CDK1 substitutes for mTOR kinase to activate mitotic cap-dependent protein translation

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Mitosis is commonly thought to be associated with reduced cap-dependent protein translation. Here we show an alternative control mechanism for maintaining cap-dependent translation during mitosis revealed by a viral oncoprotein, Merkel cell polyomavirus small T (MCV sT). We find MCV sT to be a promiscuous E3 ligase inhibitor targeting the anaphase-promoting complex, which increases cell mitogenesis. MCV sT binds through its Large T stabilization domain region to cell division cycle protein 20 (Cdc20) and, possibly, cdc20 homolog 1 (Cdh1) E3 ligase adapters. This activates cyclin-dependent kinase 1/cyclin B1 (CDK1/CYCB1) to directly hyperphosphorylate eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) at authentic sites, generating a mitosis-specific, mechanistic target of rapamycin (mTOR) inhibitor-resistant, 4E-BP1 phosphorylation pattern present in G1-arrested cells. Recombinant 4E-BP1 inhibits capped mRNA reticulocyte translation, which is partially reversed by CDK1/CYCB1 phosphorylation of 4E-BP1. elf4E binding to the elf4E-m7GTP cap complex is resistant to mTOR inhibition during mitosis but sensitive during interphase. Flow cytometry, with and without sT, reveals an orthogonal pH3120+ mitotic cell population having higher inactive p4E-BP1T37/46 saturation levels than pH3120− interphase cells. Using a Click-iT flow cytometric assay to directly measure mitotic protein synthesis, we find that most new protein synthesis during mitosis is cap-dependent, a result confirmed using the eIF4E/4G inhibitor drug 4E1RCat. For most cell lines tested, cap-dependent, a result confirmed using the eIF4E/4G inhibitor drug 4E1RCat. For most cell lines tested, cap-dependent translation levels were generally similar between mitotic and interphase cells, and the majority of new mitotic protein synthesis was cap-dependent. These findings suggest that mitotic cap-dependent translation is generally sustained during mitosis by CDK1 phosphorylation of 4E-BP1 even under conditions of reduced mTOR signaling.

Significance

Cancer cell proliferation is highly dependent on cap-dependent protein synthesis, which is generally assumed to be inhibited during mitosis. Using a viral oncoprotein that enforces mitosis, we show that CDK1 substitutes for mTOR interphase functions to hyperphosphorylate eukaryotic initiation factor 4E-binding protein (4E-BP1) to a mitosis-specific isoform. Flow cytometric assays reveal that mitotic cells have high levels of inactivated 4E-BP1 and do not generally show specific loss of cap-dependent translation compared with interphase cells. This appears to be due to cyclin-dependent kinase 1 (CDK1) activity during mitosis. Mitotic cells typically represent less than 1% of all cells in bulk culture, and mitosis-arresting drugs, such as nocodazole, can directly inhibit mitotic protein translation, potentially explaining differences between our findings and previous studies showing reduced cap-dependent translation during mitosis.


The authors declare no conflict of interest.

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MCV sT have increased mitotic rounding and a 6-fold increase in pH3S10+ mitotic cells compared with empty vector or sTmLSD transduced cells. (Fig. 1C). These APC/C E3 targets retained rapid turnover in the presence of empty vector or sTmLSD expression. CYCD1, which is not directly regulated by APC/C (27), was unaffected by MCV sT or sTmLSD expression. Similarly, MCV sT expression stabilized FLAG-tagged AURKA and endogenous CYCB1, but not CYCD1, after nocodazole release of 293 cells, whereas MCV sTmLSD expression did not (Fig. S2).

**Results**

**MCV sT Increases Mitogenesis by Targeting the Cellular APC E3 Ligase.** To search for factors contributing to MCV sT-induced transcriptional activation, the viral oncoprotein was expressed in hTERT-immortalized primary BJ-tert (BJ-T) human foreskin fibroblasts. These cells displayed a rounded phenotype in culture with increased phospho-histone H3 serine 10 (pH3S10) phosphorylation, characteristic for mitosis (Fig. 1A). Increased pH3S10 and increased expression of mitotic markers [including cyclin B1 (CYCB1) and phospho-aurora kinase B (pAURKB)] were also observed in 293 cells expressing MCV sT (Fig. S1). Immunoprecipitation of sT revealed an in vivo complex with the APC/C substrate recognition subunit cell division cycle protein 20 (Cdc20) that was dependent on an intact LSD (Fig. 1B). MCV sT also interacted with another APC/C substrate recognition subunit, cdc20 homolog 1 (Cdh1), but substantial Cdh1 binding occurred with sTmLSD having alanine substitutions at residues 91-95, suggesting that sT may bind Cdh1 at other sites in addition to the LSD. In line with these results, known APC/C E3 targets, including AURKA and AURKB, Skp2, polo-like kinase 1 (Plk1), and CYCA2, showed markedly reduced turnover in the presence of sT or sTmLSD expression. (Fig. 1C). These APC/C E3 targets retained rapid turnover in the presence of empty vector or sTmLSD expression. CYCD1, which is not directly regulated by APC/C (27), was unaffected by MCV sT or sTmLSD expression. Similarly, MCV sT expression stabilized FLAG-tagged AURKA and endogenous CYCB1, but not CYCD1, after nocodazole release of 293 cells, whereas MCV sTmLSD expression did not (Fig. S2).

