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eIF4A inhibitors suppress cell cycle feedback response and acquired resistance to CDK4/6 inhibition in cancer

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Abstract

CDK4/6 inhibitors are FDA-approved drugs for estrogen receptor-positive (ER⁺) breast cancer and are being evaluated to treat other tumor types including *KRAS* mutant non-small cell lung cancer (NSCLC). However, their clinical utility is often limited by drug resistance. Here, we sought to better understand the resistant mechanisms and help devise potential strategies to overcome this challenge. We show that treatment of CDK4/6 inhibitors in both ER⁺ breast cancer and *KRAS* mutant NSCLC cells induce feedback upregulation of cyclin D1, CDK4 and cyclin E1 mediating drug resistance. We demonstrate that rocaglates, which preferentially target translation of key cell cycle regulators, effectively suppresses this feedback upregulation induced by CDK4/6 inhibition. Consequently, combination treatment of CDK4/6 inhibitor palbociclib with the eukaryotic initiation factor (eIF) 4A inhibitor, CR-1-31-B, is synergistic in suppressing the growth of these cancer cells *in vitro* and *in vivo*. Furthermore, ER⁺ breast cancer and *KRAS* mutant NSCLC cells that acquired resistance to palbociclib after chronic drug exposure are also highly sensitive to this combination treatment strategy. Our findings reveal a novel strategy using eIF4A inhibitors to suppress cell cycle feedback response and to overcome resistance to CDK4/6 inhibition in cancer.

Introduction

CDK4/6 are key cyclin-dependent kinases that promote G1 to S phase cell cycle progression. Upon activation by complexing with D-type cyclins, CDK4/6 phosphorylate and inactivate the retinoblastoma protein (RB); this uncouples the inhibitory interaction between RB and E2F transcription factors, which initiate a transcriptional program promoting cell cycle progression. Given this pivotal role of CDK4/6, their activities are often dysregulated in cancers resulting in aberrant cell proliferation. Thus, CDK4/6 have been key targets of clinical development for cancer therapy (1-3).

Three highly selective CDK4/6 inhibitors, palbociclib (PD-0332991), ribociclib (LEE001) and abemaciclib (LY2835219), are FDA-approved for treating estrogen receptor-positive (ER⁺) advanced breast cancers, which are often characterized by dysregulated CDK4/6 activation (1-3). These inhibitors, in combination with antiestrogens, effectively target this oncogenic addiction of ER⁺ breast cancer and have significantly improved patient survival. For example, in the large phase 3 PALOMA-3 study of women with ER⁺ metastatic breast cancer, the combination of palbociclib and fulvestrant improved the median overall survival by 6.9 months compared to the fulvestrant alone after 4 years follow up (4). This is important because, of the quarter of a million new cases of breast cancer diagnosed every year in the USA, at least 65% will be ER⁺, and approximately one-third of these cases will become metastatic at some point and can benefit from CDK4/6 inhibitors. In addition to breast cancer, CDK4/6 inhibitors have shown promising antitumor activities for other cancer types dependent on CDK4/6 activity, such as *KRAS*-mutant non-small cell lung cancer (NSCLC) (5,6). It is anticipated that CDK4/6 inhibitors will become standard of care for a variety of malignancies.

Despite these encouraging clinical outcomes, ~ 20% of patients will not respond to CDK4/6 inhibitors and of those initially responding, half developed drug resistance with progression within 25 months (4,7). One known resistance mechanism is RB inactivation, as CDK4/6 inhibitors require an active RB pathway to elicit antitumor effects (8,9). Other possible mechanisms including cyclin E-CDK2 activation (10,11), CDK6 amplification (12) and enhanced MAPK signalling (13) have been reported. Adaptive response to CDK4/6 inhibition likely contributes to the development of resistance. For example, treatment of CDK4/6 inhibitors in breast cancer cells induces upregulation of cyclin D1 expression, which is dependent on AKT signalling (10). Similarly, CDK4/6 inhibition in pancreatic cancer cells results in upregulation of cyclin D1 and E1, which can be suppressed by PI3K/mTOR inhibitors (14). Furthermore, it has been shown that breast cancer cells can activate autophagy in response to palbociclib and autophagy inhibition sensitizes these cells to CDK4/6 inhibition (11). Thus, effective strategies targeting these adaptive responses may induce durable cell cycle exit in combination with CDK4/6 inhibitors.

One class of drugs capable of effectively suppressing multiple targets is translation inhibitors. Translation of many oncogenes, including those promoting cell cycle progression, is likely mainly through a cap-dependent mechanism(15). This is mediated by the eukaryotic initiation factor (eIF) 4F complex, which is composed of the cap binding protein eIF4E, a scaffolding protein eIF4G and the ATP-dependent RNA helicase eIF4A. Upon binding to the mRNA cap structure, eIF4F remodels the mRNA 5' leader region and recruits binding of a 40S ribosome (and associated initiation factors); a step that is rate-limiting for translation. Hence, mRNAs must compete for access to eIF4F and one determinant of competitive efficiency is structural barrier (secondary structure, protein:RNA complexes) within 5' leader regions. Many

mRNAs with roles in tumor initiation (eg, *Myc*), tumor maintenance (eg, cyclins, *Mcl-1*), and drug response are eIF4F-responsive mRNAs, making eIF4F a potent anti-cancer target(15-17). In addition, given the large preponderance of human tumors that have activated PI3K/mTOR signaling(18) this is expected to lead to elevated eIF4F levels, making eIF4F a tumor-selective vulnerability.

There are small molecule compounds that target the regulation of eIF4F assembly (i.e. mTOR inhibitors) or phosphorylation status of eIF4E (i.e. MNK inhibitors) (15). However, these compounds have pleiotropic effects since their target kinases are known to regulate multiple downstream pathways in addition to translation initiation. In contrast, targeting eIF4A can selectively and directly inhibit translation initiation. Several potent eIF4A inhibitors have been identified from the rocaglate family of small molecules, among which are silvestrol and CR-1-31-B (19,20). Rocaglates are among the more potent eIF4F inhibitors characterized since they elicit two responses: they cause eIF4A to clamp onto polypurine RNA sequences in 5' leader regions to block initiation(21) and they deplete eIF4F of its eIF4A subunit (22). Silvestrol-responsive mRNAs include oncogenes involved in the inhibition of apoptosis such as *MCL1* and *BCL2*, and in the regulation of cell cycle progression, notably *CCND1*, *CCND3*, *CCNE1* and *CDK6* (23,24). Therefore, silvestrol and CR-1-31-B, while also being well tolerated in animals, suppress translation of these oncogenic mRNAs and block proliferation in breast, prostate, lymphoma, and acute lymphoblastic leukemia cancer models (15,22,23,25).

In this study, we employed rocaglates to target cell cycle feedback response induced by CDK4/6 inhibition in ER⁺ breast cancer and *KRAS*-mutant NSCLC cells. We found that targeting eIF4A is synergistic with CDK4/6 inhibitors in suppressing proliferation of these cancer cells and can overcome acquired resistance to CDK4/6 inhibition.

Materials and Methods

Cell culture and Viral Transduction

MCF-7, T47D, and CAMA-1 were acquired from ATCC and cultured in DMEM with 6% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotics, and 2 mM L-glutamine, at 37°C and 5% CO₂. A549, H358, H2030 and H1944, were acquired from ATCC and cultured in RPMI-1640 media with 6% fetal FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine, at 37°C and 5% CO₂. Cell lines were regularly tested for mycoplasma using the Mycoalert Detection Kit (Lonza). Identity of all cell lines was verified by Short Tandem Repeat (STR) profiling. From thawing, cells recovered for two passages and were passaged maximum 10 times when experiments were performed.

Lentiviral transduction was performed following the guidelines outlined at <http://www.broadinstitute.org/rnai/public/resources/protocols>. Cells were infected for 30 hours and then selected with puromycin for 2 days.

Compounds and Antibodies

Palbociclib (S1116), abemaciclib (S7158) and ribociclib (S7440) were purchased from Selleck Chemicals (Houston, Texas, US). CR-1-31-B and silvestrol were synthesized as previous described (26,27). Antibodies against HSP90 (H-114), Cyclin D1 (M20), Cyclin D3 (DCS28), Cyclin A2 (BF683), Cyclin E1 (HE12), Cyclin E2 (A-9), CDK2 (D-12), CDK4 (DCS-35), CDK6 (C-21), p16 (C-20), p21 (H164), and p27 (C-19) were purchased from Santa Cruz Biotechnology; p-RB (S795) and Cyclin D2 (D52F9) were purchased from Cell Signaling; RB (554136) was purchased from BD Pharmingen; eIF4A1 (ab31217) was purchased from Abcam.

Plasmids

Individual shRNA vectors used were obtained from the Mission TRC library (Sigma): sh*CCND1* #1 (TRCN0000295876), sh*CCND1* #2 (TRCN0000288598), sh*CDK4* #1 (TRCN0000000362), sh*CDK4* #2 (TRCN0000196698), sh*CCNE1* #1 (TRCN0000045301), and sh*CCNE1* #2 (TRCN0000045302). Overexpression vectors were obtained from the TRC3 ORF collections from TransOMIC and Sigma: pLX304-*GFP*, pLX304-*CCND1*, pLX317-*GFP*, and pLX317-*CDK4*. These above plasmids are provided by the Genetic Perturbation Service of Goodman Cancer Research Centre and Biochemistry at McGill University.

