

cdc2–cyclin B regulates eEF2 kinase activity in a cell cycle- and amino acid-dependent manner

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The calcium/calmodulin-dependent kinase that phosphorylates and inactivates eukaryotic elongation factor 2 (eEF2 kinase; eEF2K) is subject to multisite phosphorylation, which regulates its activity. Phosphorylation at Ser359 inhibits eEF2K activity even at high calcium concentrations. To identify the kinase that phosphorylates Ser359 in eEF2K, we developed an extensive purification protocol. Tryptic mass fingerprint analysis identified it as cdc2 (cyclin-dependent kinase 1). cdc2 co-purifies with Ser359 kinase activity and cdc2–cyclin B complexes phosphorylate eEF2K at Ser359. We demonstrate that cdc2 contributes to controlling eEF2 phosphorylation in cells. cdc2 is activated early in mitosis. Kinase activity against Ser359 in eEF2K also peaks at this stage of the cell cycle and eEF2 phosphorylation is low in mitotic cells. Inactivation of eEF2K by cdc2 may serve to keep eEF2 active during mitosis (where calcium levels rise) and thereby permit protein synthesis to proceed in mitotic cells. Amino-acid starvation decreases cdc2's activity against eEF2K, whereas loss of TSC2 (a negative regulator of mammalian target of rapamycin complex 1(mTORC1)) increases it. These data closely match the control of Ser359 phosphorylation and indicate that cdc2 may be regulated by mTORC1.

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Introduction

Eukaryotic elongation factor 2 (eEF2) mediates the translocation step of peptide-chain elongation (Merrick and Nyborg, 2000). Phosphorylation of Thr56 in eEF2 prevents eEF2 from interacting with the ribosome, thereby inactivating it (Carlberg *et al.*, 1990). eEF2 is phosphorylated by eEF2 kinase (eEF2K) (Nairn *et al.*, 1985; Ryazanov *et al.*, 1988). The phosphorylation of eEF2 is modulated under a range of

conditions, suggesting that eEF2K is a key regulator of translation elongation (Herbert and Proud, 2006) and, thus, protein synthesis.

eEF2K is an unusual calcium/calmodulin (Ca/CaM)-dependent protein kinase. Consequently, conditions associated with elevated calcium levels, such as muscle contraction, are associated with a rapid increase in eEF2 phosphorylation (Rose *et al.*, 2005). By slowing translation elongation, this may conserve energy for use by the contractile machinery. However, there are other situations, such as mitosis, where calcium levels rise but translation is inhibited at initiation rather than elongation, and where activation of eEF2K therefore appears inappropriate (Fan and Penman, 1970; Pyronnet and Sonenberg, 2001; Whitaker, 2006; Wilker *et al.*, 2007). Indeed, specific mRNAs continue to be translated during mitosis and it is thus important to keep the elongation machinery active.

Stimuli that activate protein synthesis elicit the inactivation of eEF2K and dephosphorylation of eEF2 (reviewed in Herbert and Proud (2006); Wang and Proud (2006)). In the case of insulin, both effects are blocked by rapamycin, which interferes with some functions of the mammalian target of rapamycin complex 1 (mTORC1) (Wullschlegel *et al.*, 2006), indicating that insulin's effects on eEF2K are mediated through mTORC1. However, it should be noted that not all the effects of mTORC1 are blocked by rapamycin. For example, the phosphorylation of certain regulatory sites (Thr37/46) in eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) is quite insensitive to this drug (Gingras *et al.*, 2001; Wang *et al.*, 2005), even though multiple lines of evidence indicate that they are controlled by mTORC1. Indeed, mTORC1 can directly phosphorylate these sites *in vitro*, albeit in a quite rapamycin-insensitive manner (McMahon *et al.*, 2002). Their phosphorylation is profoundly decreased by depriving cells of amino acids, especially leucine, which strongly impairs mTORC1 signalling. Other targets for mTORC1, such as the S6 kinases, are strongly affected both by amino-acid starvation and by rapamycin (Avruch *et al.*, 2001).