**MCV sT Induces mTOR-Independent δ-4E-BP1 Hyperphosphorylation.** We next examined the role of MCV sT in 4E-BP1 hyperphosphorylation. 4E-BP1 hyperphosphorylation isoforms are named α through δ according to ascending molecular mass (Fig. 24, Left) (28). Most notable was the appearance of the highest molecular mass form, δ, containing phosphorylation marks at heavy chain. (C) APC/C target proteins (AURKA/B, Cdc20, Skp2, Plk1, CYCA2, and clasin1) are stabilized by MCV sT expression. BJ-T cells were treated with CHX (100 μg/mL) to inhibit new protein synthesis and harvested at the indicated time points. The half-lives of proteins regulated by APC/C are extended by expression of MCV sT but not empty vector or MCV sTmLSD controls. CYCD1 is not directly regulated by Cdh1, and its half-life was unchanged by MCV sT expression. A representative α-tubulin loading control is shown. Representative results are shown from three independent experiments.

Surprisingly, sT-induced δ-4E-BP1 hyperphosphorylation is not dependent on mTOR activity (21). The sT LSD region is known to bind the Fbw7 E3 ligase to promote cell proliferation, but Fbw7 targeting is not sufficient to explain either cell transformation or 4E-BP1 hyperphosphorylation (24).

We show here that MCV sT, through its LSD domain, also promotes mitogenesis and 4E-BP1 hyperphosphorylation by functioning as a promiscuous E3 ligase inhibitor that targets cellular anaphase-promoting complex/cyclosome (APC/C) E3 ligase activity. During sT-induced mitosis, sT-induced CDK1/CYCB1 rather than mTOR directly phosphorylates 4E-BP1 to the mitosis-specific δ isoform. Using a flow cytometry-based method to directly measure mitotic cap-dependent protein synthesis for the first time, to our knowledge, we do not detect a general shift from cap-dependent to cap-independent protein translation in mitotic cells compared with interphase cells. Mitotic cells actually show higher saturation levels of p4E-BP1T37/T46+ consistent with 4E-BP1 inactivation, than interphase cells. Consistent with this, and in contrast to previous studies, we find that δ-4E-BP1-positive mitotic cells show high levels of cap-dependent protein translation that is reduced by the cap translation inhibitor 4E1RCat. When accentuated or sustained, high levels of mitotic cap-dependent protein translation may play a role in cancer cell transformation and contribute to mTOR inhibitor resistance in subsets of cancers.
CDK1/CYC81 Directly Phosphorylates 4E-BP1, in the Presence and Absence of st, to the δ Isoform During Mitosis. The 4E-BP1 phosphorylation is induced by microtubule assembly inhibitors such as nocodazole and paclitaxel that arrest cells in mitosis (15, 16).

To assess the role of various kinases on mitotic 4E-BP1 phosphorylation, nocodazole-treated HeLa mitotic cell lysates were reacted with recombinant GST–4E-BP1 and kinase inhibitors, including PP242 (mTORC1 and mTORC2), RO-3306 (CDK1), and VX-680 (pan AURK) (Fig. 3A). GST–4E-BP1 was robustly phosphorylated at authentic sites by mitotic HeLa lysates, and this was reversed by inhibition of CDK1 but not by mTOR or AURK inhibition.