Cell viability assays

Cells were seeded at a density of 200-2,000 cells per well into 96-well plates and treated with drugs as indicated 24h post seeding. Media and drugs were refreshed every 3 days. CellTiter-Blue viability assay (Promega) was utilized to measure cell viability and fluorescence (560/590 nm) was recorded in a microplate reader. Cells were grown for 5-8 days depending on cell size, shape, and density.

Colony formation assays

$2-20 \times 10^3$ cells were seeded in 6-well plates. For drug assays, 24h post seeding, inhibitors were added to the cells. Media and drugs were refreshed every 3 days. Cells were grown for 10-18 days depending on cell size, shape, and density. At end point, cells were fixed with 4% formalin and stained with 0.1% w/v crystal violet before being photographed. All colony formation assays were fixed horizontally.

Drug washout assays

4-50 x 10² cells were seeded in 6-well plates. 24 hours post seeding, cells were treated with inhibitors for 6 days, refreshed every 3 days. After 6 days of treatment, cells recovered in regular media for 6 additional days until being fixed and stained.

Immunoblots

24 hours post seeding (6 or 12-well plates), cells were washed with cold phosphate buffer saline (PBS), lysed with protein sample buffer, and collected. For drug assays, 24 hours post seeding, the medium was replaced with media containing inhibitors. Cells were collected 24-72 hours post treatment.

RNA isolation and qRT-PCR

RNA isolation was performed using Trizol (Invitrogen). Synthesis of cDNAs and qRT-PCR assays were carried out as described (28). Relative mRNA levels of each gene shown were normalized to the expression of the house-keeping gene *ACTB*. The sequences of the primers for assays using SYBR® Green master mix (Roche) are as follows:

*ACTB*_Forward: 5' – GTTGTCGACGACGAGCG – 3'

*ACTB*_Reverse: 5' – GCACAGAGCCTCGCCTT – 3'

*CCND1*_Forward: 5' – GGCGGATTGGAAATGAACTT – 3'

*CCND1*_Reverse: 5' – TCCTCTCCAAAATGCCAGAG – 3'

*CCNE1*_Forward: 5' – TCTTTGTCAGGTGTGGGGA – 3'

*CCNE1*_Reverse: 5' – GAAATGGCCAAAATCGACAG – 3'

*CDK4*_Forward: 5' – GTCGGCTTCAGAGTTTCCAC – 3'

*CDK4*_Reverse: 5' – TGCAGTCCACATATGCAAC – 3'

Cell cycle analysis

Cells were treated with inhibitors for 3 days and then washed with PBS containing 1% FBS and fixed with cold 70% ethanol for 30 min on ice. Cells were washed with PBS and treated with 25 $\mu\text{g ml}^{-1}$ Ribonuclease A and stained with 50 $\mu\text{g ml}^{-1}$ propidium iodide solution for 30 min in the dark. 5×10^3 stained cells were then analyzed by Guava easyCyte HT System (MilliporeSigma) to determine proportion of cells in G₁, S, or G₂ phase of the cell cycle.

Senescence-associated- β -galactosidase staining

Cells were seeded at low density ($2\text{-}40 \times 10^2$ cells) and treated with inhibitors for 7 days, refreshed every 3 days. Cells were then washed with PBS and fixed with 0.5% glutaraldehyde solution for 15 minutes at room temperature. Cells were washed with PBS and then with PBS/MgCl₂ pH 6.0 solution twice, followed by staining with X-Gal staining solution (0.2M K₃Fe(CN)₆, 0.2M K₄Fe(CN)₆ 3H₂O, X-Gal stock (40X)) in PBS/MgCl₂ in the dark at 37°C for 8 hours. Cells were washed 3 times with PBS and were photographed. Senescent cells were quantified by counting 100 cells in three different fields for each replicate.

Co-Immunoprecipitation assay

Cells were resuspended in ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 1 mM dithiothreitol (DTT) and protease/phosphatase inhibitors) and mechanically sheered passing through a needle syringe. After 30 minutes incubation on ice, samples were centrifuged at

14,000g for 15 minutes at 4°C to collect the supernatant. 3µg of IgG or CDK4 (DCS-35, Santa Cruz) antibodies were added to 2mg of pre-cleared cell lysate in 500µl of lysis buffer and incubated overnight at 4°C with continuous rocking. Protein immunocomplexes were then incubated with 40µl protein G sepharose beads (Protein G Sepharose 4 Fast Flow, GE Healthcare) at 4°C for 2 hours. Precipitated proteins were washed three times with lysis buffer and eluted with sodium dodecyl sulfate (SDS) loading buffer at 95°C for 10 minutes and analyzed by Western Blot.

Overlap of TE down genes and Gene ontology analysis

Two publicly available datasets of cancer cell lines treated with silvestrol were identified and utilized as follows: in the silvestrol-treated KOPT-K1 cells, 281 genes were identified through RNA-seq whose mRNA translation efficiency was decreased, at a cut-off at $P < 0.03$ (Z -score > 2.5) (23). In the silvestrol-treated MDA-MB-231 cells, 284 genes were identified through RNA-seq whose mRNA translation efficiency was decreased, at a cut-off at Z -score below -1.5 (24). Gene ontology biological process was performed using the Gene Set Enrichment Analysis (29) provide by Broad Institute on the 33 overlapping TE down genes from these two datasets. The enriched genetic signatures were ranked according to p value, with the top five signatures shown.

***In vivo* xenografts**

All animal procedures (Animal Use Protocol) were approved by the Institutional Animal Care Committee according to guidelines defined by the Canadian Council of Animal Care and were conducted at the Rosalind & Morris Goodman Cancer Centre at McGill University. The persons

who performed all the tumor measurements and the IHC analysis for the endpoint tumor samples were blinded to the treatment information.

MCF-7 orthotopic xenograft

For MCF-7 xenograft experiments, estrogen pellets were synthesized (30) and were subcutaneously implanted into 4- to 6- week-old female nude mice (Charles River) prior to injection of cancer cells. 5×10^6 MCF-7 cells were injected at a ratio of 1:1 cells to Matrigel (BD Biosciences) into the 4th right mammary fat pad. Once palpable tumors reached an average volume of 50-100mm³, tumor-bearing mice were randomized into four groups ($n = 4-5$) and treated with a combination of either vehicle (5.2% TWEEN 80 and 5.2% polyethylene glycol 400) or 45mg kg⁻¹ palbociclib via oral gavage, together with control (50mM sodium lactate) or 0.35 mg kg⁻¹ CR-1-31-B via intraperitoneal injection. Mice in the palbociclib alone or palbociclib plus CR-1-31-B group were treated with palbociclib daily for 28 days. Mice in the CR-1-31-B alone or CR-1-31-B plus palbociclib group were treated daily with CR-1-31-B for the first 12 days and recovered for the following 2 days, before continuing daily CR-1-31-B treatment. Tumor volumes were measured with callipers every other day and calculated by the formula $((L \times W^2)/2)$. At the end of treatment, mice were euthanized and tumors were collected for weighing.

H358 subcutaneous xenograft

5×10^6 H358 cells were subcutaneously injected at a ratio of 1:1 cells to Matrigel (BD Biosciences) into 6- to 8- week-old male NSG mice (bred in house). Once palpable tumors reached a volume of ~200 mm³ (day 5), tumor-bearing mice were randomized into four groups ($n = 4-5$) and treated daily with a combination of either vehicle (PBS) or 45mg kg⁻¹ palbociclib via

oral gavage, together with control (50mM sodium lactate) or 0.2 mg kg⁻¹ CR-1-31-B via intraperitoneal injection. Mice were treated for a week and recovered for the following week, before continuing daily treatment for 3-4 more weeks. Mice were weighed before measuring tumor volume, and tumor volumes were measured with callipers twice a week and calculated by the formula $((L \times W^2)/2)$.

Immunohistochemistry

Isolated xenograft tumors were fixed in 10% formalin before being paraffin embedded, cut into four micron thick sections, and stained using an IntelliPath automated immunostainer (Biocare Medical). Sections were incubated with the primary antibodies: Phospho-Rb Ser807/811 (9308; Cell Signaling), 1/200 dilution; cyclin D1 (SP4; Cell Marque), 1/250 dilution; cyclin E1 (HE12; Abcam), 1/100; CDK4 (108357; Abcam), 1/500 dilution, and Ki-67 (16667; Abcam), 1/100 dilution. All sections were scanned using an Aperio Scanscope Scanner (Aperio Vista, CA) and images were extracted with Aperio ImageScope.

H-scores of cyclin D1, CDK4, cyclin E1, and pRB staining were calculated using the formula: (3x percentage of strongly stained nuclei + 2x percentage of moderately stained nuclei + 1x percentage of weakly stained nuclei), giving a H-score range of 0 to 300. For each treatment condition, sections from three independent tumors were stained and from which the average H-score was calculated from 4 independent fields.

Generation of palbociclib-resistant clones

2 x 10⁴ MCF-7 or H358 cells were seeded in a 100mm dish and kept in 300nM (MCF-7) or 100nM (H358) of palbociclib for two weeks and then cultured in 900 nM (MCF-7) or 300nM

(H358) of palbociclib for six weeks to obtain resistant clones originating from a single cell. Resistant colonies were isolated and continued to be cultured in 900 nM (MCF-7) or 300nM (H358) of palbociclib.