eEF2K is subject to phosphorylation *in vivo* at several sites (Browne and Proud, 2002; Wang and Proud, 2006). Phosphorylation of eEF2K at certain sites decreases its activity, whereas phosphorylation at others increases it (Herbert and Proud, 2006). The phosphorylation of eEF2K at Ser359 is of particular interest. First, the phosphorylation of this site strongly decreases the activity of eEF2K even at high calcium concentrations (Knebel *et al.*, 2001), that is, desensitizes eEF2K to the activating effects of elevated Ca²⁺ levels. Second, Ser359 is partially phosphorylated under basal conditions. The phosphorylation of this site is decreased by starving cells of amino acids, suggesting that mTORC1 may have an important function in controlling this site. Third, although p38 MAP kinase δ (also termed SAPK4 δ) can phosphorylate Ser359 *in vitro* (Knebel *et al.*, 2001), this enzyme is not known to be active basally or to be regulated by amino acids. It therefore appeared likely that Ser359 was

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also phosphorylated by another protein kinase, which might be controlled by the amino-acid status of the cells and perhaps by mTORC1. Our aim was to identify this kinase, a potential new link to the control of translation elongation.

Results

Development of an assay for kinase activity against Ser359 in eEF2K

To allow us to easily and specifically monitor kinase activity against Ser359 in eEF2K, we needed to develop an assay procedure to detect activity against this site. There were two reasons for this. First, eEF2K itself undergoes (auto)phosphorylation (Redpath and Proud, 1993) and the resulting incorporation of radiolabel into eEF2K presents major problems for detecting the activity of eEF2K. Secondly, eEF2K undergoes phosphorylation at multiple sites and we wished to identify the kinase that specifically phosphorylates Ser359.

Our assay exploits a new phospho-specific antibody for Ser359. Its characterization is documented in Supplementary Figure S1. Neither the crude serum (Supplementary Figure S1A) nor the affinity-purified antibody (Supplementary Figure S1B) reacted with the non-phosphorylated peptide, although a strong signal was seen with its phospho-version, defining the antibody as truly phospho-specific. To assess whether Ser359 kinase activity could be detected in KB cell lysates, we incubated lysate from serum-starved cells with GST-eEF2K and ATP. Reaction products were analysed by SDS-PAGE/western blot using the Ser359-phospho-specific antibody. Kinase activity against Ser359 was detected in such lysates (Supplementary Figure S1C) but not in lysates of amino acid-starved cells, consistent with the loss of phosphorylation of Ser359 that occurs upon amino-acid deprivation (Browne and Proud, 2004). These data show that the assay detects Ser359 kinase activity whose behaviour matches that expected for a physiologically relevant eEF2K Ser359 kinase. It was first important to establish whether two potential candidates, mTORC1 and SAPK4 (Knebel *et al.*, 2001), accounted for this activity.

eEF2K is not directly phosphorylated by mTOR and does not bind raptor

It was possible that mTORC1, signalling through which is positively regulated by amino acids, directly phosphorylated eEF2K. To test this, we performed immunoprecipitations using anti-raptor (for mTORC1), anti-riCTOR (mTORC2) or anti-mTOR (mTAB1; for both complexes). When any of the mTOR complexes was incubated with eEF2K, no phosphorylation of Ser359 in eEF2K was observed (Figure 1A). In contrast, robust phosphorylation of a known mTORC1 substrate, 4E-BP1, was seen where expected (Figure 1B). These data demonstrate that the regulation of Ser359 in eEF2K by mTORC1 is indirect, implying the existence of an additional Ser359 kinase.

To examine why eEF2K is not phosphorylated by mTORC1, we examined whether it can bind to the mTORC1 component, raptor, as is the case for direct mTORC1 substrates such as 4E-BP1, which contain TOR-signalling motifs (Schalm and Blenis, 2002). Using our overlay assay (Beugnet *et al.*, 2003), we did not observe any interaction of eEF2K with raptor, or eIF2 α , a negative control (Figure 1C). Raptor did bind to 4E-BP1, a positive control. Thus, the inability of mTORC1 to

phosphorylate eEF2K may reflect the fact that, unlike 4E-BP1, eEF2K cannot bind raptor.

