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee. Female ovariectomized athymic mice (Harlan Sprague Dawley) were implanted with a 14-day-release 17β-estradiol pellet (0.17 mg; Innovative Research of America). The following day, 1×10^7 MCF-7 cells suspended in IMEM and Matrigel (BD Biosciences) at 1:1 ratio were injected subcutaneously into the right flank of each mouse. Approximately 4 weeks later, mice bearing tumors measuring ≥ 150 mm^3 were randomized to treatment with (i) vehicle (control), (ii) ribociclib (75 mg/kg/day via
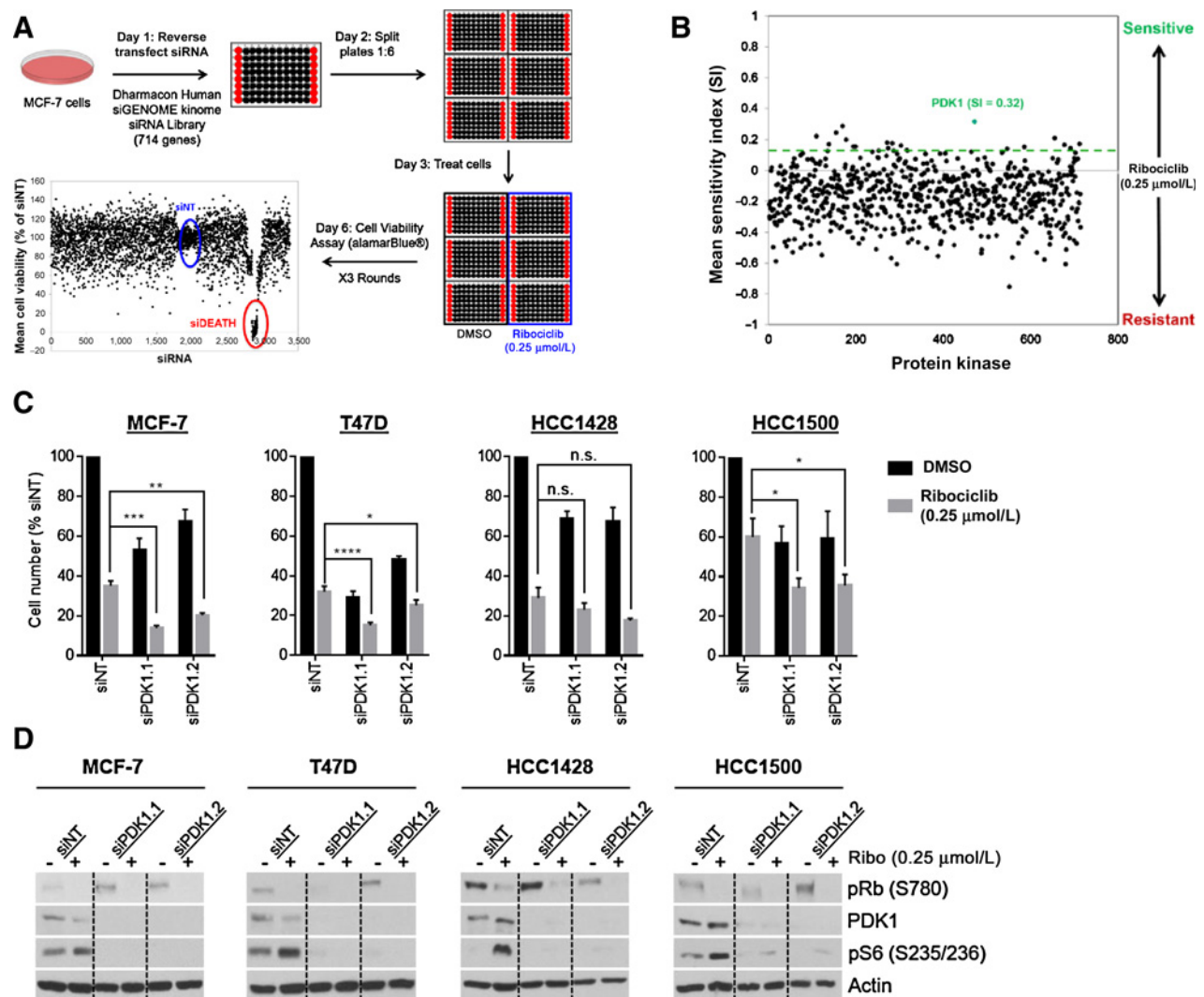
Figure 1.
Kinase screen identifies PDK1 siRNA as a sensitizer to CDK4/6 inhibitor. **A,** Overview of HTS method. MCF-7 cells were reverse transfected with siRNA in 96-well plates. Each plate contained 80 individual siRNAs, indicated in black, and supplemented with controls, indicated in red (no siRNA, siNT, and siDEATH–positive control). Transfected cells were divided into six replicate plates. Half of the plates (*n* = 3) were treated with DMSO (vehicle control) and half (*n* = 3) with 0.25 µmol/L of ribociclib. Cell viability was assessed after 72 hours of drug exposure using the AlamarBlue reagent (Invitrogen). The experiment was repeated three times. **B,** Scatter plot of the mean SI scores for 714 protein kinases and kinase-related proteins averaged across the three screening trials. A cutoff SI > 0.15 (indicated by dotted line) was used for hit selection. The position of PDK1 (SI score 0.32) is noted. **C,** ER$^+$ MCF-7, T47D, HCC1428, and HCC1500 breast cancer cell lines were transfected with one of two siRNAs targeting PDK1 (siPDK1.1 and siPDK1.2), and a siNT and treated with DMSO (vehicle control) or 0.25 µmol/L ribociclib for 72 hours. Knockdown of PDK1 decreased cell proliferation and this effect was enhanced upon simultaneous treatment with ribociclib. **D,** Immunoblot analyses of the cells following PDK1 knockdown and treatment for 72 hours with DMSO or 0.25 µmol/L ribociclib. Experiments were performed in full serum condition in the presence of endogenous estrogen (in IMEM/10% FBS/0.002% phenol red).

orogastric gavage), (iii) GSK2334470 (100 mg/kg three times per week via intraperitoneal injection), or (iv) both drugs. In a second animal experiment, mice harboring MCF-7 xenografts as above were treated with (i) vehicle, (ii) fulvestrant (5 mg per week; s.c.), (iii) fulvestrant and alpelisib (BYL719; Novartis; 35 mg/kg/day via orogastric gavage), (iv) fulvestrant and ribociclib (as above), or (v) fulvestrant, alpelisib, and ribociclib for 6 weeks. Animal weights and tumor diameters (with calipers) were measured twice weekly and tumor volume was calculated with the formula: volume = width^2 × length/2. After 6 weeks, tumors were harvested and snap-frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin followed by embedding in paraffin for IHC. Tumors were harvested 4 hours after the last dose of ribociclib or alpelisib or 24 hours after the last dose of GSK2334470 or fulvestrant. Frozen tumors were homogenized using the TissueLyser II (Qiagen).

Statistical analyses

Unless otherwise indicated, significant differences ($P < 0.05$) were determined by ANOVA using GraphPad Prism software.

Results

PDK1 siRNA oligonucleotides sensitize ER$^+$ breast cancer cells to CDK4/6 inhibitors

We used an arrayed siRNA library targeting 714 kinases and related proteins to identify targetable molecules whose down-regulation in combination with a CDK4/6 inhibitor would induce synthetic lethality in ER$^+$ MCF-7 breast cancer cells (Fig. 1A). Following siRNA transfection, in half of the plates ($n = 3$), cells were treated with an IC$_{20}$ concentration of ribociclib (0.25 μmol/L) and the other half with vehicle (DMSO). We chose to administer an IC$_{20}$ concentration to cells so that the synergy between drug and siRNA-mediated gene knockdown would be more apparent. Cell viability was measured in triplicate 6 days later using a high-throughput cell viability assay. Experiments were performed three times to allow for assessment of variation of viability data in statistical analysis. Data among the biological replicates were highly reproducible and ultimately combined for the final analysis and hit selection (Supplementary Fig. S1A).