Combination Index (CI) calculations

Cells were treated with indicated inhibitors and cell viability was measured by CellTiter-Blue. Drug combination indices were calculated through the Chou-Talalay method using the CompuSyn software (31). CI of < 1 demonstrates synergism; CI = 1 an additive effect; CI > 1 demonstrates antagonism. The fraction of cells affected was calculated as: $1 - \text{relative cell viability of treated condition compared to untreated control}$.

Densitometry

Immunoblot bands were quantified with ImageJ software and then normalized by the loading control HSP90 immunoblot band intensity. Intensity of crystal violet-stained wells from colony formation assays was quantified with ImageJ software. Each well was normalized to the untreated value from each experimental condition.

Statistical analysis

Statistical significance of biological replicates was calculated by two-tailed Student's t-test and by two-way ANOVA tests, where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Graphpad Prism 7 software was used to generate graphs and statistical analyses. All relevant assays were performed independently at least three times.

Results

CDK4/6 inhibition induces feedback upregulation of cyclin D1, CDK4 and cyclin E1 which modulate responses to CDK4/6 inhibitors in ER⁺ breast cancer

Adaptive response to CDK4/6 inhibition has been suggested to contribute to acquired resistance observed in breast cancer patients. To better characterize this response, we treated two ER⁺ breast cancer cell lines (MCF-7 and T47D) with three CDK4/6 inhibitors (palbociclib, abemaciclib, and ribociclib) and profiled the expression of key cell cycle mediators (Fig. 1A, Supplemental Table 1). We focused on factors exhibiting similar regulation in both cell lines across three drug treatments. Among those, we observed downregulation of cyclin D3 and CDK2. This is expected as *CCND3* and *CDK2* are E2F target genes(32), which would be suppressed due to reactivation of RB upon CDK4/6 inhibition. We also observed consistent upregulation of protein levels of cyclin E1 and CDK4, in addition to previously reported cyclin D1 (10,33). This is interesting and not expected as both *CCNE1* and *CCND1* are also known E2F target genes(32). We verified our findings using multiple independent shRNA vectors targeting *CCNE1* and *CDK4* (Supplemental Fig. 1A, 1B). The upregulated cyclin E1 appears to be an oncogenic low-molecular-weight isoform (34,35); this isoform, but not the full-length cyclin E1, was shown to confer palbociclib resistance (11). In addition, RT-qPCR analysis revealed that *CCND1*, but not *CDK4* or *CCNE1*, mRNA was significantly upregulated in both cell lines treated with CDK4/6 inhibitors, indicating post-transcriptional feedback regulation of CDK4 and cyclin E1 (Fig. 1B). Furthermore, this elevation of cyclin D1, cyclin E1 and CDK4 was detected across 72 hours of palbociclib treatment in MCF-7 and T47D cells (Fig. 1C), suggesting that this adaptive response is sustained.

Previous studies have implicated cyclin E1 overexpression and amplification in mediating drug resistance to CDK4/6 inhibitors (10,11). Although plausible, the causal roles of cyclin D1 and CDK4 in mediating drug resistance have not been formally established. We found that exogenous expression of cyclin D1 alone, but not of CDK4, in ER⁺ breast cancer cells (MCF-7, T47D, and CAMA-1) confers drug resistance to palbociclib in colony formation assays (Fig. 1D, E; Supplemental Fig. 2A, 2B; Supplemental Table 2). These observations suggest that cyclin D1 is limiting in these cells. Supporting this, ectopic cyclin D1 expression in MCF-7 cells led to a proportional increase of cyclin D1-CDK4 complex and elevated RB phosphorylation (Fig. 1E, F), indicating increased CDK4 kinase activities. In CAMA-1 cells, ectopic expression of cyclin D1 in combination with CDK4 induces slightly further RB phosphorylation and drug resistance, suggesting the additional contribution of exogenous CDK4 expression (Supplemental Fig. 2A, 2B). Consistent with previous reported *CCND1* function (10), cyclin D1 knockdown suppressed RB phosphorylation and sensitized MCF-7, T47D, and CAMA-1 to palbociclib in both colony formation (Fig. 1G, H; Supplemental Fig. 2C, 2D) and CellTiter-Blue cell viability assays (Supplemental Fig. 2E). These data casually establish that cyclin D1 is a key determinant of palbociclib responses and that its overexpression can cause drug resistance, which may also be enhanced by CDK4 elevation. Thus, this feedback upregulation of cyclin D1, CDK4 and cyclin E1 induced by CDK4/6 inhibitors may limit the efficiency of these anti-cancer agents.

eIF4A inhibitors suppress cell cycle feedback response induced by CDK4/6 inhibition and is synergistic in combination with CDK4/6 inhibitors against ER⁺ breast cancer cells

As an approach to simultaneously suppress these cell cycle mediators induced upon CDK4/6 inhibition, we explored the possibility of targeting translation initiation using rocaglates,

silvestrol and CR-1-31-B. As discussed earlier, these small molecule compounds selectively and directly target translation initiation by inhibiting eIF4A, a key subunit of eIF4F which is the rate-limiting factor in translation. Many oncogenic mRNAs are known to have more complex mRNA 5' leader regions and therefore be eIF4F-responsive mRNAs (15-17). In a previous study (23), treatment of KOPT-K1 T-lineage acute lymphoblastic leukemia cells with 25nM silvestrol resulted in the preferential translational suppression of 281 genes. We compared this publicly available gene set to another study(24) which identified 284 translationally suppressed mRNAs in MDA-MB-231 triple negative breast cancer treated with silvestrol. This analysis revealed an overlap of 33 genes whose mRNA translation efficiency (TE) was suppressed across both studies (Supplemental Table 3). Gene ontology analysis of these 33 genes identified the top five biological processes, four of which are cell cycle related (Fig. 2A). These unbiased findings from KOPT-K1 and MDA-MB-231 cells suggested that eIF4A inhibitors might be effective at suppressing the feedback upregulation of cell cycle regulators induced by CDK4/6 inhibition.

To confirm the effect of eIF4A inhibitors in ER⁺ breast cancer cells, we treated MCF-7 and T47D cells with CR-1-31-B and analyzed the expression of a panel of relevant key cell cycle regulators. We observed consistent suppression of cyclin D1, CDK2 and CDK4 in both cell lines at the low dose of 3.2 nM (Fig. 2B), and suppression of cyclin E1, cyclin A2 and CDK6 at higher concentrations up to 25.6 nM (Supplemental Fig. 3A). We utilized a second eIF4A inhibitor, silvestrol, which exerted a similar degree of growth suppression in both cell lines with half maximal inhibitory concentration (IC₅₀) of ~3 nM and ~1 nM for MCF-7 and T47D, respectively (Supplemental Fig. 3B), and dose-dependently suppressed cyclin D1, CDK4, and cyclin E1

(Supplemental Fig. 3C). These data indicate that rocaglates are effective in targeting these cell cycle regulators and suppressing the proliferation of ER⁺ breast cancer cells.

Next, we investigated the combination of palbociclib and CR-1-31-B in MCF-7 and T47D cells. As expected, addition of 3.2 nM CR-1-31-B reversed the palbociclib-induced upregulation of cyclin D1 and CDK4 to levels lower than basal expression and further inhibited RB phosphorylation in both cell lines (Fig. 2C). In the T47D cell line, which is more sensitive to CR-1-31-B, cyclin E1 feedback was also strongly abrogated at this low dose. Furthermore, the combination of CR-1-31-B and palbociclib further repressed cell viability of MCF-7 and T47D cells (Fig. 2D and Supplemental Table 4). Moreover, this combination of either CR-1-31-B or silvestrol with palbociclib was synergistic as determined by the combination index (31) in both MCF-7 and T47D lines (Fig. 2E, F). Similar synergism was also observed when a second CDK4/6 inhibitor, abemaciclib, was used in combination with CR-1-31-B (Supplemental Fig. 4). Furthermore, we found that CR-1-31-B enhanced G1 cell cycle arrest induced by CDK4/6 inhibition but had less effect on senescence induction (Supplemental Fig. 5A, B). Consistently, drug washout experiments (11) showed that the effect of CR-1-31-B and palbociclib single treatments are partially reversible when drugs are removed; however, the combination can better suppress the regrowth of these cells after drug removal (Supplemental Fig. 5C). These data suggest that the combination of palbociclib and CR-1-31-B can be effective in suppressing breast cancer cells predominantly through mediating cell cycle arrest.

Given that cyclin D1 and CDK4 are the two common feedback targets being suppressed at low concentrations of CR-1-31-B, we investigated whether they are the determinants of CR-1-31-B responses. As shown in Figure 2G-I, exogenous expression of cyclin D1 but not CDK4 in T47D cells conferred resistance to the growth inhibition induced by CR-1-31-B in both long-

term proliferation and cell viability assays. This is consistent with our findings that cyclin D1 is limiting in these cancer cells (Fig. 1D-H; Supplemental Fig. 2). Although these results do not rule out the contribution of CDK4 suppression (and other targets) to CR-1-31-B-induced growth inhibition, our data suggest that CR-1-31-B exerts its effect in part through targeting the dependency of cyclin D1 in these cancer cells. Together, we establish that rocaglates suppress cell cycle feedback response induced by CDK4/6 inhibition and act synergistically with CDK4/6 inhibitors to target ER⁺ breast cancer cells.