Ser359 kinase activity in amino acid-fed cells is not due to SAPK4

SAPK4 (Knebel *et al.*, 2001) is also a candidate for the Ser359 kinase activity observed in amino acid-fed cells. However, no activity whatsoever was observed in SAPK4 immunoprecipitates from such cells, even after insulin treatment. Strong SAPK4 activity was, however, detected after treatment of cells with anisomycin, which activates SAPK4 (Figure 1D; last lane; see Knebel *et al.* (2001)).

Purification and identification of the Ser359 kinase

Purified kinases can often phosphorylate on non-physiological substrates *in vitro*. Therefore, to try to ensure accurate identification of the kinase responsible for phosphorylating Ser359, we elected to attempt to purify this enzyme(s) from lysates of KB cells, which are a relatively rich source of this activity. We developed a series of purification steps using anion and cation exchangers; HiTrap Blue, a nucleotide-based affinity column; and gel filtration. At each stage, all the Ser359 kinase activity was retained on the column (data for Resource Q, Figure 2A and B; also data not shown) and eluted as a single peak, indicating that there is one major species of Ser359 kinase in amino acid-fed KB cells. Extensive work showed that a combination of Resource Q, Mono S, HiTrap Blue and Superose 6 columns gave the best enrichment and recovery of activity (Figure 2C). The data in Figure 2A and B (derived from Resource Q ion-exchange chromatography of cell lysates) provide further confirmation that basal kinase activity is not due to SAPK4: Ser359 kinase activity eluted mainly in fractions 6–10, whereas SAPK4 appeared earlier and no SAPK4 protein was detected in the most active fractions (Figure 2B, lower part).

The activity of the highly purified eEF2K Ser359 kinase decreased after incubation with the Ser/Thr phosphatase PP2A (Figure 2D), indicating that the Ser359 kinase is activated by phosphorylation and implying that it is a phosphoprotein. To identify the kinase, we therefore focused our attention on any phosphoproteins that were present in the purified material: the proteins in the most active fractions from the gel filtration column were precipitated with TCA and analysed by SDS-PAGE. Application of the phosphoprotein stain ProQ Diamond[®] (Supplementary Figure S2) revealed several bands. Each band was excised, digested with trypsin and analysed by mass spectrometry. The data (Table I) revealed multiple peptides from the cell division control protein cdc2 (also termed cyclin-dependent kinase 1 (CDK1)), but no peptides from other protein kinases. Altogether, 20 peptides matched cdc2 (covering >50% of its sequence), including one phosphopeptide. This contained phospho-Thr161, phosphorylation of which is necessary for cdc2 to be active (Solomon *et al.*, 1993). This may account for the phosphatase-sensitivity of the purified Ser359 kinase (Figure 2D).

cdc2-cyclin B phosphorylates eEF2K at Ser359 in vitro and the CDK inhibitor roscovitine decreases Ser359 phosphorylation in vivo

Western blot analysis revealed that active fractions from the Resource Q column contained cdc2 and cyclin B (Figure 3A).