To account for plate-to-plate variability, median-centered global normalization was performed across all siRNAs against the nontargeting control siRNAs (siNT) in each plate. The sensitivity index (SI) score, which measures the influence of siRNA-induced gene knockdown on drug sensitivity, was calculated for each siRNA in each of the experiments after treatment with ribociclib (Supplementary Fig. S2; Fig. 1B), as previously described (22–24). The SI scores range from –1 to +1, with positive values indicating sensitizing effects. The Z-score was calculated from the SI values. Genes with an SI ≥ 0.15 and a Z-score of >3 were considered significantly altered. In brief, individual knockdown of 15 of 714 (2.1%) kinases sensitized MCF-7 cells to ribociclib (Fig. 1B; Table 1). PDK1 was identified as the top siRNA sensitizing MCF-7 cells to ribociclib (SI = 0.32). Notably, the most sensitizing siRNAs were those that alone had minimal effect on cell viability. Addition of ribociclib significantly decreased cell viability compared with the nontargeting control, consistent with a synergistic interaction. Some siRNAs, like PIK3CA for example, inhibited cell viability per se, such that the addition of ribociclib could not reduce viability any further, as indicated by an SI score of 0.08.

Table 1. Top siRNAs that most significantly sensitized MCF-7 cells to ribociclib as compared with nonsensitizing PIK3CA siRNA

Rank	Gene	SI value[a]	Z-score[b]	Rc/Cc[c]	Rd/Cc[d]
1	PDK1	0.32	4.60	0.93 ± 0.12	0.68 ± 0.15
2	DLG1	0.29	4.36	0.98 ± 0.10	0.73 ± 0.12
3	DCAMKL1	0.25	4.08	0.93 ± 0.19	0.69 ± 0.21
4	CDKN1B	0.22	4.56	0.90 ± 0.26	0.65 ± 0.18
6	DYRK1A	0.20	3.79	1.00 ± 0.10	0.73 ± 0.09
7	HUNK	0.18	3.50	0.80 ± 0.22	0.59 ± 0.20
8	ILK	0.18	3.02	0.89 ± 0.17	0.68 ± 0.17
9	YES1	0.17	3.61	0.84 ± 0.20	0.64 ± 0.15
10	CRKL	0.17	3.94	0.84 ± 0.23	0.59 ± 0.20
11	TLK1	0.17	3.10	1.00 ± 0.17	0.79 ± 0.18
12	KCNH8	0.16	4.40	0.82 ± 0.20	0.59 ± 0.09
13	EPHA4	0.16	3.06	0.95 ± 0.18	0.74 ± 0.20
14	EPHB1	0.16	3.15	0.92 ± 0.16	0.68 ± 0.18
15	RELA	0.15	3.64	0.84 ± 0.32	0.64 ± 0.27
16	ICK	0.15	3.64	0.91 ± 0.18	0.67 ± 0.17
37	PIK3CA	0.09	3.57	0.51 ± 0.12	0.38 ± 0.10

NOTE: Data represent the mean of three different experiments performed in triplicate. Genes with an SI ≥0.15 and a Z-score >3 were considered significant. The concentration of ribociclib (0.25 μmol/L) used in the screen corresponded to an inhibitory concentration of 20% (IC$_{20}$).
[a]SI value = Expected combined effect − Observed combined effect.
[b]Z-score was calculated as follows: Z-score = (siRNA SI score − mean of all siRNAs in that plate)/(standard deviation of all siRNAs in that plate).
[c]Rc/Cc = the viability effect of siRNA without drug compared to siNT control.
[d]Rd/Cc = the viability effect of siRNA with drug compared to siNT control.

To validate the results of the screen, we knocked down PDK1 using two independent siRNAs, each in combination with 0.25 μmol/L ribociclib, in MCF-7, T47D, HCC1428, and HCC1500 ER$^+$ breast cancer cells. Individually, ribociclib treatment and PDK1 siRNA transfection inhibited proliferation of all four cell lines (Fig. 1C). However, combined inhibition of CDK4/6 (with ribociclib) and of PDK1 (with siRNA) led to a statistically significant reduction in cell proliferation in MCF-7, T47D, and HCC1500 cell lines, consistent with the results of the kinome screen. This effect was greater in PIK3CA-mutant cell lines (MCF-7 and T47D) than PIK3CA wild-type cell lines (HCC1428 and HCC1500). Knockdown of PDK1 resulted in decreased phosphorylation of S6, a downstream effector of the PDK1 target p70S6K (Fig. 1D; Supplementary Fig. S1B). To subscribe CDK4 specificity to the effects of ribociclib, we treated MCF-7 cells with CDK4 and PDK1 siRNA oligonucleotides, individually and in combination. Treatment with both siRNAs inhibited cell viability more potently than each alone while simultaneously reducing levels of PDK1, CDK4, and P-Rb (Supplementary Fig. S1C), suggesting the effects of ribociclib may extend to other CDK4/6 inhibitors.

Pharmacologic blockade of PDK1 and CDK4/6 synergistically inhibits ER$^+$ breast cancer cell proliferation

We next examined the effect of pharmacologic inhibition of PDK1 in combination with CDK4/6 inhibitors. GSK2334470 is a highly specific small-molecule inhibitor of PDK1 with a published inhibitory activity in the nanomolar range (16, 25). GSK2334470 suppresses T-loop phosphorylation and subsequent activation of the PDK1 substrates AKT, S6K, RSK2, and SGK in vitro. Treatment of MCF-7 and T47D cells with GSK2334470 resulted in a dose-dependent decrease in phosphorylation of known PDK1 substrates and downstream signal transducers such as P-S6 and P-PRAS40 (Fig. 2A). Growth of MCF-7, T47D, and HCC1500 was inhibited by ribociclib and

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GSK2334470 alone in a dose-dependent fashion; however, treatment with the combination resulted in greater growth inhibition (Fig. 2B). By the Chou–Talalay (26, 27) method, the effect of drug combination was synergistic in the cell lines we examined (Supplementary Fig. S3A). It has been proposed that results in 2D cell culture may not accurately reflect the *in situ* architecture and growth rates of cancers *in vivo* (28–30). Thus, we next extended our findings to cells growing in Matrigel in 3D culture. Under these conditions, GSK2334470 enhanced the antiproliferative effect of both ribociclib and palbociclib against MCF-7, T47D, and HCC1500 cells (Fig. 2C). Of note, the combined effect of CDK4/6 and PDK1 inhibitors in *PIK3CA* wild-type HCC1428 and HCC1500 cells was less pronounced than in *PIK3CA*-mutant MCF-7 and T47D cells despite similar reduction of P-Rb and P-S6 (Fig. 2D).

Combination therapies with CDK4/6 inhibitors are also being evaluated in other advanced solid tumors (REF 31). To test whether these findings in ER+ breast cancer cells can be translated to other tumor types, we treated triple-negative breast cancer, ovarian/endometrial, melanoma, and glioblastoma cell lines with ribociclib, GSK23334470, or the combination. Results showed that the combination induced greater inhibition of cell viability compared with each drug alone (Supplementary Fig. S3B and S3C). These observations suggest that PDK1 plays a role in mediating resistance to CDK4/6 inhibition in a variety of tumor types where CDK4/6 inhibitors are being investigated clinically (31).