Drug combination targeting eIF4A and CDK4/6 is synergistic in suppressing ER⁺ breast cancer growth *in vivo*

To validate this potential treatment strategy *in vivo*, we performed orthotopic xenograft experiments by implanting MCF-7 cells into the mammary fat pads of immunodeficient mice. Upon the establishment of tumors, we treated mice with palbociclib or CR-1-31-B at suboptimal doses, or their combination. Although palbociclib or CR-1-31-B treatment alone resulted in marginal growth suppression, their combination elicited a potent growth inhibition of MCF-7 tumors (Fig. 3A). All treatments were well tolerated as no significant weight loss was observed in the animals (Fig. 3B). Using immunohistochemistry (IHC), we analyzed protein expression of the key cell cycle regulators in tumors isolated at the treatment endpoints (Fig. 3C, Figure 3D). Palbociclib treatment alone or together with CR-1-31-B led to reduction of RB-phosphorylation, confirming the target modulation by palbociclib. IHC analysis also shows that treatments including CR-1-31-B resulted in decreased expression of cyclin D1, CDK4, and cyclin E1, which is consistent with our *in vitro* data. While palbociclib or CR-1-31-B treatment alone had minimal effects on expression of the proliferation marker Ki67, their combination resulted in strong

suppression of Ki67 expression. Taken together, these results demonstrate that combination treatment of CR-1-31-B and palbociclib is highly effective in suppressing ER⁺ breast tumor growth *in vivo*.

Combination treatment targeting eIF4A and CDK4/6 synergistically suppress proliferation of *KRAS*-mutant NSCLC cells

We next aimed to extend our findings to other cancer types where CDK4/6 inhibitors are being evaluated. *KRAS*-mutant NSCLCs have been shown to respond to CDK/6 inhibitors due to their dependency on CDK4 and these inhibitors are being further evaluated in the clinic (5,6). *KRAS*-mutant NSCLC cell lines (H358, A549, H1944, and H2030) have similar sensitivity to palbociclib (Supplemental Fig. 6) as ER⁺ breast cancer (Fig. 1G). Treatment of all three CDK4/6 inhibitors in these *KRAS*-mutant NSCLC cells also resulted in upregulation of cyclin D1, CDK4 and cyclin E1 (Fig. 4A), mirroring the feedback response observed in breast cancer cells (Fig. 1A). Furthermore, the addition of CR-1-31-B suppressed this feedback (Fig. 4B) and is synergistic in combination with palbociclib in suppressing proliferation of these NSCLC cells (Fig. 4C and Supplemental Table 4), also predominantly through cell cycle arrest (Supplemental Fig. 7A-C). In support of our MCF-7 ER⁺ breast cancer xenograft data, combination treatment with suboptimal doses of palbociclib and CR-1-31-B also significantly suppressed tumor proliferation of H358 NSCLC xenografts (Fig. 4D) while also being well-tolerated by animals (Supplemental Fig. 8). Together, our findings demonstrate that eIF4A inhibitors are effective at suppressing the adaptive response induced by CDK4/6 inhibitors and inhibiting the proliferation of *KRAS*-mutant NSCLC and ER⁺ breast cancer cells.

eIF4A inhibitors overcome acquired resistance to CDK4/6 inhibition in ER⁺ breast cancer and NSCLC cells

We next investigated whether eIF4A inhibitors are also effective in suppressing breast cancer cells that have acquired resistance to CDK4/6 inhibition. For this purpose, we first generated a panel of spontaneously resistant clones of MCF-7 cells through chronic palbociclib exposure (Fig. 5A, supplemental Fig. 9). As shown in Figure 5B and C, one frequent mechanism of acquired resistance was loss of RB expression in 6 of 11 established clones, which is consistent with the established role of RB (1-3,8). In addition, cyclin E1 was significantly upregulated in 6 clones and cyclin D1 was also upregulated in 5 clones. We also observed the upregulation of CDK6 in 2 clones (R3 and R7) and slight elevation of CDK4 in 3 clones. Thus, the cell cycle profiles of these resistant clones are in line with previous reports. In a similar manner, we also isolated two stably spontaneously palbociclib-resistant clones of H358 NSCLC cells (Fig. 5D). Indeed, both H358 R1 and R2 clones also had elevated expression of cyclin D1, cyclin E1, and CDK4, and reduced RB phosphorylation (Fig. 5E).

To test if the combination of CR-1-31-B and palbociclib is also effective in targeting the spontaneously- palbociclib-resistant cells, we selected MCF-7 clones that were representative of altered signatures – MCF-7 R3 (upregulation of cyclin E1 and CDK6), and MCF-7 R16 (upregulation of cyclin D1 and cyclin E1), and H358 R1 and R2 clones. Indeed, the addition of a low concentration (3.2 nM) of CR-1-31-B was able to suppress the feedback upregulation of cyclin D1 and CDK4 in all palbociclib-resistant clones (Fig. 5F). The inhibition of cyclin E1 upregulation was also pronounced in H358 clones at this low CR-1-31-B concentration, which is consistent with the fact that H358 parental cells are more sensitive to CR-1-31-B compared to MCF-7 parental cells (Supplemental Fig.10). Furthermore, the drug combination demonstrated

synergy in suppressing the growth of resistant populations from both cancer types (Fig. 5G, H). Thus, these results suggest that eIF4A inhibitors can also be viable therapeutic agents treat tumors acquire drug resistance to CDK4/6 inhibitors.

Discussion

In this study, we found that feedback upregulation of cyclin D1, CDK4 and cyclin E1 is a common adaptive response induced by CDK4/6 inhibition in both ER⁺ breast cancer and *KRAS*-mutant NSCLC cells. We demonstrate that rocaglates, potent inhibitors of eIF4A, can effectively suppress this feedback response and synergize with CDK4/6 inhibitors targeting these cancer cells and their sub-populations that have acquired resistance to CDK4/6 inhibition. Furthermore, we show that the combination of CR-1-31-B and palbociclib was well tolerated in animals and elicited a potent inhibition to the tumor growth *in vivo* for both cancer types.

Acquired resistance to CDK4/6 inhibitors is a major challenge that limits their clinical utilities. Cyclin E1 overexpression and amplification have been implicated in mediating drug resistance to CDK4/6 inhibitors (10,11). Here, we establish a causal role of cyclin D1 in modulating responses to CDK4/6 inhibitors in ER⁺ breast cancer cells: immunoprecipitation assays indicate that cyclin D1 is limiting in these cells; ectopic expression of cyclin D1 led to elevated RB phosphorylation and resistance to CDK4/6 inhibition while cyclin D1-knockdown sensitized these cells to palbociclib. These results are consistent with our previous findings in subtypes of ovarian and lung cancers, where cyclin D1 deficiency limits CDK4/6 activities leading to drug sensitivity to CDK4/6 inhibitors (36,37).

It is important to note that our results do not rule out other potential contributions of cyclin D1 in mediating drug resistance independent of cell cycle activities, such as controlling glucose metabolism (38), promoting NF- κ B transcriptional regulation(39) and facilitating ER transcriptional activity(40). In addition, this potential role of cyclin D1 in mediating drug resistance remains to be further validated in clinical settings.

Our data suggest that the feedback upregulation of cyclin D1, CDK4 and cyclin E1 induced by CDK4/6 inhibition may contribute to selection of drug resistant variants after prolonged drug treatment. Indeed, elevation of these cell cycle regulators is observed in palbociclib-resistant clones of MCF-7 cells and H358 cells generated through chronic drug exposure. In addition, we also observed resistance clones with RB loss which is consistent with the recent report that RB1 mutations emerged in some tumors of patients treated with palbociclib(9). While RB1 inactivation remains as a challenge, we have identified here a potential strategy to suppress the feedback upregulation of the other cell cycle regulators contributing to drug resistance.

Our unbiased analysis of previous rocaglate-responsive transcriptome studies indicated that translation of cell cycle regulators is particularly sensitive to eIF4A inhibition due to the complex nature of their 5' leader regions of their mRNAs (23,24). Indeed, eIF4A inhibitors can effectively suppress the cell cycle feedback response induced by CDK4/6 inhibition and other key cell cycle regulators, even at the low nanomolar concentrations used in this study. Thus, this selective feature of eIF4A inhibitors results in a strong synergy in combination with CDK4/6 inhibition against the breast and lung cancer cell lines examined. Consistently, ectopic expression of cyclin D1 from a cDNA lacking the complex endogenous 5' leaders conferred resistance to CR-1-31-B in breast cancer cells, indicating that the growth suppressive effect of eIF4A inhibition is in part through targeting cell cycle pathway. However, translation suppression of other target genes such as *c-MYC* (22,41), may also contribute to the potent effect of eIF4A inhibitors and their combination with CDK4/6 inhibitors which require further investigation. In addition, our data do not exclude the possibility of autophagy inhibition induced by eIF4A inhibitors, which may contribute to the synergy in combination with CDK4/6 inhibition - it has been recently shown that targeting autophagy is synergistic with palbociclib

(11) and there are emerging connections between protein synthesis, energy homeostasis and autophagy(42). Nevertheless, our data demonstrate that co-targeting CDK4/6 and eIF4A is an effective combination in better suppressing cancer cells including those that acquired resistance to palbociclib.