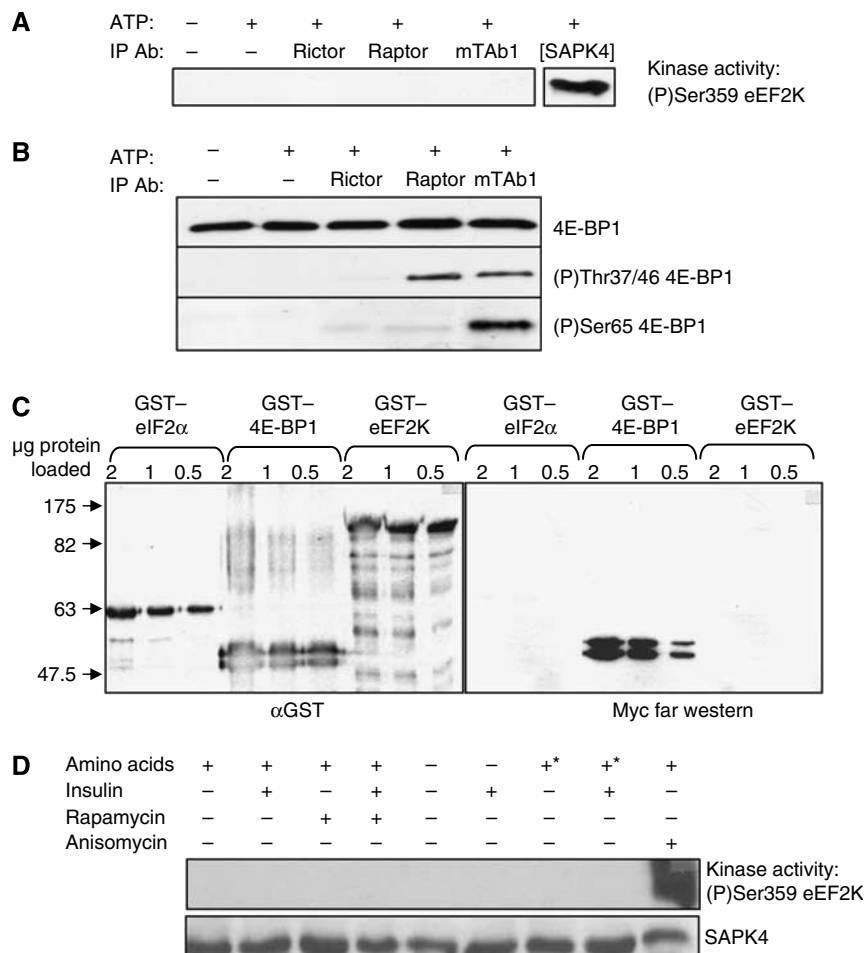


Figure 1 Neither mTORC1 nor SAPK4 is the amino acid-regulated eEF2K Ser359 kinase. **(A, B)** KB cells were harvested in CHAPS lysis buffer. mTOR was immunoprecipitated with indicated antibody bound to protein G-Sepharose. Immunoprecipitates were washed three times in lysis buffer and then used in kinase assays with **(A)** eEF2K (right-hand lane is a control using SAPK4 to phosphorylate eEF2K) or **(B)** 4E-BP1 (positive control) as substrate. Assay products were subjected to SDS-PAGE/western blot analysis with the indicated antibodies. **(C)** Raptor does not bind eEF2K: recombinant GST 4E-BP1, eEF2K and eIF2 α were subjected to SDS-PAGE, and then transferred onto PVDF membrane for 'far western analysis'. The blot was incubated with lysates from HEK293 cells expressing myc-raptor and probed with anti-myc. The membrane was then stripped and re-probed using anti-GST. **(D)** HEK293 cells were transiently transfected with DNA for myc-SAPK4. At 24 h later, cells were starved of serum for 16 h. Cells were then treated as indicated above (*D-PBS was supplemented with amino acids). Myc-SAPK4 was immunoprecipitated from HEK293 cell lysates and activity was measured versus GST-eEF2K. Kinase assay products were analysed by SDS-PAGE/western blot using the indicated antibodies.

Ser359 kinase activity also co-purified with cdc2 during the second purification step (Mono S; Figure 3B) and at all subsequent stages (data not shown), consistent with the identification of the eEF2K Ser359 kinase as cdc2. The most highly purified active fractions also contained cyclin B (Figure 3C).

The cdc2 inhibitor roscovitine completely inhibited Ser359 kinase activity both in the peak fraction from the Resource Q column and in KB cell lysates (Figure 4A and B), indicating that no other, roscovitine-insensitive, kinase(s) contribute to this activity. The Resource Q-purified Ser359 kinase also phosphorylated histone H1 (a well-established substrate for cdc2-cyclin B; see, e.g., Clarke *et al* (1992)).

cdc2 immunoprecipitates from KB cell lysates phosphorylated eEF2K at Ser359 (Figure 4C) and recombinant cdc2-cyclin B also phosphorylated eEF2K at this site (Figure 4D). Activity was again completely blocked by roscovitine. These data show that cdc2 can indeed phosphorylate eEF2K at Ser359. The sequence around Ser359 in eEF2K is

CGSPRVRTL, similar to the optimal consensus for phosphorylation by cdc2-cyclin B, S/T-P-X-K/R (X = any amino acid) (Ubersax *et al*, 2003), although CDKs can phosphorylate sites with the minimal consensus S/T-P.

