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# Cyclin-dependent kinase 4 inhibits the translational repressor 4E-BP1 to promote cap-dependent translation during mitosis–G1 transition

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**Phosphorylation of translational repressor eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) controls the initiation of cap-dependent translation, a type of protein synthesis that is frequently upregulated in human diseases such as cancer. Because of its critical cellular function, it is not surprising that multiple kinases can post-translationally modify 4E-BP1 to drive aberrant cap-dependent translation. We recently reported a site-selective chemoproteomic method for uncovering kinase–substrate interactions, and using this approach, we discovered the cyclin-dependent kinase (CDK)4 as a new 4E-BP1 kinase. Herein, we describe our extension of this work and reveal the role of CDK4 in modulating 4E-BP1 activity in the transition from mitosis to G1, thereby demonstrating a novel role for this kinase in cell cycle regulation.**

**Keywords:** 4E-BP1; cap-dependent translation; CDK4; mitosis

Cap-dependent translation is an important cellular process that controls the translation of select mRNAs typically encoding for growth factors and oncogenes [1–4]. The initiation of cap-dependent mRNA translation is governed by the availability of eIF4E, the $m^7$GpppX-cap-binding translation initiation factor [5,6]. This protein is highly regulated, primarily through the work of the 4E-BPs, which sequester eIF4E from eIF4G and the eIF4F translation initiation complex [7–13]. The activity of 4E-binding protein 1 (4E-BP1) is in turn regulated by phosphorylation, where hypophosphorylated 4E-BP1 binds strongly to eIF4E to inhibit translation, while hyperphosphorylated 4E-BP1 releases eIF4E to initiate cap-dependent translation [13–16]. For many years, the only validated kinase known to affect 4E-BP1 phosphorylation has been mechanistic target of rapamycin complex 1 (mTORC1), which was shown to hierarchically phosphorylate 4E-BP1 at T37 and T46 followed by T70 and S65 [14,15,17,18]. However, several findings have called into question the exclusivity of mTORC1 for each of these phosphorylation sites [19], namely reports demonstrating that other unknown kinases can also phosphorylate 4E-BP1 to stimulate cap-dependent translation [20–22], particularly in cases of mTOR inhibitor drug resistance [23–26].

Recently, our laboratory has developed a chemoproteomic pipeline by which to identify site-specific kinase–substrate interactions, Phosphosite-Accurate kinase–substrate cross(X)linking Assay or PhAXA [27]. Using this methodology, we discovered that cyclin-dependent kinase 4 (CDK4), which is primarily responsible for controlling the cell cycle checkpoint at the $G_1$/S transition through phosphorylation of the retinoblastoma tumor suppressor protein (Rb)

**Abbreviations**
4E-BP1, 4E-binding protein 1; CDK, cyclin-dependent kinase; FDR, false discovery rate; mTORC1, mechanistic target of rapamycin complex 1; PSMs, peptide-spectrum matches; WT, wild-type.

[28], regulates cap-dependent translation *via* phosphorylation of 4E-BP1 at both canonical mTORC1 sites (T37, T46, T70) in addition to a noncanonical site (S101) [27]. Importantly, we found that CDK4 can promote rapamycin-resistant cap-dependent translation through this function, and inhibition of CDK4 using the clinically approved CDK4/6 inhibitor palbociclib led to a significant reduction in the expression of cap-dependent transcripts c-Myc and cyclins D2 and D3 [27]. Moreover, we found that inhibition of both mTORC1 and CDK4 could cooperatively antagonize the initiation of cap-dependent translation [27].

While our report is the first to directly connect CDK4 to 4E-BP1 regulation, this is not the only CDK linked to phosphorylation of this translational repressor. CDK12, *via* phosphorylation of 4E-BP1 at S65 and T70, cooperates with mTORC1 to drive selective translation of proteins involved in maintenance of the mitotic genome [29]. Additionally, CDK1, the master regulator of the $G_2$/M transition, can substitute for mTORC1 to phosphorylate 4E-BP1 at the putative mTORC1 sites to activate cap-dependent translation during mitosis [30,31] and meiosis [32]. A mechanistic investigation of the mitotic phosphorylation of 4E-BP1 uncovered CDK1-mediated phosphorylation of the noncanonical site S83. This poorly understood phosphorylation site was demonstrated to function outside of the traditional context of eIF4E regulation and cap-dependent translation, and instead localizes 4E-BP1 to the mitotic spindle [33]. Further evaluation of cell cycle-dependent cap-dependent translation has found that eIF4G interacts with eIF4E to similar degrees in interphase and mitotic cells [34]. These findings are exciting, as it was previously thought that mitosis was associated with a decrease in cap-dependent translation [35,36], and, together, hint at a larger role for phosphorylated 4E-BP1 and cap-dependent translation in regulation of the cell cycle [37]. However, as the studies linking CDK1 and 4E-BP1 relied on the use of the CDK1 inhibitor RO-3308 [38], which is typically used as a tool compound for inducing mitotic arrest at the precipice of prophase [39], they provide only a snapshot of the regulation of 4E-BP1 during a narrow window of mitosis. Thus, we became interested in using PhAXA to uncover potentially novel kinases that regulate 4E-BP1 hyperphosphorylation in later stages of mitosis once the anaphase-promoting complex mediates degradation of cyclin B1, rendering CDK1 inactive [40]. Herein, we describe these efforts and reveal a novel function of CDK4 in driving mitotic cap-dependent translation and $G_2$/M cell cycle progression.