Our study highlights the unique ability of eIF4A inhibitors to overcome different modes of resistance to CDK4/6 inhibition, by simultaneously suppressing multiple targets that mediate resistance. Furthermore, given that these targets such as cyclin D1 are often controlled by diverse signaling pathways, targeting their translation is effective regardless the nature of upstream inputs leading to their dysregulation. Supporting this, drug combination targeting eIF4A and BRAF/MEK has also been shown to overcome different resistance mechanisms arising in BRAF^{V600}-mutant cancer models (43). Thus, our data suggest that eIF4A inhibitors, which preferentially target key cell cycle regulators, could be an effective and novel treatment option to enhance efficiency of CDK4/6 inhibitors and overcome acquired drug resistance.

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Author contributions

T.K., Y.X., J.P. and S.H. designed the study. T.K., Y.X., Z.F., and X.Z. performed *in vitro* experiments. T.K., Y.X., X.Z., R.C., A.M, V.P., and V.S. performed *in vivo* experiments. M.C.G. performed immunohistochemistry and provided pathology expertise. W.D.F. and M.P. provided advice and technical support. J.A.P synthesized CR-1-31-B. J.P. and S.H. conceived and oversaw the study. T.K., J.P., and S.H. designed the experiments, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1 - CDK4/6 inhibition induces feedback upregulation of cyclin D1, CDK4 and cyclin E1 which modulate responses to CDK4/6 inhibitors in ER⁺ breast cancer

- A) Immunoblot profiling of key cell cycle regulators upregulated after exposure to three CDK4/6 inhibitors. MCF-7 and T47D cells were treated for 24 hours with 300 nM palbociclib, 150 nM abemaciclib, or 1 μ M ribociclib.
- B) RT-qPCR analysis of *CCND1*, *CDK4*, and *CCNE1* after 24 hour treatment with 300 nM palbociclib, 150 nM abemaciclib, or 1 μ M ribociclib. mRNA fold change normalized to mRNA of *ACTB* housekeeping gene. MCF-7: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; by Student's t-test, unpaired, two-sided; (n=3).
- C) Immunoblot showing a time course of sustained upregulation of key cell cycle regulators. Cells were treated with 300 nM palbociclib for 24, 48, and 72 hours.
- D) Long-term colony formation assays showing resistance to palbociclib after overexpression of *CDK4*, *CCND1*, or their combination in MCF-7 cells. Cells were seeded and treated with palbociclib for 10-14 days. Palbociclib was refreshed every 3 days.
- E) Immunoblot analysis of overexpression of *CDK4*, *CCND1*, or their combination in MCF-7 cells.
- F) Immunoblot detection of CDK4 and cyclin D1 of co-immunoprecipitated samples from MCF-7 cells overexpression a control vector or cyclin D1. Co-immunoprecipitation was performed using an antibody specific against CDK4 or a corresponding IgG control antibody. HSP90 served as a loading control.

- G) Long-term colony formation assays showing enhanced sensitivity to palbociclib in MCF-7 and T47D after knockdown of *CCND1* utilizing two independent shRNAs. Cells were seeded and treated with palbociclib for 10-14 days. Drugs were refreshed every 3 days.
- H) Immunoblot analysis of knockdown of *CCND1* utilizing two independent shRNAs in MCF-7 and T47D cells.

Figure 2 - eIF4A inhibitors suppress the adaptive response induced by CDK4/6 inhibition and is synergistic in combination with CDK4/6 inhibitors against ER⁺ breast cancer cells

- A) Gene Set Enrichment Analysis of Gene ontology biological processes for 33 overlapping genes, whose mRNA translation inhibition is suppressed upon treatment with 25 nM silvestrol from two ribosome footprint profiling studies in MDA-MD-231 and KOPT-K1 cells.
- B) Comprehensive immunoblot profiling of altered protein expression of cell cycle mediators upon treatment with 3.2 nM CR-1-31-B. MCF-7 and T47D cells were treated for 24h.
- C) Immunoblot analysis showing suppression of cyclin D1, CDK4, and cyclin E1 upon 24 hours treatment with 3.2 nM CR-1-31-B alone or in combination with 300 nM palbociclib in MCF-7 and T47D cells.
- D) Reduced cell viability upon combination treatment with CR-1-31-B and palbociclib. Cells were treated with indicated concentrations of palbociclib and/or CR-1-31-B for 5-8 days, and cell viability was determined using CellTiter-Blue. Error bars represent mean \pm standard deviation (s.d.). CR= CR-1-31-B.

- E, F) Isobologram synergy analysis at multiple dose combinations of CR-1-31-B (E) or Silvestrol (F) with palbociclib. CI, combination index; $CI < 1.0$ suggests synergism, $CI > 1$ suggests antagonism, $CI = 1$ suggests an additive effect. CI = combination index; FA = fraction of cells affected (1- relative cell viability of treated condition compared to untreated control).
- G, H) Long-term colony formation assays (G) cell viability assays (H) of T47D cells overexpressing *CCND1* or *CDK4* and their response to CR-1-31-B treatment. In long-term colony formation assays, cells were seeded and treated with CR-1-31-B for 10-14 days. In cell viability assays T47D cells were treated for 8 days with 1.6nM of CR-1-31-B, and cell viability was determined using CellTiter-Blue. Error bars represent mean \pm standard deviation (n=3). *** $p < 0.001$ by Student's t-test, unpaired, two-sided.
- I) Western blot analysis of T47D cells overexpressing either *CCND1* or *CDK4* and treated with 3.2 nM of CR-1-31-B for 24 hours.

Figure 3 – CR-1-31-B in combination with palbociclib is synergistic in suppressing ER⁺ breast cancer growth *in vivo*

- A) Tumor growth curves of MCF-7 orthotopic xenografts in nude mice treated for 28 days with vehicle (n=5), palbociclib (45mg/kg; n=4), CR-1-31B (0.35mg/kg; n=4), or palbociclib + CR-1-31-B combination (n=5). Error bars indicate standard error of mean (SEM) values. **** $p < 0.0001$; by two-way ANOVA.
- B) Weight of nude mice over xenograft period. Mice were weighed at the beginning of drug treatment and after day 13, every 2 days thereafter.

- C) Immunohistochemistry staining was performed on formalin-fixed, paraffin embedded xenograft tumors at end point. Representative results are shown. Scale bar for pRB S807/811, cyclin D1, CDK4, cyclin E1, and Ki67: 60 μ m.
- D) H-scores of cyclin D1, CDK4, cyclin E1, and pRB immunohistochemistry staining of xenograft tumors and percentage of Ki-67-positive cells in each treatment condition. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; by Student's t-test, unpaired, two-sided.

Figure 4 – Combination treatment targeting eIF4A and CDK4/6 synergistically suppress proliferation of *KRAS*-mutant non-small cell lung cancer cells

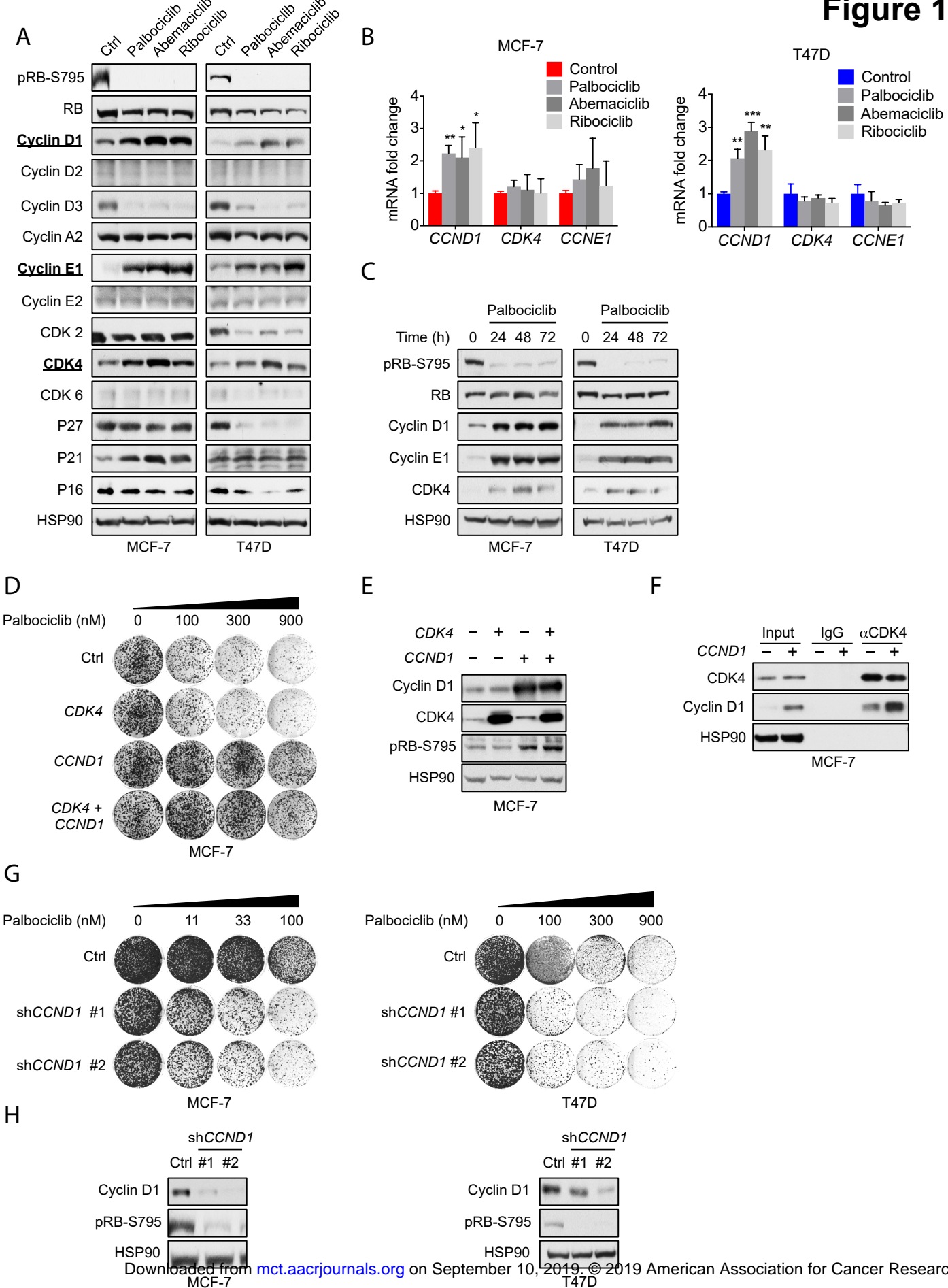
- A) Immunoblot analysis showing upregulation of cyclin D1, CDK4, and cyclin E1 of four *KRAS*-mutant NSCLC cell lines (H358, A549, H1944, and H2030) treated with CDK4/6 inhibitors. Cells were treated for 24 hours with 300 nM palbociclib, 150 nM abemaciclib, or 1 μ M ribociclib.
- B) Immunoblot showing treatment of CR-1-31-B suppresses upregulation of cyclin D1, CDK4, and cyclin E1 induced by palbociclib in *KRAS*-mutant NSCLC cells. Cells were treated for 48 hours with 300 nM palbociclib, 3.2 nM CR-1-31-B or their combination.
- C) Cell viability assays and isobologram synergy analysis of four *KRAS*-mutant non-small cell lung cancers treated with multiple combination doses of CR-1-31-B and palbociclib. Cells were treated with increasing concentrations of palbociclib and/or CR-1-31-B for 5-8 days, and cell viability was measured using CellTiter-Blue. Error bars represent mean \pm standard deviation. Combination indices were calculated for multiple doses of palbociclib and CR-1-31-B for all four NSCLC cell lines.