To study further the phosphorylation and regulation of eEF2K by cdc2, we first tested the effect of cdc2-mediated phosphorylation on its activity. Incubation of eEF2K with cdc2-cyclin B and ATP led to a marked decrease in its activity against eEF2 (Supplementary Figure S3A), consistent with earlier data (Knebel *et al*, 2001) that phosphorylation at Ser359 inhibits eEF2K activity. To test whether Ser359 is the major cdc2 site in eEF2K, we mutated Ser359 to Ala (using a newly created kinase-dead version of eEF2K (Lys170Met; to be reported elsewhere) to avoid complications arising from the propensity of eEF2K to autophosphorylate (Redpath and Proud, 1993). This mutation markedly decreased, but did not entirely abolish, cdc2-catalysed phosphorylation of eEF2K (Supplementary Figure S3B), showing that, although Ser359 is the major site for cdc2, it is not the only one. The S359A

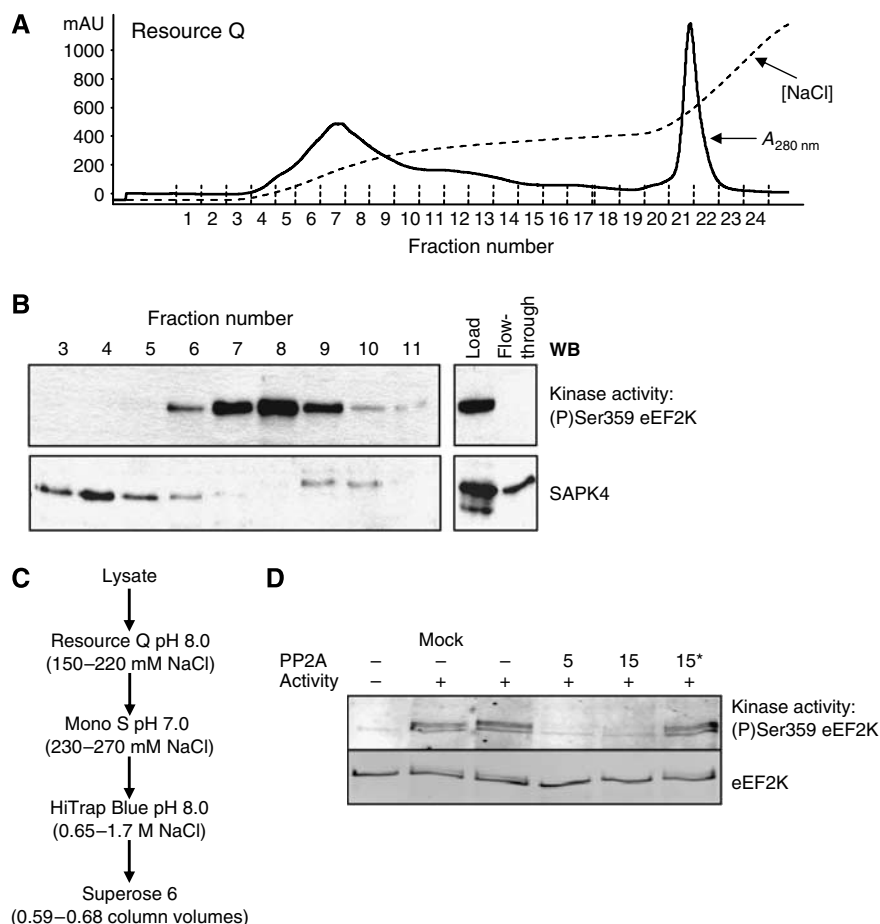


Figure 2 Purification of eEF2K Ser359 kinase from KB cell lysates. **(A, B)** Resource Q FPLC was performed and 1-ml fractions were collected. **(A)** The elution profile, that is, the absorbance at 280 nm and the ionic strength of the elution buffer, is shown. **(B)** Western blot of kinase assay products (probed with anti-(P)Ser359 eEF2K) for the indicated fractions, the load and flowthrough. Samples were also analysed by western blot for SAPK4, as indicated. **(C)** Scheme outlining the large-scale purification procedure for the Ser359 kinase. **(D)** The activity of the eEF2K Ser359 kinase decreases upon incubation with protein phosphatase PP2A. Purified kinase activity from the Superose 6 gel filtration fraction 13 was incubated at room temperature for 15 min with or without 50 mU/ml of PP2A. Phosphatase activity was stopped by adding 1 μ M microcystin. Kinase assays were then performed against GST-eEF2K as described in Materials and methods section. 'Mock'—kinase was pre-incubated at 4°C prior to assay. *Phosphatase was inactivated by adding 1 μ M microcystin prior to incubation with Ser359 kinase.