In addition to cell-cycle arrest, CDK4/6 inhibitors can induce senescence through regulation of FoxM1-mediated transcription (32). Consistent with this, we observed a decrease in FoxM1 levels and an increase in senescence-associated (SA)-β-galactosidase–positive cells upon treatment with ribociclib, which was unaffected by the PDK1 inhibitor (Fig. 2E and F). Treatment with GSK2334470 alone or in combination with ribociclib induced apoptosis as measured by increased Annexin V staining (Fig. 2G) and PARP cleavage (Fig. 2H), compared with DMSO- or ribociclib-treated MCF-7 cells. These findings suggest that inhibition of PDK1 with GSK2334470 induces apoptosis without counteracting the effect of ribociclib on tumor cell senescence, resulting in the synergistic growth inhibition of ER+ breast cancer cells.

Inhibition of PI3K/PDK1 enhances the antitumor effect of ribociclib *in vivo*

To validate the efficacy of combined inhibition of CDK4/6 and PDK1 *in vivo*, we established MCF-7 xenografts in athymic ovariectomized female nude mice supplemented with a 14-day release, 17β-estradiol pellet to support initial tumor establishment. Once tumors reached a volume of ≥ 150 mm^3, and the estrogen pellet had expired, a state mimicking estrogen deprivation in patients treated with anti-estrogens, mice were randomized to treatment with vehicle, ribociclib, GSK2334470, or the combination of both drugs. After 6 weeks of treatment, the combination induced a statistically superior antitumor effect compared with each drug alone (Fig. 3A). The combination of ribociclib and GSK2334470 induced clear objective tumor regressions as early as 2 weeks in all mice with three xenografts treated with the combination achieving a complete response. After 6 months of follow-up on no therapy, none exhibited a tumor recurrence (Supplementary Fig. S4A). During treatment, mice in all four groups displayed stable body weight (Supplementary Fig. S4B) and no signs of toxicity.

We next assessed pharmacodynamic biomarkers of drug action by IHC of tumor sections and immunoblot analysis of tumor lysates. Treatment with ribociclib modestly increased S235/236 P-S6 after 6 weeks of treatment, whereas GSK2334470 alone or in combination with ribociclib reversed this effect (Fig. 3B and C). Both agents decreased Rb phosphorylation at the S780 CDK4 site (33), whereas treatment with GSK2334470 alone or in combination with ribociclib decreased T308 P-AKT (Fig. 3C). These data suggest that activation of S6 compensates for inhibition of CDK4/6 and that the combined blockade of PDK1 and CDK4/6 limits such activation.

PI3K activates PDK1, which then activates AKT and mTOR to increase tumor cell proliferation and survival (11). Although PDK1 inhibitors remain in preclinical development (16–18), pharmacologic blockade of the PI3K/PDK1 signaling pathway can be achieved with PI3K inhibitors currently in registration clinical trials (34). Furthermore, because CDK4 inhibitors are approved for use in combination with anti-estrogens in ER+ breast cancer (1, 4), we extended our findings to such combination ± the PI3Kα-specific inhibitor alpelisib (BYL719; ref. 35) in ovariectomized nude mice bearing ER+ MCF-7 xenografts (Fig. 3D). Similar to the results with GSK2334470, addition of alpelisib markedly increased the antitumor effect of fulvestrant/ribociclib against established MCF-7 xenografts and inhibited ER, PR, and S473 P-AKT (Fig. 3D–F).

CDK4/6 inhibition results in upregulation of phosphorylated PDK1

PDK1 siRNA was the top hit sensitizing MCF-7 cells to ribociclib in the siRNA kinome screen (Fig. 1B; Table 1). Furthermore, pharmacologic inhibition of PDK1 markedly enhanced the antitumor effect of ribociclib *in vivo* while blocking S6 activation observed in tumors treated with ribociclib alone (Fig. 3A–C), suggesting that PDK1 function counteracts the response to CDK4/6 inhibitors. To test this possibility, we examined the expression of PDK1 and its known targets after short- and long-term treatment with CDK4/6 inhibitors. In MCF-7 cells treated with ribociclib, we observed an induction in total PDK1 protein levels followed by subsequent increase in S241 P-PDK1 and concomitant increase in the PDK1 targets: S227 P-RSK2, T227 P-70S6K, T320 P-SGK3, and cyclin D1 (Fig. 4A). The levels of PDK1 mRNA were variable with ribociclib treatment; however, there was a statistically significant increased seen after 72 hours of treatment (Supplementary Fig. S5A), suggesting the induction of PDK1 requires, in part, new mRNA synthesis. In contrast, treatment with palbociclib and abemaciclib showed an induction of S241 P-PDK1 as early as 1 hour after drug exposure (Supplementary Fig. S5B) without an increase in total PDK1 protein. We also examined PDK1 mRNA and protein levels in HCC1428, HEC1B, and LN229 to determine the effects of CDK4/6 inhibition in different cancer types. HCC1428 did not show an induction of PDK1 mRNA or protein, whereas HEC1B, a human endometrial adenocarcinoma cell line, showed an increased in PDK1 mRNA but not PDK1 protein. In contrast, the glioma cell line LN229 showed an increase in PDK1 mRNA with concomitant increase in P-PDK1 levels, but not total PDK1 (Supplementary Fig. S5C). These results suggest that there is heterogeneity in the response to CDK4/6 inhibition across breast cancer cell lines and different cancer types.