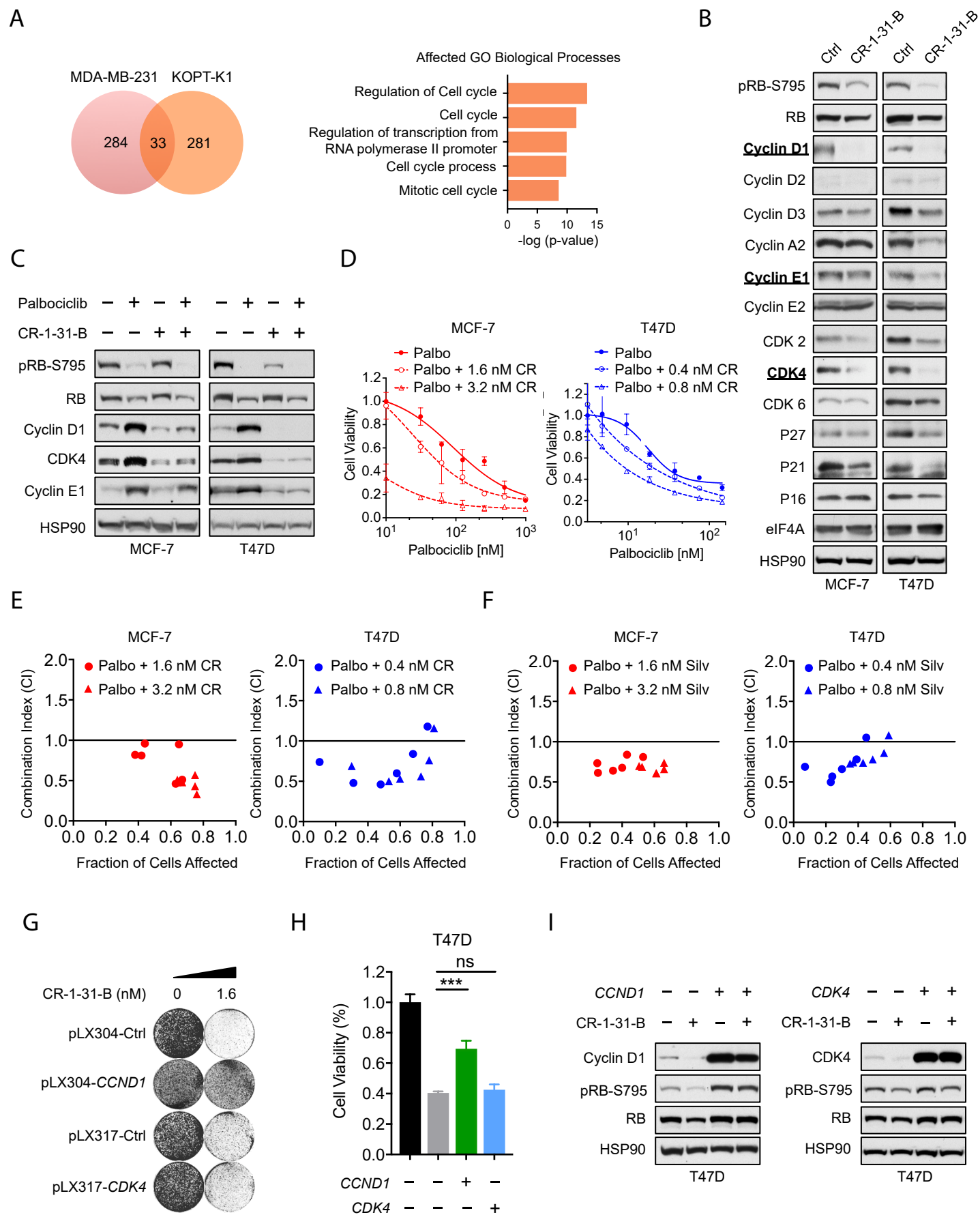
- D) Tumor growth curves of H358 subcutaneous xenografts in NSG mice treated with vehicle (n=5), palbociclib (45mg/kg; n=4), CR-1-31B (0.2mg/kg; n=4), or palbociclib + CR-1-31-B combination (n=4). Mice treated daily starting on day 5 (arrow) for one week and recovered for one week. Following recovery, mice were treated daily until end-point. Error bars indicate standard error of mean (SEM) values. **** $P < 0.0001$; by two-way ANOVA.

Figure 5 – CR-1-31-B overcomes acquired palbociclib resistance in ER⁺ breast cancer cells and KRAS mutant NSCLC cells

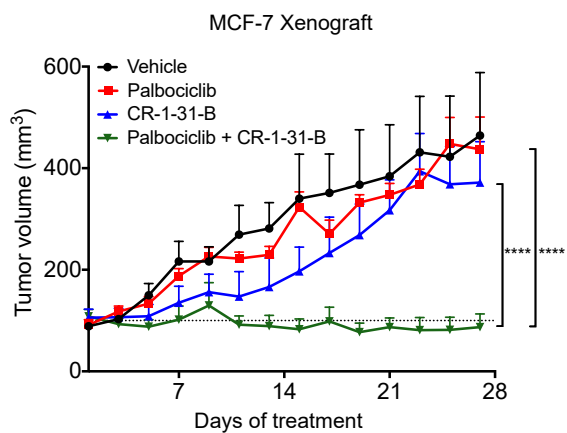
- A) Colony formation assays of MCF-7 R3 and R16 clones and their resistance to palbociclib compared to the parental line. Cells were seeded and treated with palbociclib for 10-14 days at the indicated concentrations. Palbociclib was refreshed every 3 days.
- B) Immunoblot analysis showing definitive protein expression adaptations mediating acquired resistance to palbociclib in MCF-7 cells.
- C) Heat map of protein expression fold change (log₂) of key cell cycle proteins in the MCF-7 palbociclib-resistant clones. Protein expression in panel B was quantified by densitometry and normalized to the intensity of the HSP90 loading control and then by immunoblot density of the MCF-7 parental line.
- D) Colony formation assays of two H358 NSCLC clones and their resistance to palbociclib. Cells were seeded and treated with palbociclib for 12-16 days at the indicated concentrations. Palbociclib was refreshed every 3 days.
- E) Immunoblot showing elevated protein expression of cyclin D1, cyclin E1, and CDK4 in H358 R1 and R2 clones relative to the parental line.

- F) Immunoblot indicating the addition of CR-1-31-B is sufficient to consistently suppress cyclin D1 and CDK4 protein across four MCF-7 and H358 palbociclib-resistant clones. MCF-7 R3, R16 and H358 R1 and R2 cells were treated with either 300 nM palbociclib or 3.2 nM CR-1-31-B alone, or in their combination for 24 hours.
- G, H) Isobologram synergy analysis of MCF-7 R3 and R16 and H358 R1 and R2 palbociclib-resistant clones treated with multiple combination doses of CR-1-31-B and palbociclib.