## Materials and methods

### Reagents

Palbociclib isethionate (Selleckchem, Houston, TX, USA) was dissolved in water. Rapamycin (Alfa Aesar, Tewksbury, MA, USA) and SP600125 (ApexBio, Houston, TX, USA) were dissolved in DMSO. Human recombinant insulin was purchased from Sigma (St. Louis, MO, USA). Nocodazole and 3XFLAG peptide were purchased from ApexBio. All reagents were used as received.

### Cell culture

HEK293T and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Corning, Tewksbury, MA, USA) supplemented with 10% FBS, glutamine, penicillin, and streptomycin (Gibco, Gaithersburg, MD, USA). U2 OS cells were kindly provided by Beth Lawlor and cultured according to ATCC guidelines. MDA-MB-231 cells were a kind gift from Nouri Neamati and grown in RPMI-1640 media supplemented with 10% FBS and glutamine. MDA-MB-468 and MCF-7 cells were a kind gift from Max Wicha. MDA-MB-468 cells were grown in DMEM (Corning) supplemented with 10% FBS and glutamine. MCF-7 cells were cultured according to ATCC guidelines. Cells were grown at 37 °C with 5% $CO_2$ in a humidified incubator and passaged at least twice before use for experiments and no more than 10 times before returning to low-passage stocks. All cell lines were authenticated by STR profiling and regularly tested for mycoplasma contamination.

### Immunoblotting

Cells were lysed directly in-well using RIPA buffer (10 mM Tris/HCl, 150 mM NaCl, 1% Triton, 1% sodium deoxycholate, 0.1% SDS, pH 7.2) supplemented with 10 $\mu g \cdot mL^{-1}$ aprotinin, 5 $\mu g \cdot mL^{-1}$ leupeptin, 7 $\mu g \cdot mL^{-1}$ pepstatin, 10 mM NaF, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, and 2 mM sodium pyrophosphate). Lysates were then sonicated thoroughly on ice. Protein concentrations were normalized by the bicinchoninic acid assay (Pierce, Waltham, MA, USA), resolved on 4–20% Tris/glycine gels (Invitrogen, Waltham, MA, USA), transferred to 0.45-μm poly(vinylidene difluoride) (Thermo, Waltham, MA, USA) using Towbin's buffer (low amperage for ~ 4 h at 4 °C), blocked with 5% nonfat milk in TBST, and then probed with primary antibodies overnight at 4 °C. Antibodies used in this study were as follows: Actin-HRP (sc-47778) from Santa Cruz Biotechnology (Dallas, TX, USA); and CDK4 (12790), Cyclin D2 (3741), 4E-BP1 (9644), p4E-BP1 (T37/46) (2855), p4E-BP1 (S65/101) (9451), p4E-BP1 (T70) (9455), Rb (9313), pRb (S780) (3590), eIF4E (9742), eIF4G (2498), and pS6 (240/244) (2215) from Cell Signaling Technology (Danvers, MA, USA).

### Chemoproteomics

The Phosphosite-Accurate kinase–substrate cross(X)linking Assay (PhAXA) was carried out as previously described [27], with changes to the database search and relative protein quantification. Protein identification and quantification were performed using MAXQUANT (version 1.6.7.0) [41,42]. MS/MS spectra were searched with Andromeda against the reference human database from UniProt (02-02-2014 download) appended with common contaminants and the automatically generated reverse database for the decoy search, which was used to calculate the false discovery rate (FDR). Carbamidomethylation of cysteine (57.021464 Da) was set as a fixed modification; acetylation of protein N termini (42.010565 Da) and oxidation of methionine (15.994915 Da) were set as variable modifications. Other search parameters included fixed main-search MS1 error of 4.5 p.p.m., with 0.5 Da mass deviation allowed for fragment ions, and minimum peptide length of 7; two missed cleavages were allowed. Match between runs was enabled with a match time window of 0.5 min.

### Data analysis

Relative quantification of proteins was achieved with the MaxLFQ algorithm using default settings. Proteins identified with a FDR of < 1% were further filtered by removal of known contaminants and decoy proteins, and those proteins identified by fewer than three peptide-spectrum matches (PSMs) in both samples, as well as those identified by a single peptide. This final list of proteins was loaded into PERSEUS (version 1.6.5.0) [43] and LFQ intensities were $\log_2$-transformed before imputing missing values column-wise, based on a normal distribution (downshift of 1.8 and a width of 0.3).

### $m^7$GDP cap affinity assay

The cap pull-down assay was carried out as previously described [44,45].