To extend these findings to primary human cancers, we examined tumor explants obtained from patients with newly diagnosed ER+ breast cancer undergoing surgical resection. Tumor
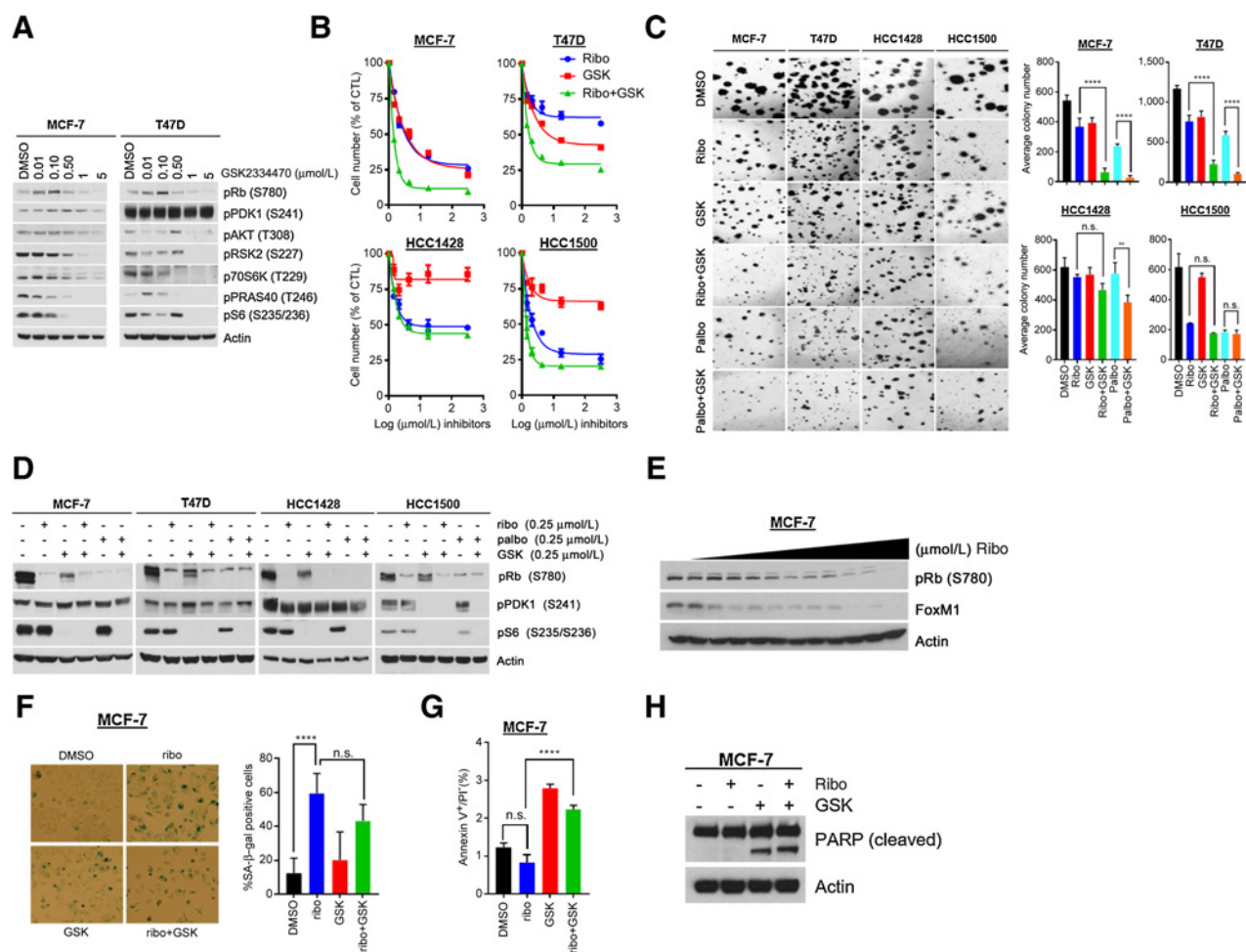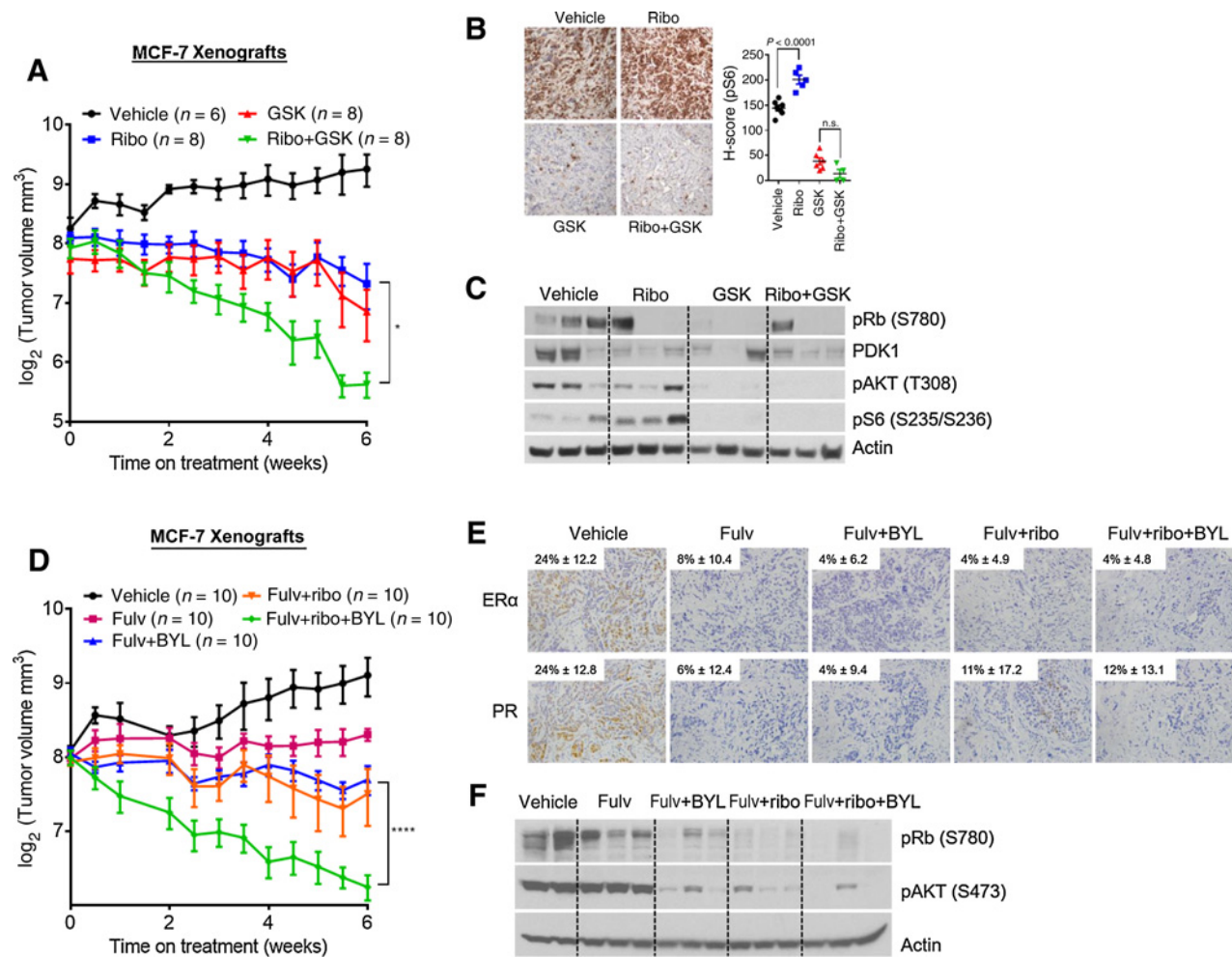
Figure 2.
Combined PDK1 and CDK4/6 inhibition reduces ER^+ breast cancer cell proliferation. **A,** ER^+ MCF-7 and T47D cells were treated for 24 hours with DMSO or increasing concentrations of GSK2334470. Lysates were prepared for immunoblot analysis with the indicated antibodies. GSK2334470 treatment diminished the expression of downstream targets of PDK1. **B,** Cells were treated with DMSO or increasing concentrations of ribociclib, GSK2334470, or the combination for 7 to 10 days, after which nuclei were stained with DAPI and counted on the Molecular Devices ImageXpress instrument. Data are presented as the percent of cells remaining compared with the control (CTL, DMSO-treated). In all cell lines, combined inhibition of PDK1 and CDK4/6 was most effective than each drug alone. **C,** Cells seeded in Matrigel were treated with DMSO or 0.25 μmol/L ribociclib ± 0.25 μmol/L GSK2334470. After 10 to 21 days, colonies were stained with the MTT reagent, photographed (left), and counted (right) using the GelCount reader. In MCF-7 and T47D cells, specifically, combined inhibition of PDK1 and CDK4/6 was more effective than single-agent inhibition. **D,** Cells were treated with DMSO, 0.25 μmol/L ribociclib ± 0.25 μmol/L GSK2334470, or 0.25 μmol/L palbociclib ± 0.25 μmol/L GSK2334470 for 24 hours, after which lysates were prepared for immunoblot analysis with the indicated antibodies. Only combined inhibition of PDK1 and CDK4/6 led to concomitant decreases in P-Rb and P-S6. **E,** MCF-7 cells were treated for 72 hours with DMSO or increasing concentrations of ribociclib. Immunoblot analysis of the lysates showed that ribociclib decreased P-Rb and FoxM1 levels. **F,** MCF-7 cells were treated with DMSO or 1 μmol/L ribociclib ± 1 μmol/L GSK2334470 for 72 hours and analyzed for senescence by β-galactosidase staining. Ribociclib alone or in combination with GSK2334470 induced senescence compared with DMSO-treated cells. Data represent the average percent of SA-β-galactosidase–positive cells per 5 high-power fields. **G,** MCF-7 cells were treated with DMSO or 1 μmol/L ribociclib ± 1 μmol/L GSK2334470 for 72 hours, stained with Annexin V and PI, and analyzed by FACS. GSK2334470 alone or in combination with ribociclib increased the percent of apoptotic cells compared with DMSO-treated cells. **H,** MCF-7 cells were treated with DMSO or 1 μmol/L ribociclib ± 1 μmol/L GSK2334470 for 72 hours. Immunoblot analysis of these lysates revealed PARP cleavage only when cells were treated with GSK2334470 alone or in combination with ribociclib. Unless noted, media and drugs were replenished every 2 to 3 days (**, $P < 0.01$; ****, $P < 0.0001$ by ANOVA).