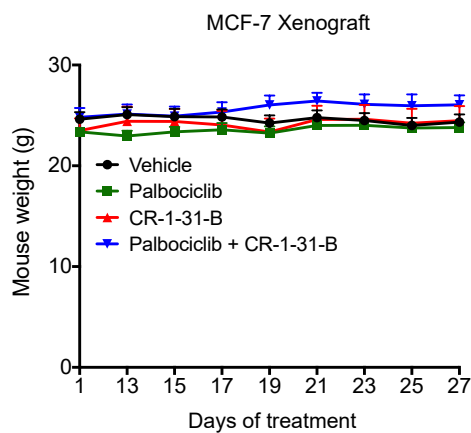




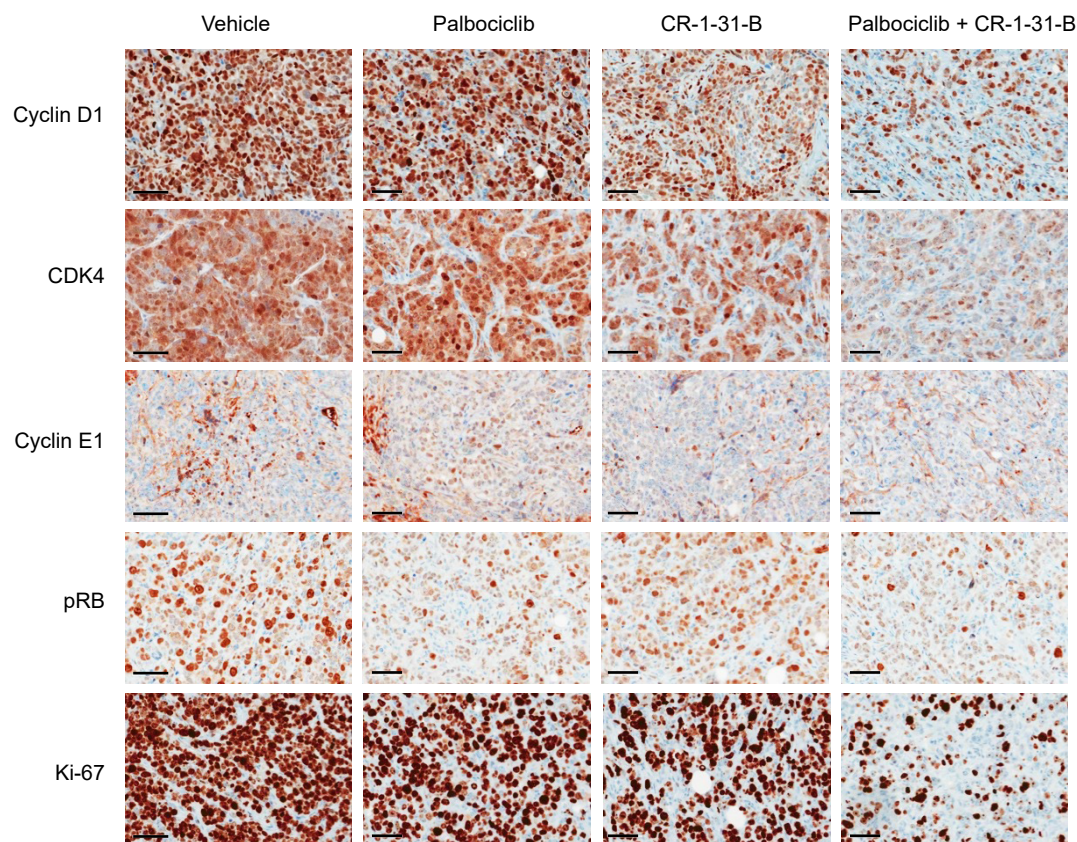
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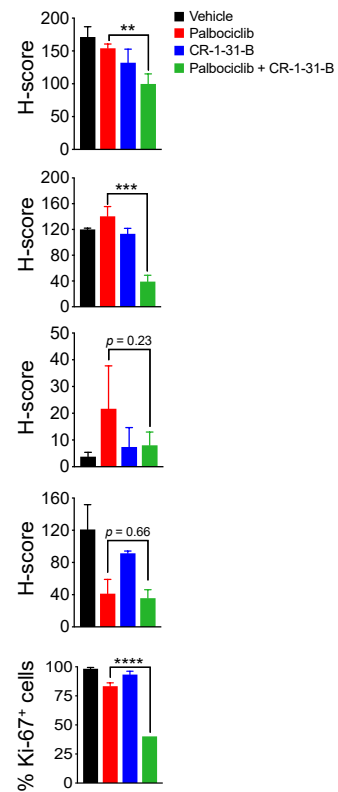
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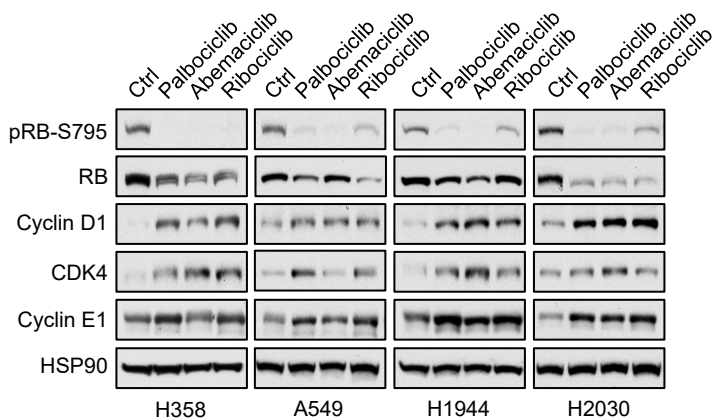
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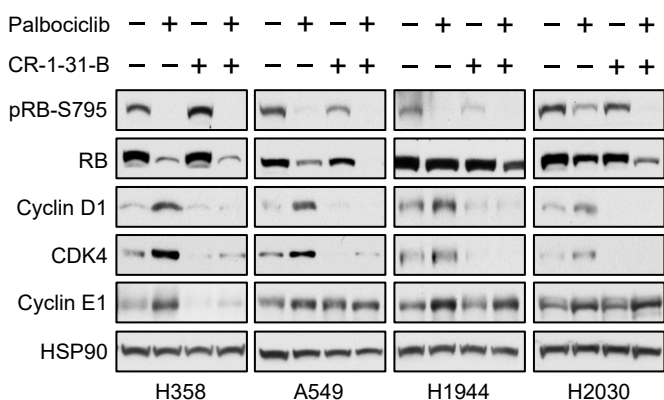
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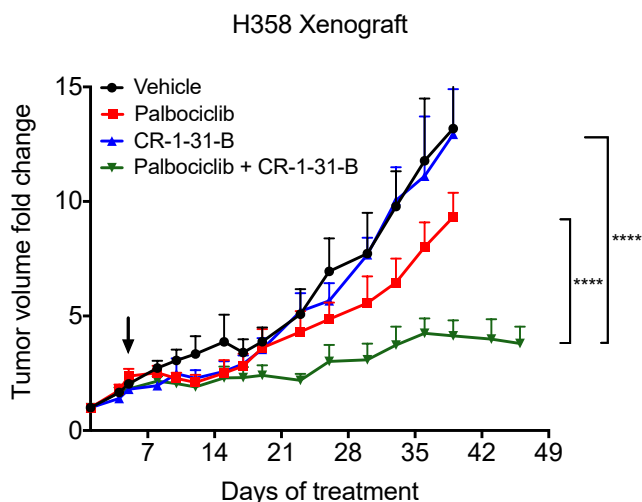
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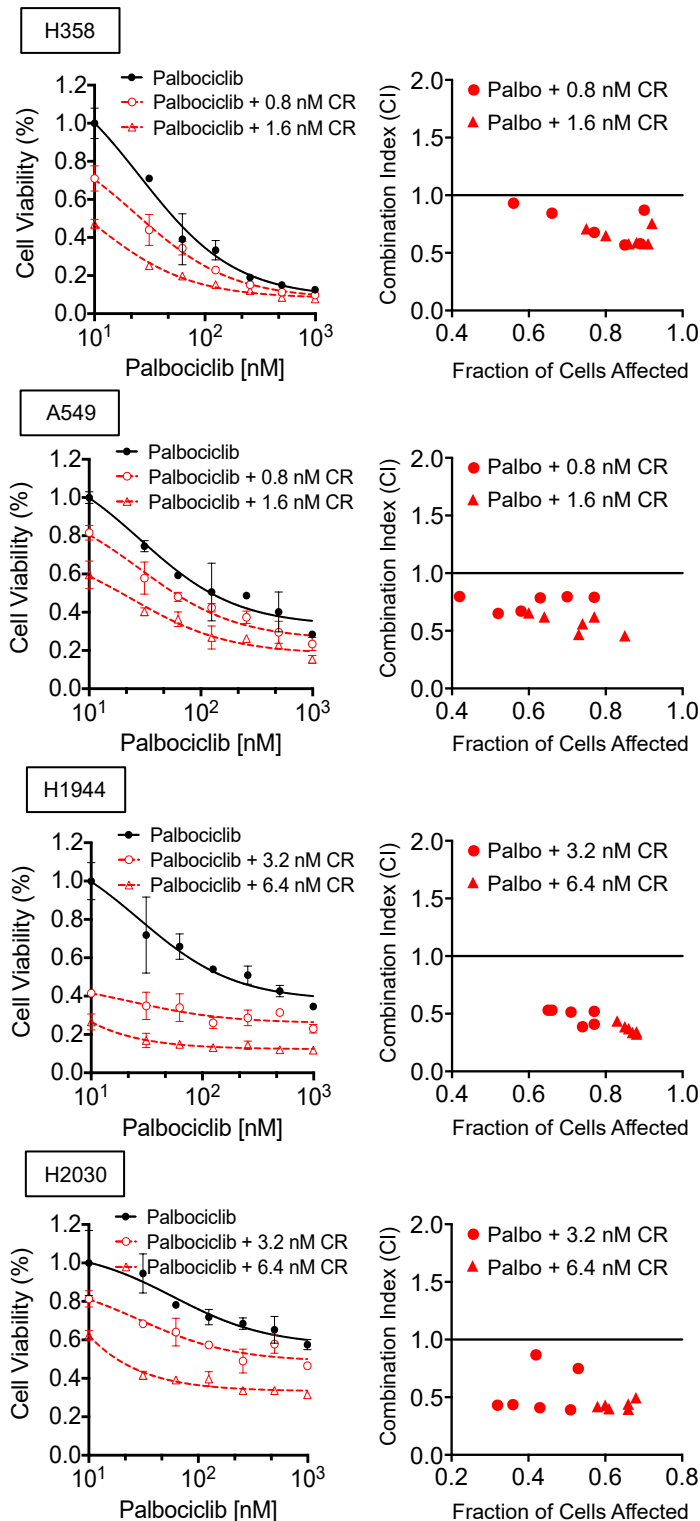