### NanoBiT assay

eIF4E was digested out of HaloTag-eIF4E using SgfI and PmeI and then ligated into pFN33K (Promega, Madison, WI, USA) to obtain the LgBiT-eIF4E construct. 4E-BP1 was cloned into pFN35K (Promega) using the same method to obtain the SmBiT-4E-BP1 construct. HaloTag-4E-BP1 and HaloTag-eIF4E have been described elsewhere [27,46]. pFN33K LgBiT-eIF4E and pFN35K SmBiT-4E-BP1 (50 ng each) were reverse-transfected into MCF-7 and MDA-MB-468 cells in a 96-well white opaque plate using Lipofectamine LTX with PLUS reagent. After 16 h, cells were arrested with nocodazole (500 nM). Twenty hours later, cells were treated with rapamycin (100 nM) and/or palbociclib (5 μM). After 2-h incubation, Nano-Glo Live Cell reagent (Promega #N2011; 25 μL) was added and total luminescence was read within 40 min on a BioTek (Winooski, VT, USA) Cytation 3 reader.

### Flow cytometry

Cells were harvested with trypsin, washed once with ice-cold 1× PBS containing 1% FBS, and resuspended in ice-cold 1× PBS, and then, 100% ice-cold ethanol was added dropwise to a final concentration of 70%. Cells were fixed at −20 °C for 4 h and then stored at 4 °C for 18–96 h. Fixed cells were washed twice with 1× PBS containing 1% FBS, resuspended in 1× PBS containing propidium iodide (50 $\mu g \cdot mL^{-1}$; Sigma) and RNase A (100 $\mu g \cdot mL^{-1}$; Fisher), and incubated at 37 °C for 30 min. Cells were then filtered and analyzed using a CytoFLEX flow cytometer (Beckman, Indianapolis, IN, USA). Cell cycle distributions were analyzed using FLOWJO (v10, FlowJo, LLC, Ashland, OR, USA).

### Statistical analysis

Two-sided *t*-tests were performed using PRISM (v7, GraphPad Software, San Diego, CA, USA); equal variance between samples being compared was established. Graphs show mean ± SEM or ±SD as described in the figure legends.

## Results and Discussion

Given our limited knowledge regarding 4E-BP1 phosphorylation in mitosis, coupled with previous reports which demonstrated that this post-translational modification is mTOR-independent [30,33], we used PhAXA-based chemoproteomic profiling [27] to identify candidate 4E-BP1 kinases. HEK293T cells were transiently transfected with wild-type (WT) or T46C mutant FLAG-4E-BP1 probes, arrested in prometaphase using nocodazole, and released into media containing insulin before harvesting and proceeding with PhAXA analysis [27]. Four protein kinases were identified: mTOR, CDK4, Erk2, and PRKDC, with mTOR and CDK4 showing the greatest level of enrichment, in line with our previous results using asynchronous cells (Fig. 1A, B) [27]. Pull-down of CDK4 from lysate was then confirmed *via* PhAXA and western blot [27], and enrichment was observed from samples expressing both T37C and T46C mutant probes (Fig. 1C).

The discovery of CDK4 was unexpected given the relative lack of information linking CDK4 to regulation of mitosis and/or cytokinesis. Thus, we examined a panel of cell lines to determine the role of CDK4 in mediating mitotic phosphorylation of 4E-BP1. To probe this, prometaphase-arrested cells were released

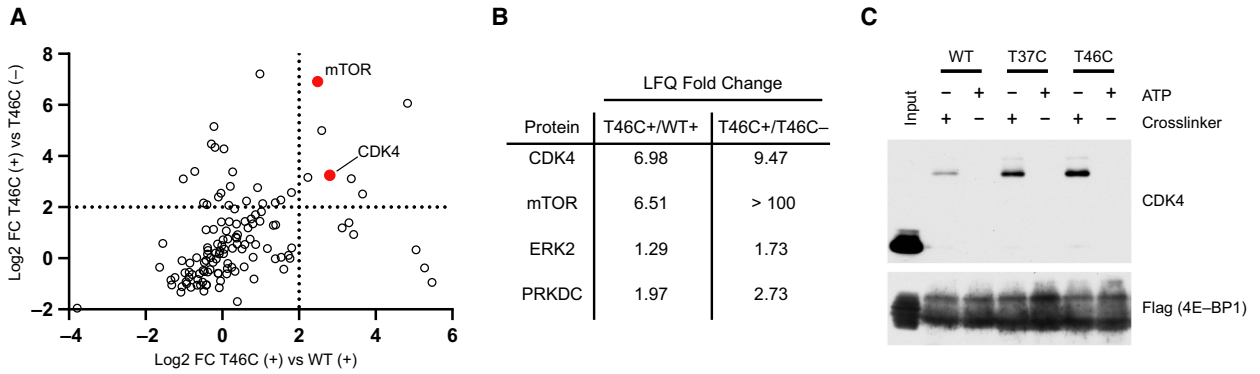


**Fig. 1.** PhAXA identifies putative 4E-BP1 kinases in cells released from a nocodazole arrest. (A) $Log_2$ fold change of LFQ ratios for the 3XFLAG-4E-BP1 (T46) probe relative to appropriate controls. '+' refers to samples treated with crosslinker, while '−' refers to ATP only controls. Fold changes were capped at 100 for this graph. Dotted lines represent fourfold enrichment. (B) Table of all protein kinases identified by three or more PSMs in at least one sample. (C) CDK4 is enriched from lysate generated from nocodazole-arrested cells expressing 3XFLAG-4E-BP1 WT, T37C, and T46C mutants. A representative input is included to show the mass shift of CDK4 upon crosslinking to 4E-BP1.