explants were treated *ex vivo* with palbociclib for 48 hours. Consistent with the data with cell lines, treatment with palbociclib resulted in an increase in PDK1, S235/236 P-S6, and cyclin D1 levels in 2 of 3 primary tumor explants (Fig. 4B). Similarly, serial tumor samples from two patients with metastatic breast cancer treated with palbociclib for 7 days as part of a clinical trial (NCT01522989) showed an increase in PDK1, S235/236 P-S6, and cyclin D1 levels compared with the baseline (pretreatment) biopsy (Fig. 4C).

We also treated MCF-7, T47D, HCC1428, and HCC1500 cells long-term with progressively increasing concentrations (up to 1 μmol/L) of ribociclib to develop cells that may recapitulate the acquired resistance observed in patients treated with CDK4/6 inhibitors. The ribociclib-resistant cells (MCF-7/RR, T47D/RR, HCC1428/RR, and HCC1500/RR) exhibited an IC_{50} at least 20-fold higher than that of their parental counterparts and displayed cross-resistance to the CDK4/6 inhibitors palbociclib and abemaciclib (Supplementary Fig. S5D). The

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Figure 3.
Pharmacologic inhibition of PI3K/PDK1 enhances the effect of ribociclib *in vivo*. **A,** MCF-7 cells were injected subcutaneously into athymic ovariectomized female mice, each supplemented with a short-term, 14-day release 17β-estradiol pellet. Mice bearing tumors ≥150 mm^3 were randomized to vehicle, ribociclib, GSK2334470, or the combination of ribociclib and GSK2334470 for 6 weeks. Data are presented as log$_2$ of mean tumor volume in mm^3 (*, $P < 0.05$ vs. single-agent ribociclib or GSK2334470). Numbers in parenthesis represent the number of mice per treatment arm. **B,** Representative images of tumor sections from **A** and quantitative comparison of P-S6 histoscores (H-score). GSK2334470 ± ribociclib inhibited P-S6; single-agent ribociclib increased P-S6 levels. **C,** Xenografts from **A** were homogenized after the last dose of drug treatment and tumor lysates were subjected to immunoblot analysis for the indicated antibodies. **D,** MCF-7 cells were injected into mice as in **A**. Mice bearing tumors ≥150 mm^3 were randomized to vehicle, fulvestrant, BYL719 and fulvestrant, ribociclib and fulvestrant, or fulvestrant, BYL719, and ribociclib for 6 weeks. The triple combination was most effective at decreasing tumor volume compared with single-agent therapy or double combinations. Data are presented as log$_2$ of mean tumor volume in mm^3 (****, $P < 0.0001$ vs. fulvestrant, fulvestrant and BYL719, or fulvestrant and ribociclib). **E,** Representative images of tumor sections from **D** and quantitative comparison of ER and PR histoscores (H-score) confirming target inhibition with fulvestrant. **F,** Xenografts from **D** were homogenized after the last dose of drug treatment and tumor lysates were subjected to immunoblot analysis for the indicated antibodies.

increased content of S241 P-PDK1 observed upon short-term treatment was sustained in the resistant cell lines (Fig. 5A). Furthermore, immunoblot analysis of ribociclib-resistant cells showed incomplete inhibition of Rb phosphorylation in the presence of drug and increased levels of S227 P-RSK2 (target of PDK1), T308 P-AKT (target of PDK1), S235/236 P-S6 (downstream effector of the PDK1-activated kinase P70S6K) compared with parental drug–sensitive cells (Fig. 5A). Cell-cycle analysis revealed that CDK4/6 inhibition failed to induce G$_1$ cell-cycle arrest and a reduction in S-phase in MCF-7/RR and T47D/RR as compared with MCF-7 and T47D parental cell lines (Fig. 5B and C). Consistent with these observations, MCF-7/RR and T47D/RR cells continued to proliferate in the presence of

ribociclib (Supplementary Fig. S5E). Importantly, however, genetic and pharmacologic inhibition of PDK1 in combination with ribociclib resensitized MCF-7/RR and T47D/RR cells to ribociclib (Supplementary Fig. S6A and S6B; Fig. 5D). We also examined inhibitors along the PI3K/AKT/mTOR signaling pathway in combination with ribociclib and observed similar growth inhibition of MCF-7/RR and T47D/RR cells as with combined PDK1 and CDK4/6 inhibition (Supplementary Fig. S6C). Furthermore, combined CDK4/6 and PDK1 inhibition significantly reduced the percentage of ribociclib-resistant cells in S-phase (Fig. 5C), which, unlike in parental cells, was relatively unaffected by either single agent. These results suggest that enhanced PDK1 expression and PI3K/PDK1/AKT/
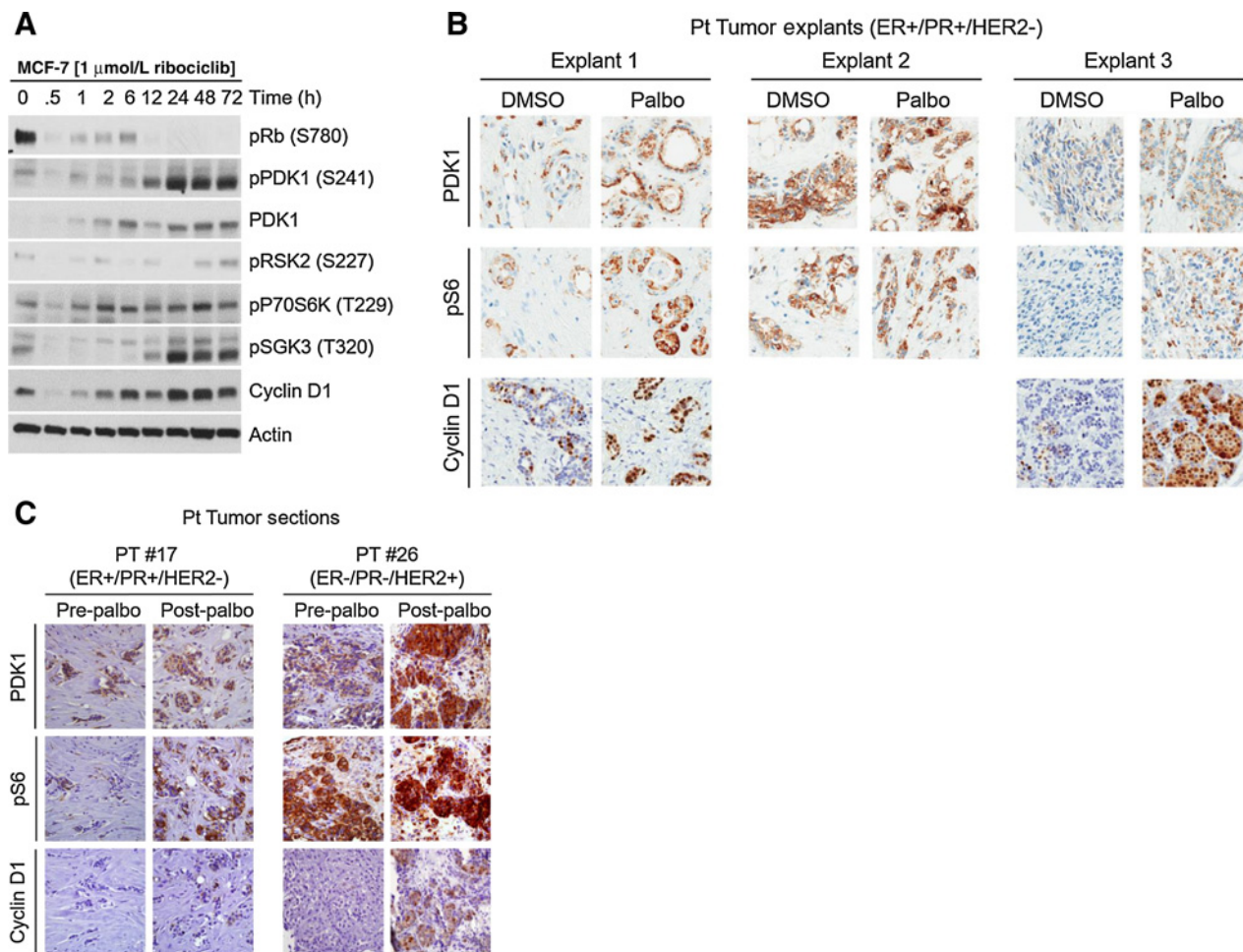
Figure 4.
CDK4/6 inhibition increases PDK1 expression in ER$^+$ breast cancer cells and in primary tumor explants. **A,** MCF-7 cells were treated with ribociclib over a time course up to 72 hours. Cell lysates were prepared and subjected to immunoblot analyses with the indicated antibodies as described in Materials and Methods. **B,** Patient tumor explants were treated with DMSO or palbociclib for 48 hours. Representative IHC for PDK1, S235/236 P-S6, and cyclin D1 is shown. Tumor explant 2 exhibited high basal levels of PDK1 and P-S6, whereas explants 1 and 3 exhibited drug-induced increases in PDK1, P-S6, and cyclin D1 levels. **C,** PDK1, P-S6, and cyclin D1 IHC analysis of serial primary tumor sections from two patients before treatment and on the 7th day of treatment with palbociclib.