Evidence that CDK1 is responsible for δ-4E-BP1 mitotic phosphorylation was also obtained by treatment of nocodazole-arrested HeLa cells with the CDK1 inhibitor RO-3306 (Fig. S34). 4E-BP1 hyperphosphorylation could not be fully restored by RO-3306/ MG132 cotreatment. A technical issue in using mitotic kinase inhibitors to assess 4E-BP1 phosphorylation is the occurrence of mitotic slippage, a side effect of kinase inhibition concurrently causing enforced exit from mitosis with general loss of mitotic kinase activities (30, 31). Mitotic slippage can be prevented by simultaneous inhibition of APC/C-mediated protein degradation with the proteasome inhibitor MG132, which in effect “freezes” the mitotic phenotype. Like RO-3306, treatment of nocodazole-arrested HeLa cells with the AURK inhibitor VX-680 also eliminated δ-4E-BP1 phosphorylation (Fig. S3B). Unlike RO-3306, however, this was completely reversed by cotreatment with VX-680/ MG132, suggesting that AURK inhibition effects on 4E-BP1 phosphorylation are due to mitotic slippage. Extensive in vitro phosphorylation studies also failed to reveal evidence for direct 4E-BP1 phosphorylation by purified AURKB. To confirm direct 4E-BP1 phosphorylation by CDK1/CYC81, we generated an in vitro phosphorylation reaction using purified CDK1/CYC81 and GST–4E-BP1 (Fig. 3B). CDK1 phosphorylation of 4E-BP1 was ATP-dependent and -inhibitable by RO-3306. CDK1 phosphorylation occurred at the previously described T70 residue (10) as well as at authentic 4E-BP1 phosphorylation sites, including T37/T46 and S65/S101, which are known to regulate 4E-BP1 binding to eIF4E.

Mitotic δ-4E-BP1 phosphorylation was also examined in nocodazole-arrested 293 cells in the presence of CDK1 and mTOR inhibitors (Fig. 3C). MG132 was added to nocodazole-arrested cells 30 min before RO-3306 treatment to prevent CDK1 inhibition-induced mitotic slippage (30). In this experiment, phosphorylate mitotic cells comprised ∼10% of the total asynchronous (no cell cycle arrest) cell population (Fig. 3 C, Left, and Fig. S1). MCV st expression promotes formation of PP242-resistant δ-4E-BP1 that is lost after treatment with RO-3306. Notably, S6235/S236 phosphorylation, a known phosphorylation mark for mTORC1 kinase activity (32, 33), is nearly ablated by PP242 but not by RO-3306. These results are consistent with st induction of δ-4E-BP1 through CDK1 rather than mTOR kinase activity.

Distinctive 4E-BP1 phosphorylation patterns were seen during nocodazole (prometaphase) and mimosine (late G1) cell cycle arrest (Fig. 3C). During nocodazole arrest, the δ-4E-BP1 isoforms became prominent even in the absence of MCV st expression. In contrast, δ-4E-BP1 isoforms were nearly absent under all conditions for cells arrested in G1 by mimosine. Whereas δ-4E-BP1 was resistant to mTOR inhibition, CDK1 inhibition during nocodazole mitotic arrest ablated δ-4E-BP1. These results were confirmed in HeLa cells treated with nocodazole and kinase inhibitors (Fig. S3).

To confirm these findings in the absence of chemical inhibitors, we used mechanical shake-off to isolate mitotic cells from st-expressing BJ-T cells (Fig. S4). This maneuver enriched the mitotic cell fraction from ∼2% to ∼66% as determined by flow cytometry with propidium iodide (PI) and pH3/S10 phosphorylation (Fig. S4A). Shake-off cells exclusively expressed the δ-4E-BP1 isoform, whereas adherent cells expressed only α-γ isofoms of 4E-BP1 (Fig. S4B). In vitro lambda phosphatase treatment of st-expressing and nocodazole-arrested 293 cell lysates showed that the high-molecular-mass 4E-BP1 isoforms, including the α-γ and δ isoforms, are formed as a result of phosphorylation rather than another type of posttranslational modification (Fig. S5).

Although PP242-inhibitable mTOR kinase activity contributes to mitotic 4E-BP1 phosphorylation, particularly for lower molecular mass α and β forms (Fig. 3C), mTOR may be dispensable for mitotic 4E-BP1 hyperphosphorylation under some conditions. U2OS cells were arrested at the G2/M boundary for 24 h using 10 μM RO-3306 (31, 34) (Fig. 3D). After RO-3306 removal, cells progressed through mitosis, with most exiting mitosis 3 h after RO-3306 release. PP242 pretreatment markedly reduced pS6S235/S236 but not δ-4E-BP1, consistent with mTOR-independent phosphorylation of 4E-BP1 during mitosis. The 293 cells failed to arrest in G2 with RO-3306 and could not be examined.

The 4E-BP1 δ Isoform Is Induced in Mitosis During Normal Cell Cycling. Nocodazole-arrest experiments suggest that δ-4E-BP1 accumulates during mitosis even in the absence of MCV st expression. To confirm this in the absence of drug treatment, 293 cells were synchronized by double-thymidine block and release, harvested

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at sequential time points, and immunostained for pH3S10 and p4E-BP1T37/T46 (Fig. 4A). For each time point after release, cells were pretreated with PP242 or DMSO vehicle control 1 h before harvesting.