into media containing vehicle control, rapamycin, and/or palbociclib, and inhibition of 4E-BP1 phosphorylation was monitored by western blot. As expected, rapamycin, the allosteric mTORC1 inhibitor, had no effect on nocodazole-induced mitotic phosphorylation of 4E-BP1 (Fig. 2). However, cells released from nocodazole arrest in media containing the clinically approved CDK4/6 inhibitor palbociclib [47,48] showed a marked decrease in phosphorylation, as demonstrated by the disappearance of the β and γ bands that indicate hyperphosphorylated 4E-BP1 (Fig. 2). In MDA-MB-468 cells, 4E-BP1 phosphorylation was unaffected by CDK4 inhibition, similar to our previous findings in asynchronously proliferating cells. The lack of a response is likely due to the intrinsic resistance of this cell line to CDK4/6 inhibitors, resulting from an inability to assemble functional cyclin D–CDK4/6 complexes [49]. Interestingly, in CDK4/6 inhibitor-sensitive cells, palbociclib-induced downregulation of mitotic 4E-BP1 phosphorylation was markedly increased by combined treatment with rapamycin (Fig. 2). These findings indicate that mTOR and CDK4 act in concert to regulate 4E-BP1 phosphorylation following release from prometaphase arrest.

To verify that palbociclib treatment was on-target, the phosphorylation state of 4E-BP1 was compared to that of Rb at S780, the canonical CDK4 substrate. A broad range of sensitivity to palbociclib was observed across the cell lines tested, ranging from < 330 nM in U2 OS cells to ~ 1.25 μM in HeLa cells (Fig. 3A). In each case, however, the effective concentration of palbociclib required to inhibit both 4E-BP1 and Rb phosphorylation at S780 correlated with the relative expression of CDK4 in each cell line (Fig. 3B). A similar effect was seen in HEK293T cells, which required a concentration of > 1.25 μM to fully inhibit 4E-BP1 phosphorylation and, along with HeLa cells, have the highest relative expression of CDK4 at the protein level (Fig. S1). While there are clearly other factors that affect CDK4 inhibitor sensitivity, including Rb status and D-cyclin expression levels among others [50], this correlation was noteworthy.

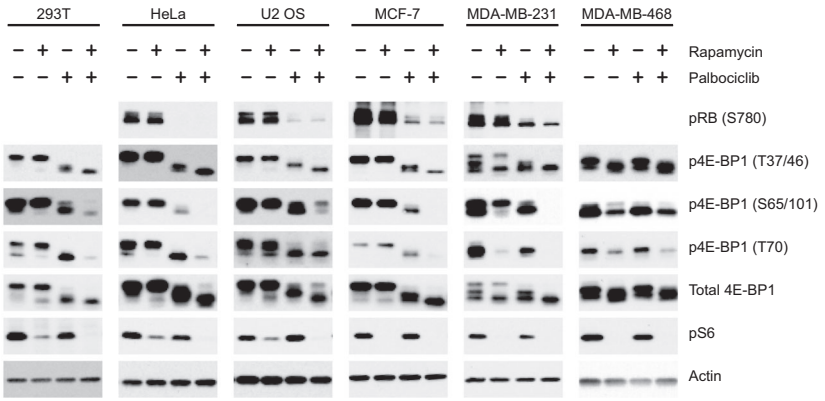


**Fig. 2.** Mitotic phosphorylation of 4E-BP1 is palbociclib-sensitive. Nocodazole-arrested cells (500 nM, 20 h) were treated with rapamycin (100 nM) and/or palbociclib (5 μM) in fresh media containing insulin (150 nM) for 2 h. Blots for pRb in HEK293T and MDA-MB-468 cells are not shown as they are Rb inactive and null, respectively.