mTOR signaling mediate acquired resistance to CDK4/6 inhibition by maintaining progression through the cell cycle.

PDK1 promotes cell-cycle progression in CDK4/6-resistant cell lines through increased CDK2/cyclin E/cyclin A

Phosphorylated PDK1 levels were increased upon CDK4/6 inhibition (Fig. 4A; Supplementary Fig. S5B). Ribociclib-resistant cells progressed through the cell cycle in presence of ribociclib (Fig. 5B and C). This continued progression into S-phase suggested the ribociclib-resistant cells may exhibit increased or sustained expression of S-phase cyclins and/or CDKs. Indeed, cyclin A, cyclin E, and activated T160 P-CDK2 levels were reduced upon ribociclib treatment in parental drug–sensitive cells but not, or not as potently, inhibited in all three drug-resistant cell lines (Fig. 6A). In addition, MCF-7, T47D, and HCC1428 ribociclib-resistant cells exhibited sustained phosphorylation of AKT at S477/T479 (Fig. 4A), a CDK2-dependent phosphorylation site required for full kinase activity, which is limited to the S-phase of the cell cycle (19). Consistent with recent studies (36), we also

observed sustained increased expression of cyclin D1 in all three cell lines with acquired resistance to CDK4/6 inhibition (Fig. 6A).

CDK2 regulates cell-cycle progression through its interactions with both cyclin E and cyclin A. CDK2/cyclin E kinase activity is important for the G_1 to S transition and phosphorylation of Rb (37). During S-phase and persisting through G_2, active CDK2/cyclin A complexes phosphorylate E2F to promote transcription (38). To determine whether CDK2 promotes cell survival and proliferation in the context of CDK4/6 inhibitor resistance, we treated our ribociclib-resistant cells with dinaciclib (SCH 727965), a potent inhibitor of CDK2, CDK5, CDK1, and CDK9 with IC$_{50}$ of 1, 1, 3, and 4 nmol/L in cell-free assays, respectively (39). MCF-7/RR, T47D/RR, and HCC1428/RR cells continued to proliferate in the presence of ribociclib and also showed relative insensitivity to dinaciclib (Fig. 6B). Inhibition of PDK1 with GSK2334470 or of CDK2 with dinaciclib resensitized the drug-resistant cells to ribociclib (Fig. 6B). The combination of ribociclib/GSK2334470 inhibited MC-7/RR, T47D/RR, and HCC1428/RR cell proliferation more potently than ribociclib/dinaciclib.

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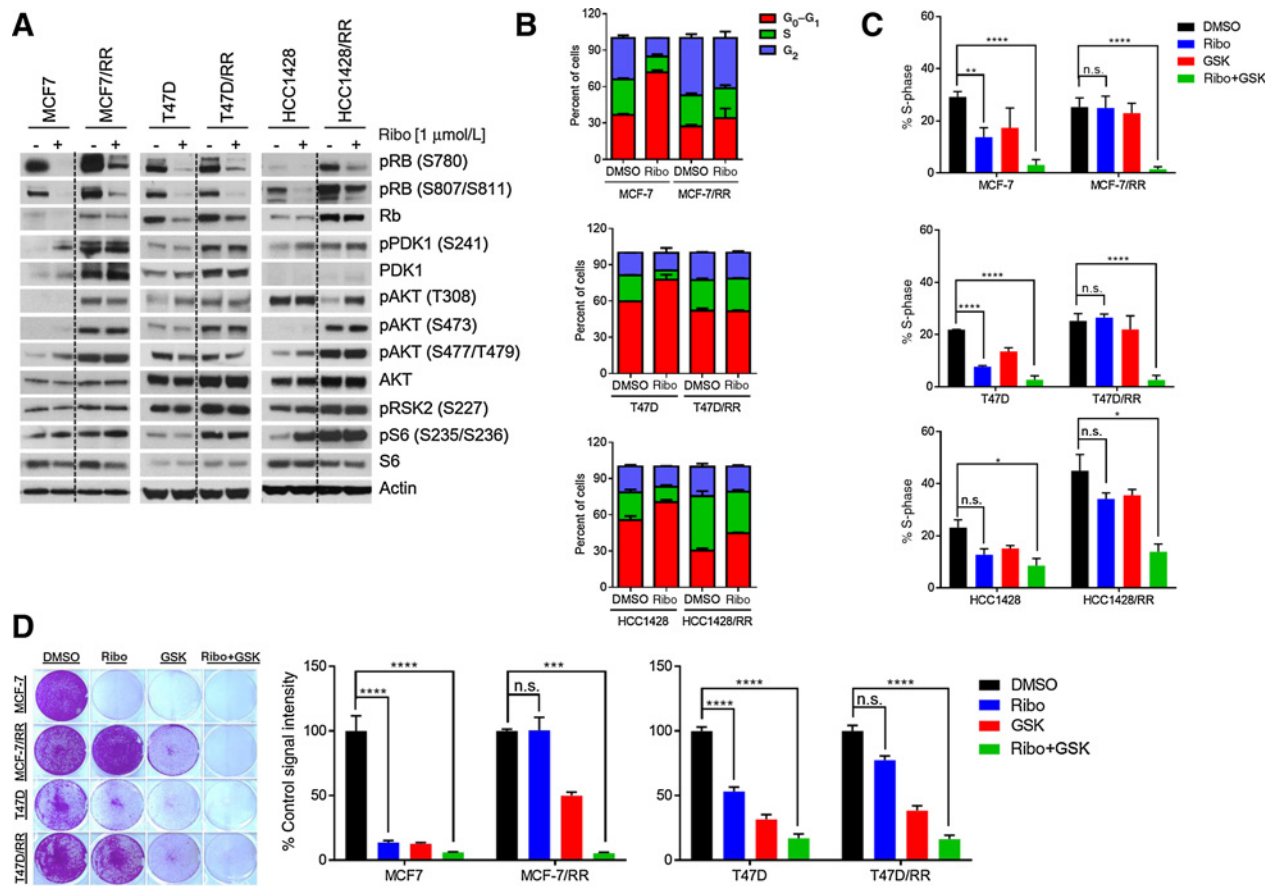
Figure 5.
PDK1 inhibition restores sensitivity to CDK4/6 blockade in drug-resistant cells. **A,** Lysates from parental MCF-7, T47D, HCC1428, and ribociclib-resistant cells treated $\pm$ 1 μmol/L ribociclib were analyzed by immunoblot with the indicated antibodies. Resistant cells were removed from drug for 24 hours prior to ribociclib treatment for this analysis. **B,** Parental and ribociclib-resistant (RR) cells were serum starved for 24 hours, treated with 1 μmol/L ribociclib for 24 hours, stained with propidium iodide, and then analyzed by FACS. **C,** Cells were serum starved for 24 hours as in **A**, treated with drugs for 24 hours, stained with propidium iodide, and then analyzed by FACS. In all cases, combined treatment with ribociclib and GSK2334470 markedly reduced the percent of cells in S-phase (*, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.0001$). **D,** MCF-7 and T47D parental and ribociclib-resistant cells were seeded at low density in 12-well plates and treated with fresh media and drugs every 2 to 3 days. Resistant cells were removed from ribociclib for 24 hours prior to drug treatment. Cells were treated with 1 μmol/L of each drug in all experiments. After 10 to 21 days, cell monolayers were stained with crystal violet and subjected to image analysis as indicated in Materials and Methods. Each bar represents the mean image signal intensity $\pm$ SD of triplicate wells (***, $P \leq 0.001$; ****, $P \leq 0.0001$).