Flow cytometry showed peak pH3S10-integrative entry occurring reproducibly at 10 h, which began to diminish by 12 h after release (Fig. 4A and Fig. S6). This same pattern occurred with PP242 pretreatment, although mitotic entry was more abundant at 8 h postrelease. Unexpectedly, pH3S10+ mitotic 293 cells formed an orthogonal population with the highest per-cell saturation levels of p4E-BP1T37/T46 compared with any other stage of the cell cycle. PP242 pretreatment reduced p4E-BP1T37/T46+ staining in interphase cells at 2–8 h (note leftward shift for p4E-BP1T37/T46+ staining among pH3S10+ cells) consistent with mTOR regulation of 4E-BP1. At peak mitotic entry (8–10 h postrelease), however, pH3S10+ cells were resistant to loss of p4E-BP1T37/T46+ staining with PP242 treatment.

Immunoblotting performed on these same cell fractions at each time point (Fig. 4B) showed prominent α-γ-E-BP1 phosphorylation at early time points (0–6 h), which was sensitive to mTOR inhibition. The δ isoform emerged 8–12 h after release, corresponding to maximum pH3S10+ and p4E-BP1T37/T46+ staining, and was resistant to PP242 inhibition. Similar results, but with a less abundant orthogonal pH3S10+/p4E-BP1T37/T46+ cell population, were seen in U2OS cells (Fig. S7).

**CDK1/CYC81 Activates Cap-Dependent Translation During Mitosis**

According to the existing model for 4E-BP1-regulated protein synthesis, high levels of p4E-BP1T37/T46+ are predicted to promote cap-dependent translation during pH3S10+ mitosis (35). We directly examined this by using cap-binding assays for mitosis-enriched
and -depleted cells and by using a flow cytometry method designed to directly measure single-cell cap-dependent protein synthesis. We performed m7GTP cap resin pulldown assays to assess the functional correlates of our flow cytometry and immunoblot findings. Highly enriched mitotic BJ-T cells expressing MCV sT, isolated by shake-off (nonadherent), showed m7GTP cap binding to eIF4G that was unaffected by PP242 treatment (Fig. 5A). In contrast, although interphase-enriched BJ-T cells (adherent) had comparable levels of eIF4G, eIF4G cap binding remained sensitive to PP242. Input 4E-BP1 protein from mitosis-enriched BJ-T cells (Fig. 5B). Similar analyses using double thymidine block and release synchronization of 293 cells, however, revealed that PP242 reduced new protein synthesis for both mitotic and interphase cells (Fig. S9), suggesting that PP242 resistance may be cell line specific.

We next generated capped, polyadenylated luciferase reporter mRNA using T7 polymerase (38, 39) and performed in vitro translation in commercial rabbit reticulocyte lysates in medium containing methionine analog L-azidohomoalanine (AHA) to measure cap-dependent translation (Fig. 5C). Addition of 4E1RCat (40), a cap-dependent translation inhibitor that prevents eIF4F formation, virtually abolished translation. Addition of recombinant GST–4E-BP1 reduced cap-dependent translation in the reticulocyte lysates to 20% of buffer control (Fig. 5B). This inhibition was reduced to 45% of buffer control when GST–4E-BP1 was phosphorylated (p4E-BP1) by a CDK1/CYCBI kinase reaction. This reversal of inhibition was antagonized by the CDK1 inhibitor RO-3306. Measurement of cap-dependent protein synthesis during mitosis was directly determined for HeLa and U2OS cells after G2 release and synchronization using our AHA assay in cells treated with 4E1RCat (Fig. 5D). Costaining for pH3S10 allowed segregation of cells into mitotic (pH3S10+) and interphase (pH3S10−) populations. Non-specific AHA incorporation was determined using the ribosome translation elongation inhibitor CHX (Fig. 5D, vertical lines), and new protein synthesis was reflected by AHA fluorescence above this baseline. Like BJ-T cells, fewer (27%) mitotic HeLa cells were positive for new protein synthesis compared with interphase (46%) HeLa cells (Fig. 5D). In contrast, percentages of mitotic and interphase U2OS cells with new protein synthesis were identical (42%) for mitotic and interphase cells. For both cell lines, however, nearly all new protein synthesis in both mitosis and interphase was cap-dependent and -sensitive to 4E1RCat treatment. Preliminary analyses revealed that MG132 treatment nonspecifically inhibited protein synthesis as previously reported (41), preventing us from accurately measuring the effects of CDK1 inhibition on mitotic translation under conditions that inhibit mitotic slippage. Using direct AHA uptake, however, we could confirm that nucodazole treatment specifically inhibits mitotic protein synthesis (Fig. S10).