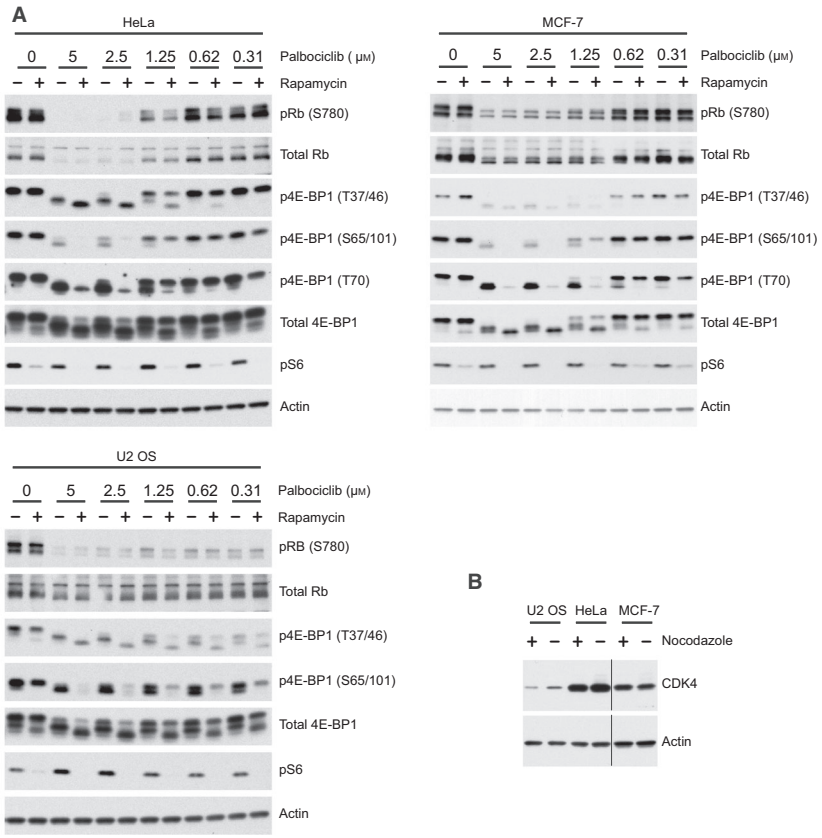


**Fig. 3.** 4E-BP1 phosphorylation mirrors pRb in a dose-dependent manner. (A) Nocodazole-arrested cells were treated with rapamycin (100 nM) and/or palbociclib (5 μM–313 nM) in fresh media containing insulin (150 nM) for 2 h. (B) Western blot of relative CDK4 expression in cell lines used in (A). Samples were run on the same gel and are from the same exposure, with unnecessary lanes removed for clarity.

While CDK4 inhibition clearly reduced mitotic phosphorylation of 4E-BP1, we were unsure of its impact on cap-dependent translation. Thus, we utilized the $m^7$GDP cap-binding assay (Fig. 4B) to measure assembly of the eIF4F translation initiation complex [51]. Mitotic MCF-7 and HeLa cells treated with rapamycin or palbociclib alone showed a marginal increase in 4E-BP1 associated with cap-bound eIF4E; yet, the eIF4G–eIF4E interaction was only modestly affected by these conditions (Fig. 4A). With the combination, however, inhibition of eIF4F complex formation was enhanced (Fig. 4A). This benefit was observed only in cells released from nocodazole arrest, as short treatments with palbociclib had very little effect on eIF4F assembly in asynchronous cells (Fig. 4A). As expected, MDA-MB-468 cells did not respond to CDK4 inhibition, but displayed a predictable response to rapamycin (Fig. 4A).

As further confirmation of the impact of CDK4 on cap-dependent translation, we analyzed the expression of cyclin D2, an established cap-dependent transcript [52], in mitotic and asynchronous U2 OS cells treated with palbociclib and/or rapamycin. In asynchronous cells, inhibition of CDK4 had no effect, whereas rapamycin markedly reduced the levels of this protein (Fig. 4C). Conversely, in cells recently released from a prometaphase block, rapamycin had negligible effect, while inhibition of CDK4 resulted in a reduction of cyclin D2 at the protein level (Fig. 4C). Because cyclin D2 expression was found to be higher in nocodazole-arrested cells than in asynchronous cells, this may allude to a requirement for CDK4/6 activity postmetaphase.

Although the $m^7$GDP cap affinity assay is a robust way of analyzing the ratio of 4E-BP1- and eIF4G-bound eIF4E, it is performed in lysate, thus rendering any intricacies in subcellular localization of these proteins indeterminable. Therefore, a split-nanoluciferase-based assay was developed to quantify the eIF4E–4E-BP1 interaction in live cells (Fig. 5A) [53]. Using this assay, we found that inhibition of CDK4 caused a large increase in this interaction in MCF-7 cells, an effect that was compounded by tandem inhibition of mTORC1 with rapamycin (Fig. 5B). As expected, no change in the eIF4E–4E-BP1 interaction was observed in MDA-MB-468 cells upon CDK4/6 inhibition. Of note, attempts to develop a related assay for measuring the eIF4E–eIF4G interaction were unsuccessful, likely due to the large size of eIF4G (220 kDa) and the relative orientation of the N and C termini of the proteins. Together, these results