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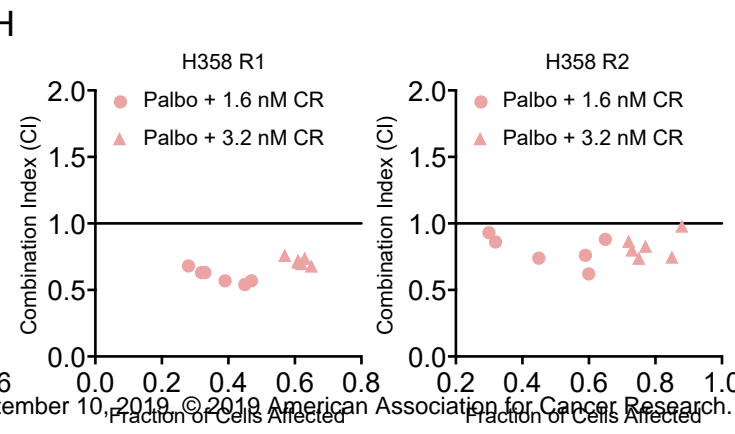
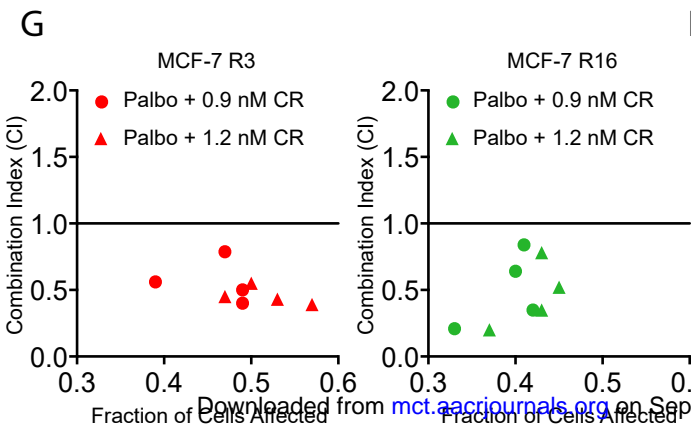
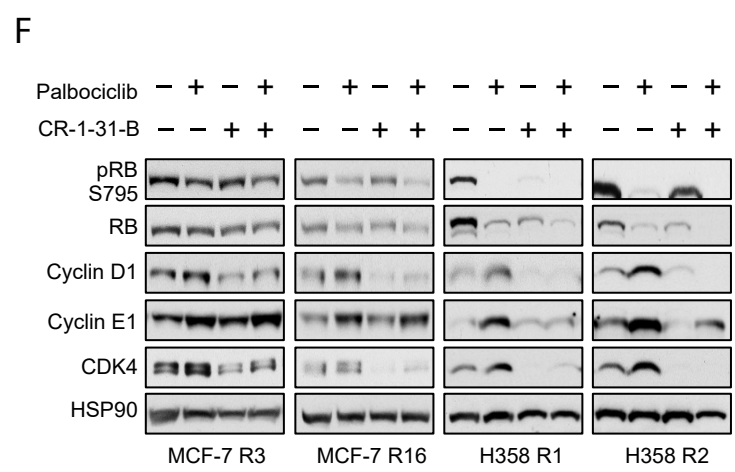
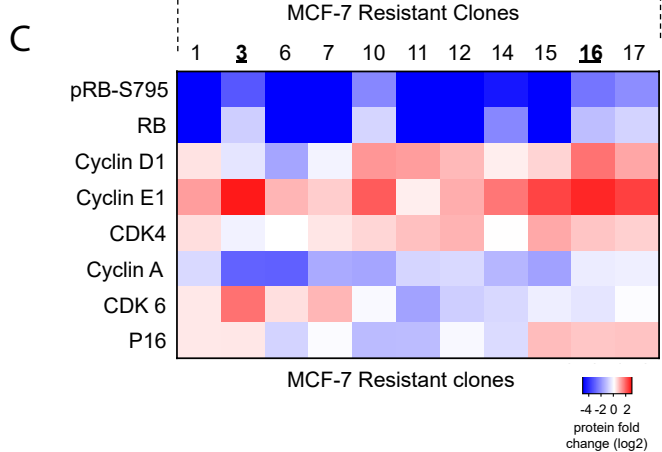
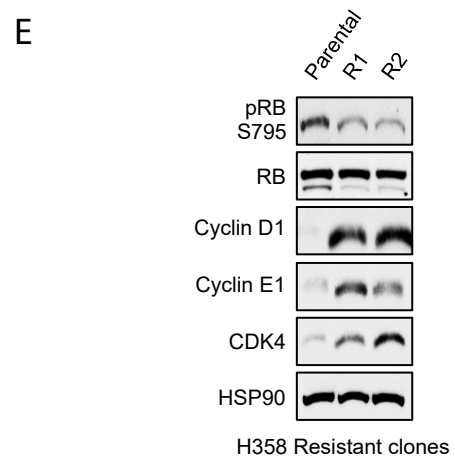
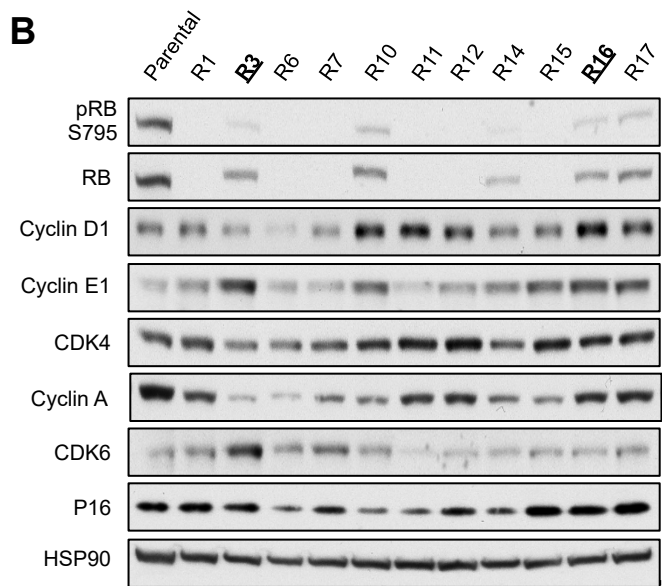
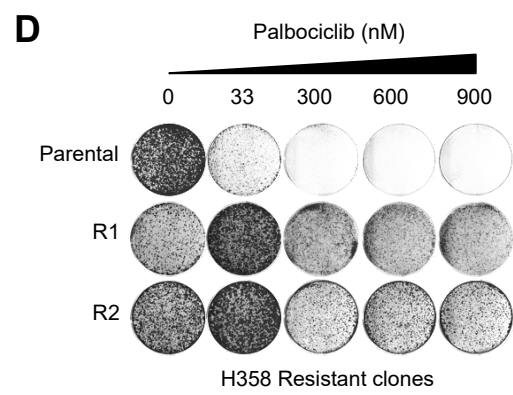
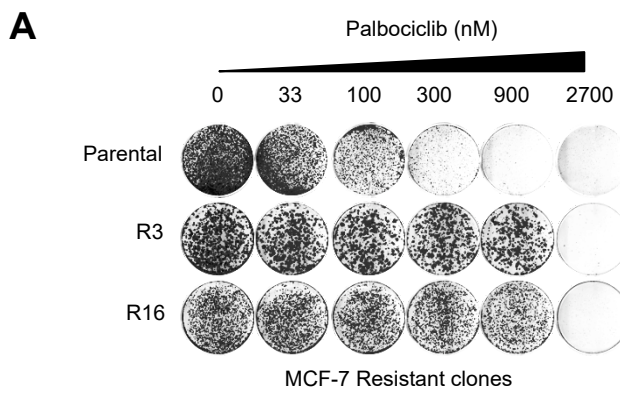


D



C





Molecular Cancer Therapeutics

eIF4A inhibitors suppress cell cycle feedback response and acquired resistance to CDK4/6 inhibition in cancer

Tim Kong, Yibo Xue, Regina Cencic, et al.

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