Furthermore, the combination of ribociclib/GSK2334470, but not ribociclib/dinaciclib, completely abrogated phosphorylation of Rb, S6, and RSK2 and expression of cyclin D1, cyclin A, and cyclin E (Fig. 6C). Finally, addition of dinaciclib to ribociclib/GSK2334470 did not result in further inhibition of cell proliferation. Taken together, these results suggest the PI3K/PDK1 signaling pathway mediates acquired resistance to CDK4/6 inhibition via an aberrant upregulation of S-phase cyclins and CDKs, which can be blocked with a PDK1 inhibitor.

Discussion

The combination of an anti-estrogen and the CDK4/6 inhibitor palbociclib has recently emerged as an effective option for the treatment of advanced ER$^+$ breast cancer (1, 4). Despite the impressive results of randomized trials with palbociclib, some patients do not respond to therapy and those who do eventually progress, underscoring the need to discover mechanisms of de

novo or acquired resistance to CDK4/6-targeted drugs. Using a kinome-wide siRNA screen, we identified PDK1 as the top RNA whose downregulation sensitized ER$^+$ breast cancer cells to CDK4/6 inhibition. RNAi-mediated knockdown of *PDK1* or *CDK4* inhibited growth of ER$^+$ breast cancer cells, but dual knockdown synergistically inhibited cell proliferation and suppressed PI3K/PDK1/AKT/mTOR signaling. Pharmacologic inhibition of PDK1 with GSK2334470 in combination with CDK4/6 inhibitors synergistically inhibited proliferation and increased apoptosis of ER$^+$ breast cancer cells and xenografts. In MCF-7 cells with acquired resistance to ribociclib, we observed an upregulation of phosphorylated and total PDK1 protein levels and subsequent activation of AGC kinases as a mechanism of escape from CDK4/6 inhibition. The upregulation of PDK1, P-S6, and cyclin D1 upon inhibition of CDK4/6 was confirmed in primary breast tumor biopsies. Furthermore, we observed aberrant cell-cycle progression in the ribociclib-resistant cells via upregulation of the S-phase cyclins/CDKs (P-CDK2, cyclin E, and cyclin A) with

Figure 6.
Expression of cell-cycle cyclins and CDKs is sustained in ribociclib-resistant cells. **A,** MCF-7, T47D, HCC1428 parental, and ribociclib-resistant (RR) cells were treated ± ribociclib for 24 hours. Cell lysates were then prepared and analyzed by immunoblot with the indicated antibodies. **B,** Cells were plated in triplicate in 12-well plates and treated with ribociclib, GSK2334470, and dinaciclib alone or in combination as indicated. Cells were trypsinized and counted on days 0, 1, 3, 5, and 7 of treatment. Media and drugs were replenished on days 3 and 5. Ribociclib/GSK2334470 or the triple combination of ribociclib/GSK2334470/dinaciclib was most effective at inhibiting cell growth compared with single agent or ribociclib/dinaciclib (*, $P \leq 0.05$). **C,** Ribociclib-resistant cells were treated with ribociclib, GSK2334470, dinaciclib, or combinations of these drugs for 24 hours; cell lysates were analyzed by immunoblot for the indicated proteins. Resistant cells were removed from ribociclib for 24 hours prior to drug treatment. The combination of ribociclib/GSK2334470 or the triple combination was most effective at diminishing expression of cyclins and CDKs.
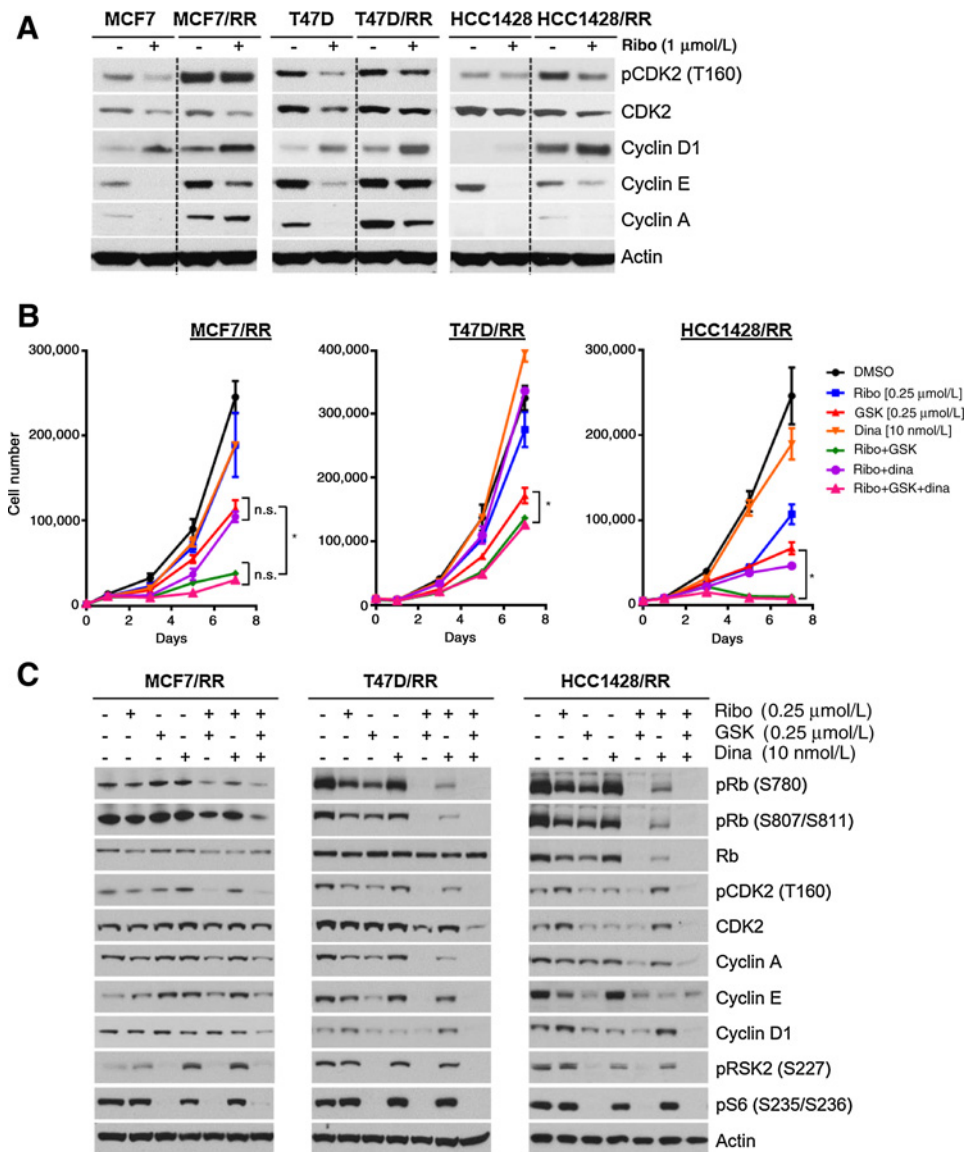
a concomitant increased in phosphorylated AKT at S477/T479, a CDK2-dependent phosphorylation required for full AKT kinase activity and limited to the S-phase of the cell cycle (19). Pharmacologic inhibition of PDK1 or CDK2 resensitized the ribociclib-resistant cells to CDK4/6 inhibitors; however, ribociclib/GSK2334470 inhibited MCF-7/RR and T47D/RR cell proliferation better than ribociclib/dinaciclib in part through complete abrogation of P-Rb, P-S6, P-RSK2, P-CDK2, cyclin A, cyclin E, and cyclin D1.