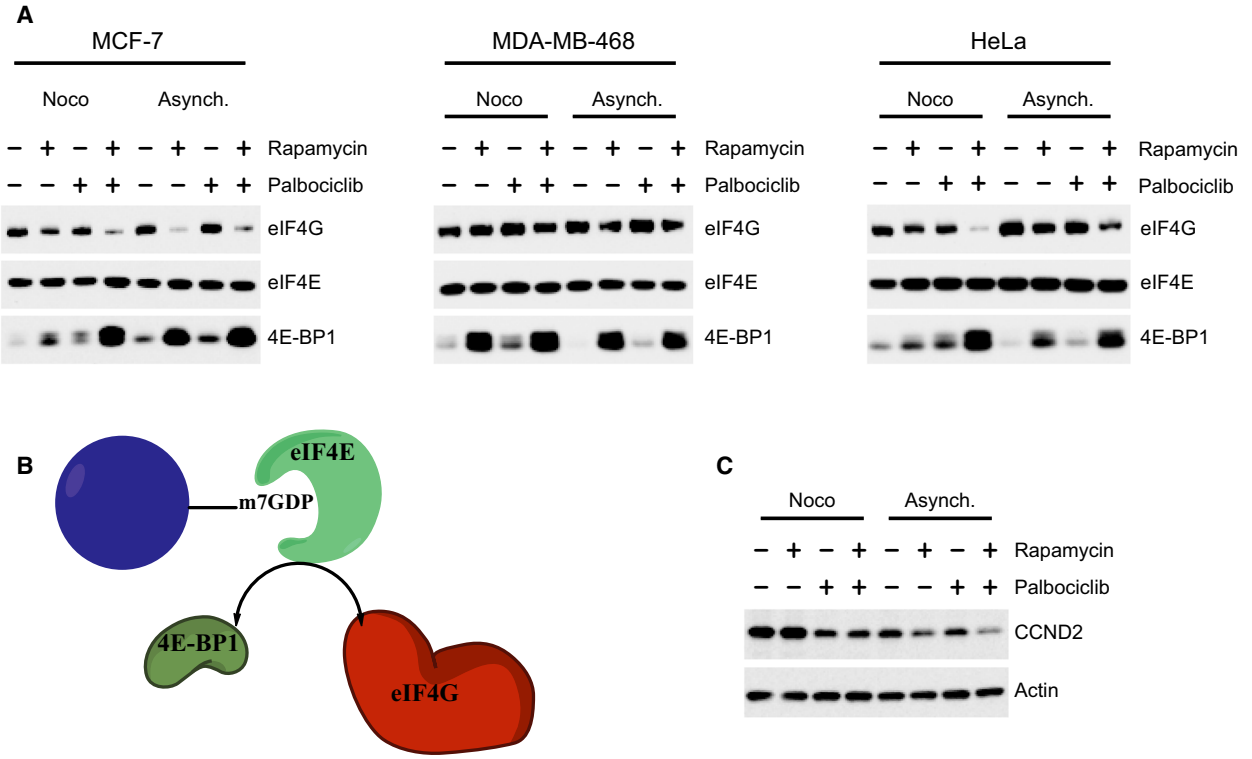


**Fig. 4.** mTOR and CDK4 coregulate mitotic cap-dependent translation. (A) $m^7$GDP cap affinity assay. Palbociclib and rapamycin cooperate to increase eIF4E–4E-BP1 association and decrease eIF4E–eIF4G association in palbociclib-sensitive cells. (B) Schematic for the cap affinity assay. (C) Palbociclib results in the reduction of cap-dependent translation of cyclin D2 in nocodazole-arrested U2 OS cells. U2 OS cells were used for this experiment as they are one of the few cell lines in our panel to express satisfactory amounts of cyclin D2. All experiments were performed in biological triplicate; representative images are shown. Replicates of the data shown in (A and C) can be found in Fig. S2.

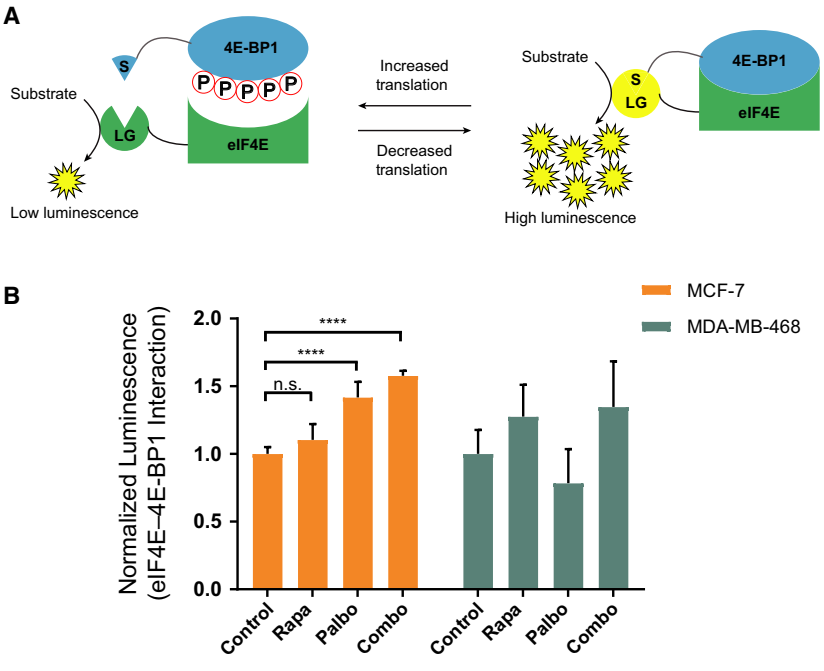


**Fig. 5.** mTOR and CDK4 regulate eIF4E–4E-BP1 association in mitotic cells. (A) Schematic for the nanoluciferase assay monitoring the eIF4E–4E-BP1 interaction in live cells. S = SmBiT; LG = LargeBiT. For data shown in panel (B), the error is plotted as ±SEM, $n = 5$.

demonstrate that CDK4-mediated phosphorylation of 4E-BP1 can promote mitotic cap-dependent translation in the absence of mTORC1 signaling.