**Discussion**

Tumor viruses have been central to cell biology because their oncoproteins allow interrogation of specific cell proliferation and survival pathways. Among many critical findings, viral oncoproteins have been essential to the discovery of cellular oncoproteins (42) and the tumor suppressor p53 (43–45), the characterization of the G1/S checkpoint (46) and the Akt-mTOR pathway (47), and identification of common innate immune and tumor suppressor signaling networks (48). MCV sT, an oncoprotein for MCC, induces mTOR-resistant 4E-BP1 hyperphosphorylation and cell transformation (21), which led us to investigate mTOR-independent 4E-BP1 signaling and cap-dependent translation in mitosis.

In addition to targeting Fbw7 (24), MCV sT inhibits APC/C E3 ligases and induces mitogenesis in sT-expressing cells. One consequence of this is increased mitotic CDK1/CYCB1 activity that is responsible for 4E-BP1 phosphorylation and 6-4E-BP1 formation. Caution is appropriate in interpreting our data, as mitotic kinase inhibition can cause mitotic slippage and exit from the mitotic phenotype. Considerable effort by our group was devoted to evaluating AURKA and AURKB as potential mitotic kinases, because AURK inhibitors (e.g., VX-680, MK-5108, and AZD-1152) also reduce 4E-BP1 hyperphosphorylation during mitosis. This was reversible, however, by cotreatment with MG132 to prevent APC/C-mediated mitotic egress, and we have no evidence that AURKs are directly responsible for 4E-BP1 phosphorylation.
In contrast, there is considerable evidence from this study and others (10, 16) to indicate that CDK1/CYCB1 is a bona fide kinase for 4E-BP1. This study suggests an alternative pathway for CDK1/CYCB1-regulated cap-dependent translation during mitosis (Fig. 6). We find that mitotic 4E-BP1 is highly phosphorylated at the priming residues T37 and T46 in pH310- cells, which runs counter to what would be predicted if cap-dependent translation is reduced during mitosis through an mTOR-related mechanism. The high-molecular-mass δ-4E-BP1 isoform is specific to mitosis, and our data indicate that this results from CDK1-mediated phosphorylation. Although δ-4E-BP1 can form under mitotic conditions in which mTOR is inhibited, it seems likely that mTOR cooperates with CDK1/CYCB1 to generate the mitotic δ-4E-BP1 by phosphorylating lower molecular mass ε-δ isoforms that may be precursors to the δ-4E-BP1 isoform. Another limitation to our study is that we measure only 4E-BP1 phosphorylation but not δ-4E-BP1 dephosphorylation or turnover. These are likely to affect steady-state p4E-BP1 levels as well.

Our findings contrast with studies suggesting that loss of mTOR activity leads to inhibition of mitotic elf4F4G cap-association and cap-dependent translation. We see cap-dependent protein translation is sustained during mitosis using a pulse flow cytometry approach. Pharmacological (4E1RCat) cap-dependent translation inhibition provides evidence that this effect is generalizable. AHA pulse labeling allows direct measurement of translation in mitotic cells, which avoids confounding issues stemming from bulk culture measurements. Although most mitotic translation was cap-dependent in all of the cell lines tested by AHA uptake, differences in relative mitotic and interphase translation were present between cell lines. Like [35S]methionine incorporation studies, AHA incorporation measurements require incubation of cells in low-methionine media.

We suspect that technical issues, which have only recently been resolved, explain differences between our studies and those of others. Measurement of mitotic protein translation (both cap-dependent and -independent) has relied on separation of mitotic and interphase cells in bulk culture, using nocodazole-induced mitotic enrichment. We confirm that nocodazole inhibits mitotic translation for synchronized 293 cells. This has been ascribed by Coldwell et al. (15) to inhibitory phosphorylation of eIF2 and elf4F4GII by nocodazole downstream to 4E-BP1 regulation. This is consistent with our findings that nocodazole both promotes δ-4E-BP1 and inhibits mitotic translation. We have not tested other mitotic-arrest compounds (e.g., paclitaxel) to determine if...
These changes generally cannot be accurately measured by mTOR typically acts in concert with CDK1/CYCB1 to promote downstream to 4E-BP1. We also find evidence that in most cells mitosis (51). Thus, PP242 may affect mitotic translation by acting mitotic protein translation and may be missed in our study of early regulation by 14-3-3 eIF4B (49) and eEF2 elongation factor (50). Further, eIF4B somal biosynthesis as well as direct phosphorylation of trans-lation of 4E-BP1. mTOR regulates translation through ribo-somal translation and is useful for accentuating mitotic reg-ulation of 4E-BP1. mTOR regulates translation through ribo-somal biosynthesis as well as direct phosphorylation of trans-lation machinery components downstream from 4E-BP1, such as eIF4B (49) and eEF2 elongation factor (50). Finally, newly developed classes of cap-dependent translation inhibitors such as 4E1R Cat now allow direct determination of cap-dependent translation. When used in combination with AHA incorporation, direct measure-ment of mitotic cap-dependent translation can be determined.