A prior study showed that CDK4/6 inhibitors can restore sensitivity to PI3K inhibitors in *PIK3CA*-mutant cells (40) and that early adaptation and acquired resistance to CDK4/6 inhibition can be prevented by cotreatment with PI3K inhibitors (36). Using an open-ended screen, our study identified PDK1 as the top RNA sensitizing ER$^+$ breast cancer cells to CDK4/6 inhibition. *PIK3CA* siRNAs were included in the siRNA library used in the screen and the combination of ribociclib and alpelisib potently inhibited cell growth *in vitro* and *in vivo*. Of note, however, *PIK3CA* siRNA did not score as sensitizing to ribociclib based on our

calculation of the sensitivity index, which measures the influence of siRNA-induced gene knockdown on drug sensitivity (22, 24, 41). On the basis of the sensitivity index method, sensitizing siRNAs have a small effect on cell viability alone with a greater effect in combination with ribociclib. Thus, we suspect that because *PIK3CA* siRNAs had a significant effect on MCF-7 cell viability by themselves, they were not identified by our screen.

These results are in partial agreement with those recently reported by Herrera-Abreu and colleagues (36). In their study, PI3K inhibitors prevented resistance to CDK4/6 inhibitors but did not restore sensitivity to CDK4/6 inhibitors once resistance has been acquired (36). In contrast, we show in the study herein that inhibition of PDK1 with GSK2334470 can resensitize ribociclib-resistant cells to CDK4/6 inhibition similar to parental drug–sensitive cells. CDK4/6 inhibitors are being explored in other solid tumors (31). There are preclinical studies that support the combination of a CDK4/6 with BRAF and MEK inhibitors in melanoma (42) and colorectal cancer (43), respectively. CDK4/6 inhibition can also overcome resistance to vemurafenib in

$BRAF^{V600E}$-mutant melanoma cell lines (44). As a result, there are several clinical trials in registration exploring the combination of MAPK and PI3K pathway inhibitors in combination with CDK4/6 inhibitors in advanced solid tumors (www.clinicaltrials.gov). Our studies suggest the combination of CDK4/6 and PDK1 inhibitors may be effective in these tumor types.

PDK1 functions downstream of PI3K and is crucial for the activation of AKT and many other AGC kinases including PKC, S6K, SGK, and RSK (11, 13). Our data suggest PDK1 may be a compelling therapeutic target to inhibit AKT and non-AKT targets. This is further supported by a recent study demonstrating complete inhibition of PI3K/PDK1/AKT/mTOR signaling when a PDK1 inhibitor is added to a PI3K inhibitor in $PIK3CA$-mutant breast cancer cells resistant to PI3K inhibition (45). These data also suggest some plausible reasons for the overall lack of substantial tumor regressions in in patients with $PIK3CA$-mutant breast cancer treated with single-agent PI3K inhibitors (46). Our studies suggest that the combination of PDK1 and CDK4/6 may be effective in both $PIK3CA$ wild-type and mutant breast cancers as well as other solid tumors in which CDK4/6 inhibitors are being explored clinically.

The ribociclib-resistant cells we generated displayed cross-resistance to the CDK4/6 inhibitors palbociclib and abemaciclib. Furthermore, the ribociclib-resistant cell lines failed to display G_1 arrest, a reduction in S-phase, and senescence compared with parental drug–sensitive cells upon drug treatment. The resistant cells exhibited significantly higher levels of phosphorylated CDK2 and cyclin E, consistent with a previous report (36). In this study, we also showed that ribociclib-resistant cells have increased levels of cyclin D1-, cyclin A-, and CDK2-dependent S477/T479 P-AKT. Interestingly, although pharmacologic inhibition of PDK1 or of CDK2 resensitized the ribociclib-resistant cells to CDK4/6 inhibition, the combination of ribociclib/GSK2334470 inhibited MCF-7/RR, T47D/RR, and HCC1428/RR cell proliferation better than ribociclib/dinaciclib. Furthermore, ribociclib/GSK2334470 but not ribociclib/dinaciclib completely abrogated P-Rb, P-S6, P-RSK2, P-CDK2, cyclin A, cyclin D1, and cyclin E, levels, further suggesting the PI3K/PDK1 pathway maintains cell-cycle progression in cells with acquired resistance to CDK4/6 inhibitors.

Finally, the combination of GSK2334470 and ribociclib inhibited growth of established MCF-7 xenografts in nude mice more potently than either drug alone. Previous clinical studies with nonspecific PDK1 inhibitors have been disappointing due to dosing issues (47). However, with the discovery of more selective PDK1 inhibitors like GSK2334470 (16, 25) or SNS-229 and SNS-510 (18), they may prove to be more efficacious when combined with CDK4/6 inhibitors. Our studies suggest cotargeting of PI3K/PDK1 and CDK4/6 may overcome resistance to CDK4/6 inhibitors and is worthy of further translational and clinical investigation in patients with ER$^+$ breast cancer.

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Correction: Kinome-Wide RNA Interference Screen Reveals a Role for PDK1 in Acquired Resistance to CDK4/6 Inhibition in ER-Positive Breast Cancer

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In the original version of this article (1), the stated disclosure of the author Carlos L. Arteaga is incorrect. The error has been corrected in the latest online HTML and PDF versions of the article. The authors regret this error.

Reference

1. Jansen VM, Bhola NE, Bauer JA, Formisano L, Lee K-M, Hutchinson KE, et al. Kinome-wide RNA interference screen reveals a role for PDK1 in acquired resistance to CDK4/6 inhibition in ER-positive breast cancer. Cancer Res 2017;77:2488–99.

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