Finally, to characterize the effect of CDK4 inhibition on progression through mitosis into $G_1$, the cell cycle distribution was analyzed at several time points following release from a prometaphase arrest. As cell lines, we chose MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cells that demonstrate high, medium, or no sensitivity to palbociclib, respectively [45,52]. MCF-

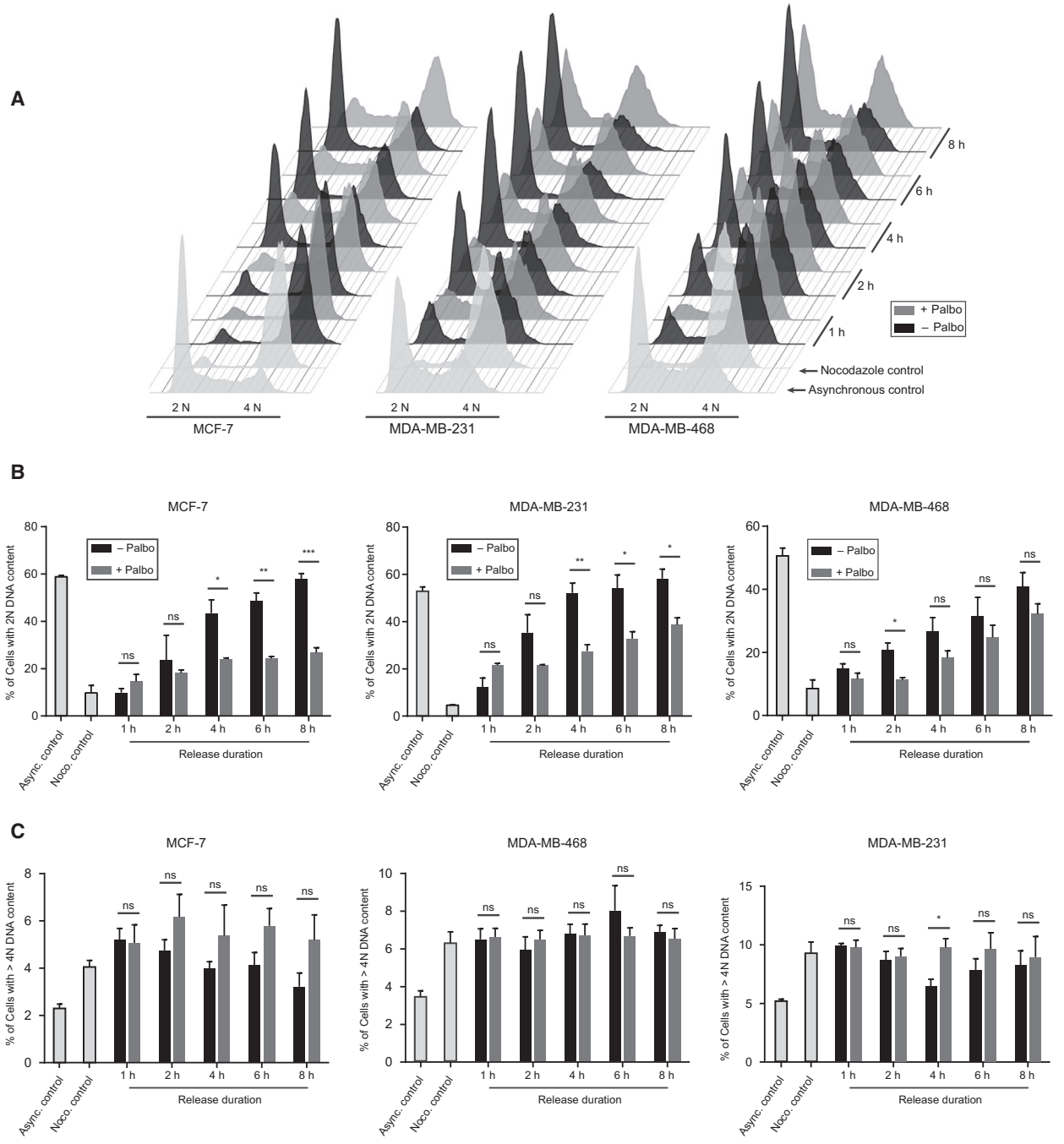


**Fig. 6.** CDK4 inhibition results in a $G_2$ block in palbociclib-sensitive cells. (A) Full cell cycle profiles of cells arrested with nocodazole and then released into media with or without palbociclib (5 μM) for the indicated time points. (B) Quantification of the percentage of cells from (A) with 2N DNA content. (C) Quantification of the percentage of cells from (A) with > 4N DNA content. Data are represented as the mean ± SE of three biological replicates.