Both nocodazole and PP242 are nonetheless important in-hibitors to measure 4E-BP1 phosphorylation and translation during mitosis. As indicated, nocodazole does not interfere with δ-4E-BP1 formation and is useful for accentuating mitotic reg-ulation of 4E-BP1. mTOR regulates translation through ribo-somal biosynthesis as well as direct phosphorylation of trans-lation machinery components downstream from 4E-BP1, such as eIF4B (49) and eEF2 elongation factor (50). Finally, newly developed classes of cap-dependent translation inhibitors such as 4E1R Cat now allow direct determination of cap-dependent translation. When used in combination with AHA incorporation, direct measure-ment of mitotic cap-dependent translation can be determined.

In Vitro Phosphorylation Assays. Recombinant GST–4E-BP1 (0.2 μg) (Signal-Chem) was incubated in a 25-μL reaction containing 1 μg protein kinase buffer (NEB) and 20 units of recombinant CDK1/CYCB1 (NEB) or 10 μg of mitotic HeLa cell lysate, supplemented with 200 μM ATP and/or 5 μM active site kinase inhibitors, for 30 min at 30 °C. HeLa cells were arrested in mitosis by treatment with 0.5 μM nocodazole for 16 h and enriched by mechanical shake-off for lysis in nonadenaturing lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 2 mM Na3VO4, and 2 mM NaF). The reactions were stopped by adding 5x SDS sample buffer to 1x concentration and boiling for 5 min. Reaction samples were then subjected to SDS/PAGE and immuno-blotting. For in vitro protein dephosphorylation, 293 cell extracts were incubated with lambda phosphatase in protein maltemp phosphatase reaction buffer (NEB) supplemented with 2 mM MnCl2 for 30 min at 37 °C. Reactions were stopped by adding 2x SDS sample buffer and then subjected to SDS/ PAGE and immunoblotting.

Diagrams:

- **Interphase**
  - mTOR
  - 4E-BP1
  - Raptor
  - α-γ 4E-BP1 (PP242-sensitive)
  - δ-4E-BP1 (PP242-resistant)

- **Mitosis**
  - CDK1/CYCB1
  - 4E-BP1
  - Cap-dependent translation

**Fig. 6.** Model for cell cycle-dependent 4E-BP1 regulation of cap-dependent translation. Interphase 4E-BP1 is inhibited by mTORC1 kinase, whereas CDK1/CYCB1 is primarily responsible for δ-4E-BP1 inactivation during mitosis.

Materials and Methods

Plasmids, antibodies, primers, and standard methods are described in SI Materials and Methods.
reaction was then stopped by adding 10 μL of luciferase lysis buffer to the mixture. Translation was measured as firefly luciferase activity.

**Nascent Protein Synthesis Analysis.** BJ-T stably cells were labeled with an azide-linked methionine analog AHA (Life Technologies) at 25 μM for 45 min in the presence or absence of PP242 (5 μM), followed by mitotic shake-off to separate mitotic cells and interphase cells. To analyze mitotic cap-dependent translation in U2OS and HeLa cells, cells were arrested at the G2/M boundary by 10 μM RO-3366 treatment for 24 h (34). After 30 min of RO-3366 removal, cells were labeled with AHA (25 μM) for 90 min in methionine-depleted DMEM (Corning Cellgro) after optimization of preexperiments. Translation cells were labeled with AHA (25 μM) for 90 min in methionine-depleted DMEM (Corning Cellgro) after optimization of preexperiments. Translation inhibitors (4E1R Cat (50 μM) or CHX (100 μg/mL)) or DMSO (0.1%) were added to cells with AHA. Cells were trypsinized and fixed in 10% (vol/vol) formalin for 5 min. Fixed cells were permeabilized in PBS containing 0.1% saponin and 1% FBS for 30 min at room temperature. Cells were harvested and labeled with the Alexa Fluor 488 alkyne using the Click-iT cell reaction buffer kit (Life Tech./trans) trans. ANA incorporation in cells was analyzed by flow cytometry as a measure for nascent protein synthesis in interphase and mitotic cells.

**Statistical Analysis.** One-sided t-test was performed for densitometric analysis of m7GTP pulldown eluates and two-sided t-test (unequal variances) for in vitro translation assays. A P value less than 0.05 was considered to be significant.

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Supporting Information

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SI Materials and Methods

Cell Culture and Transfection/Transduction. The 293, 293FT, U2OS, HeLa, and BJ-T cells were maintained in DMEM (Corning Cellgro) supplemented with 10% FBS. The 293 and 293FT cells were transfected using Lipofectamine 2000 (Invitrogen) and harvested after 48 h.