7 cells treated with palbociclib showed a profound $G_2$ block relative to control cells, as they were unable to fully transition into $G_1$ (Fig. 6A,B). In the CDK4 inhibitor-resistant MDA-MB-468 cell line, palbociclib had very little effect after an initial $G_2$ delay. As expected, the MDA-MB-231 cell line showed an intermediate $G_2$ block based on its intermediate potency for CDK4/6 inhibition [54]. Of note, these results were not influenced by a change in polyploidy (Fig. 6C).

In context of the canonical role of CDK4, our findings are quite interesting, as CDK4 is believed to be primarily responsible for controlling the checkpoint at the $G_1$/S transition [28]. However, several previous studies have demonstrated CDK4 activity outside of this phase of the cell cycle [55–58]. First, cyclin D–CDK4 complexes have been observed to persist throughout the cell cycle although their function is entirely unknown [55,56]. Moreover, it has previously been found that cyclin D3–CDK4 activity is essential for progression through $G_2$ [57,58] and that ionizing radiation-induced activation of p16 leads to a $G_2$ phase delay *via* inhibition of CDK4 [57]. In fact, our own analysis of pRb phosphorylation at S780 showed an increase in CDK4 activity in the majority of cells released from a prometaphase arrest across a panel of cell lines (Fig. S3). These data support an essential role of cyclin D–CDK4 in progression into $G_1$ from $G_2$/M and provide evidence that pharmacological inhibition can result in a $G_2$ block even in p16-null cancer cell lines, such as MCF-7 [59]. Although the exact mechanism of this $G_2$ delay is not fully understood, it is possible that inhibition of Rb phosphorylation is the primary factor governing this phenotype. Alternatively, an inability to translate critical anabolic proteins due to inhibited cap-dependent translation may play a role. However, the fact that palbociclib treatment slows $G_1$ entry in Rb-null MDA-MB-468 cells at early time points following release suggests that another CDK4 activity may be important for regulation of this transition. This could be due to an altered transcriptional program induced by inhibition of phosphorylation of FOXM1, a master transcription factor that regulates expression of genes essential for mitosis [60]. While it is unclear at what stage of the cell cycle these cells are being blocked, in later stages of mitosis or simply a failure to undergo cytokinesis, it is clear that a postmitotic checkpoint is in part regulated by CDK4, and gaining a better understanding of the mechanism behind this warrants further consideration.

## Conclusions

In conclusion, using a chemoproteomic approach, we have discovered that CDK4 phosphorylates 4E-BP1 during the M-to-$G_1$ transition, thereby maintaining cap-dependent translation. These findings shed further light on the cell cycle-dependent phosphorylation of 4E-BP1, the regulation of which was previously reported to be mediated by CDK1 and CDK12 in mitotic cells [29,61,62], and PLK1 and CDK1 in cells undergoing meiosis [22,32,63–65]. Given this newly discovered role of CDK4, it is likely that inhibition of mitotic 4E-BP1 phosphorylation is a previously unknown function of CDK4/6 inhibitors such as palbociclib, and may explain the synergy between these drugs and mTOR inhibitors [66–72]. It is important to try to reconcile these observations with those previously observed that report CDK1 as the principal kinase that phosphorylates 4E-BP1 during mitosis [30,33]. It is possible that given the differences in experimental design, CDK1 phosphorylates 4E-BP1 in prometaphase, but in later stages of mitosis and/or cytokinesis, CDK4 assumes that role. It is also possible that S101 phosphorylation, which we previously identified as strictly CDK4-dependent and important for the global phosphorylation of 4E-BP1 [27], renders CDK1 effectively null toward the reported CDK1 sites. Finally, although mTORC1 is inactive during mitosis [35,61,62,73], we have found that inhibition of both CDK4/6 and mTORC1 provides the most robust decrease in 4E-BP1 phosphorylation in prometaphase-released cells. To explain this cooperativity, we are investigating the possibility that CDK4 inhibitors induce mTORC1 reactivation in response to mitosis-associated dephosphorylation of 4E-BP1. Thus, our working hypothesis is that mTOR, CDK1, and CDK4 all act in concert to regulate cap-dependent translation during G2, mitosis, and the transition into $G_1$. Future efforts will be focused on the investigation of this interplay among kinases throughout the cell cycle.

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## Author contributions

ALG and DCM conceived the project. DCM, AM, and ALG designed the experiments. DCM and AM

performed the experiments. DCM, AM, and ALG analyzed the data. DCM and ALG wrote the manuscript.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Palbociclib-sensitive 4E-BP1 phosphorylation correlates with CDK4 expression.
**Fig. S2.** Replicate experiments from Main Text Figure 4.
**Fig. S3.** Cells arrested in mitosis exhibit increased CDK4 activity.