Kinase Inhibitors. The following active-site kinase inhibitors were dissolved in DMSO and used for kinase inhibition and in vitro phosphorylation experiments: mTOR kinase inhibitor PP242 (Selleckchem), CDK1 kinase inhibitor RO-3306 (Calbiochem), and pan Aurora kinase inhibitor VX-680 (Selleckchem).

Plasmids and Transfections. Plasmids pcDNA6.sTco (wild-type MCV sT, codon optimized) and pcDNA6.sTmlSD that were used for transient transfection experiments are previously described (1, 2). To efficiently express SV40 sT, codon-optimized SV40 sT [GenBank accession no. KM359729 (3)] was generated by overlapping PCR.

Immunoblotting and Antibodies. Cells were lysed in lysis buffer (50 mM Tris-·HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 2 mM Na3VO4, 2 mM NaF, and 0.1% SDS) containing protease inhibitors (Roche). Lysates were resolved by 12% SDS/PAGE and transferred to nitrocellulose. Membranes were blocked with 5% milk in 1× TBS and incubated with primary antibodies overnight at 4 °C. Blots were subsequently incubated with IRDye-labeled anti-rabbit or anti-mouse secondary antibodies and analyzed on the Odyssey infrared scanner (LI-COR Biosciences). The following primary antibodies were used in this study: total 4E-BP1, phospho-4E-BP1 T37/46, phospho-4E-BP1 S65, eIF4E, eIF4G, phospho-S6 S235/S236, total S6, phospho-histone H3 S10, total histone H3,cdc25C, phospho-Aurora A/B/C, total Aurora A, total Aurora B, Skp2, Cdc20, Claspin (Cell Signaling), total Aurora C,phospho-MPM2 (Millipore), Cdh1 (Calbiochem), CYCA, CYCD1, c-Myc (Santa Cruz Biotechnology), HA (Covance), FLAG (Sigma-Aldrich), 800CW goat polyclonal anti-rabbit IgG, and 680CW goat polyclonal anti-mouse IgG (LI-COR Biosciences). Previously described CM8E6 (2) and CM5E1 (1) were used to detect MCV sT. For CHX chase assays, BJ-T cells were treated with 100 μg/mL CHX and harvested at different time points for immunoblotting.

Immunoprecipitation. The 293 cells cotransfected with sT constructs and myc-cdh1, HA-cdc20, or pcDNA6 empty vector were harvested after 48 h and lysed in IP lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 2 mM Na3VO4, and 2 mM NaF) supplemented with protease inhibitors (Roche). Precleared lysates were incubated with either anti-myc tag or anti-HA antibodies overnight at 4 °C. Immune complexes were precipitated with protein A/G Sepharose beads (Santa Cruz) for 1 h at 4 °C. Beads were collected, washed with lysis buffer, and boiled in 1× SDS loading buffer. Samples were subjected to SDS/PAGE and immunoblotting.


![Fig. S1. MCV sT increases mitogenesis in 293 cells. (A) MCV sT expression increases phospho-histone H3 S10 (pH3 S10)-positive mitotic cells. (B) MCV sT expression in 293 cells increases mitotic marker expression including pAURKA and pAURKB, CYCB1, and pH3 S10. Transfected 293 cells were split into two fractions for cell cycle profile (A) and mitosis marker immunoblotting (B).](image-url)
Fig. S2. MCV sT stabilizes APC/C targets (AURKA and CYCB1) in nocodazole-arrested 293 cells. The 293 cells cotransfected with FLAG-tagged AURKA and MCV sT, sTmLSD, or empty vector were arrested with nocodazole (0.5 μM) for 15 h and then treated with CHX after nocodazole washout and harvested at different time points for immunoblotting. Asynchronous cells for each transfection were used as a control for nocodazole arrest. MCV sT but not sTmLSD or empty vector stabilizes AURKA and CYCB1 proteins in metaphase-arrested 293 cells. MCV sT increased FLAG-AURKA and CYCB1 expression in asynchronous cells, consistent with sT induction of increased mitogenesis.
Fig. S3. Mitotic slippage with mitotic kinase inhibition. (A) CDK1 inhibition during nocodazole/MG132 treatment fails to fully restore δ-4E-BP1 hyperphosphorylation. Notably, residual 4E-BP1 phosphorylation during RO-3306 treatment is further reduced by PP242 treatment, suggesting that mTOR phosphorylation may partially restore 4E-BP1 phosphorylation under conditions of CDK1 inhibition. Cdc25C is a direct phosphorylation target for CDK1. (B) The same experiment as in A was repeated using the pan-AURK inhibitor VX-680. Treatment with VX-680 reduces 4E-BP1 hyperphosphorylation in nocodazole-arrested HeLa cells by inducing mitotic exit. When HeLa cells were arrested with nocodazole (0.5 μM) for 16 h and treated with the proteasome inhibitor MG132 (10 μM) to prevent APC/C-mediated mitotic exit, VX-680 no longer prevents 4E-BP1 hyperphosphorylation but does inhibit AURKB-mediated phosphorylation of H3S10.
Fig. S4. δ-4E-BP1 isoform expression in sT-expressing mitotic cells. (A) BJ-T cells transduced with MCV sT can be enriched for mitotic and nonmitotic cell populations by mechanical shake-off. Nonadherent cells are enriched for pH3\(^{S10}\) positivity from 1.8% to 66% after shake-off, whereas remaining pH3\(^{S10}\) positivity was reduced to less than 1% for adherent cells. (B) Immunoblotting for p4E-BP1 reveals that δ-4E-BP1 is present only in the mitotic fraction, confirmed by mitotic markers pAURKA, pAURKB, pH3\(^{S10}\), and pMPM2. Adherent cells, positive for CYCE1, are negative for δ-4E-BP1. Representative result is shown of three independent experiments.

Fig. S5. 4E-BP1 α, β, γ, and δ isoforms are entirely lost after λ phosphatase treatment, consistent with these posttranslational modifications resulting from phosphorylation.

Fig. S6. Flow cytometry, with PI and pH3\(^{S10}\) staining, of 293 cells synchronized by double-thymidine release as in Fig. 4A.
**Fig. S7.** Induction of δ-4E-BP1 isoform during mitosis in synchronized U2OS cells. (A) Flow cytometry, with PI and pH3—all staining, of U2OS cells synchronized by double-thymidine release indicates maximum mitotic entry 10 h postrelease, in the presence and absence of mTOR inhibition by PP242. Dual pH3-all and p4E-BP1—all-positive mitotic cells form an orthogonal cell population that peaks at 10 h and is reduced by 12 h postrelease, similar to 293 cells shown in Fig. 4A. (B) Protein lysates from A were immunoblotted for p4E-BP1 and pH3-all. The δ-4E-BP1 isoform is apparent 6–12 h after release, corresponding to pH3-all positivity. This 4E-BP1 isoform is resistant to PP242 in U2OS cells.
Fig. S8. elf4F formation on the m⁷GTP cap is inhibited by CDK1 inhibition in mitosis-enriched HeLa cells. HeLa cells were enriched or depleted for mitosis by G2/M boundary arrest synchronization and shake-off. For mitosis-enriched cells, 4E-BP1 binding to the m⁷GTP resin was increased by RO-3306 treatment alone. RO-3306 but not PP242 significantly inhibits elf4F pulldown by m⁷GTP resin in HeLa cells. Near-complete inhibition, however, was present with combined PP242 and RO-3306 (PP + RO), suggesting cooperativity for mTOR and CDK1 in mitosis-enriched cells. For mitosis-depleted HeLa, PP242 alone inhibits elf4F binding and activates 4E-BP1 binding to the m⁷GTP resin. Error bars are SEM; asterisks denote significant comparisons by one-sided t test with $P < 0.05$, and n.s. denotes nonsignificant change. Quantitative LICOR immunoblotting shown is representative for one of three independent experiments used to generate average and SEM values for cap binding.
Fig. S9. The 293 cell-nascent protein synthesis is sensitive to PP242. Double-thymidine release was performed for 293 cells. Drug treatment (PP242 at 5 μM and CHX at 100 μg/mL) was given at 8.5 h and AHA (25 μM) at 9 h, 15 min postrelease, and then cells were harvested at 10 h. The protein synthesis inhibitor CHX served as a negative control for AHA incorporation, and pH3S10 was used to measure mitotic activity. New protein synthesis is similar for both phospho-pH3S10-positive and -negative mock-treated cells, indicating that protein synthesis is not inhibited during mitosis for 293 cells. Unlike BJ-T, PP242 reduced AHA incorporation for both mitotic and nonmitotic populations.

Fig. S10. Nocodazole inhibits mitotic protein translation. Double-thymidine–released 293 cells were treated with or without nocodazole and pulse-labeled with AHA for 45 min prior to 10-h post-thymidine-release harvesting point. AHA incorporation is present for both mitotic (pH3S10+) and interphase (pH3S10−) 293 cells but is markedly reduced when pH3S10+ cells are treated with 0.5 μM nocodazole. No significant change in AHA incorporation was noted for pH3S10− cells with nocodazole treatment. Dotted lines represent threshold between pH3S10+ and pH3S10− cells, with active or inhibited new protein synthesis. I, interphase pH3S10− cells; M, mitotic pH3S10+ cells.