Table I Mascot search results

Band ID	Accession	Protein	Peptides matched
1	P06493	Niban-like protein (Meg3)	19
	O43583	Glutamyl-tRNA synthetase	15
2	P02768	Serum albumin precursor	9
	P49748	Very-long chain-specific acyl-coA dehydrogenase, mitochondrial precursor	7
4	P50579	Methionine aminopeptidase 2	6
	Q9UQ80	Proliferation-associated protein 2G4	24
	P38919	Probable ATP-dependent helicase DDX48	7
5	P064493	Cell division control protein 2	20
	O43583	Density-regulated protein	8
6	Q9UQ80	Proliferation-associated protein 2G4	25
	P38919	Probable ATP-dependent helicase DDX48	9

The peptide masses for tryptic peptides from excised gel bands (see Supplementary Figure S2) were subjected to analysis by MS. Data presented show the first two significant hits from the Mascot (<http://matrixscience.com>) database searches performed on resulting peptide masses of each band (1–6).

mutation abolished the ability of cdc2 to inactivate eEF2K (Supplementary Figure S3A), showing that the minor cdc2 sites do not affect eEF2K's activity.

Scansite (<http://scansite.mit.edu/>) does predict a second potential cdc2 site in eEF2K at Ser329, albeit with lower confidence than Ser359. We therefore also mutated this residue

to alanine. The S359A/S329A mutant showed no further decrease in phosphorylation by cdc2 (data not shown), indicating that additional sites are phosphorylated, at least *in vitro*.

It was crucial to establish whether cdc2 actually regulates the phosphorylation of eEF2K and its substrate, eEF2, in cells. Because the Ser359 phospho-specific antibody was

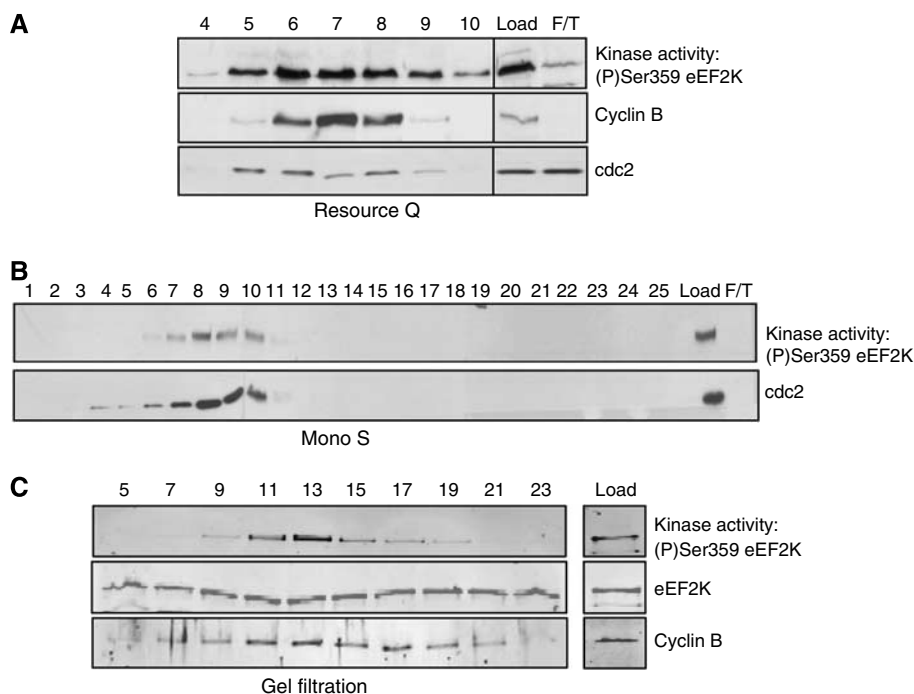


Figure 3 Ser359 kinase activity co-purifies with cdc2-cyclin B. (A) Resource Q; (B) Mono S and (C) gel filtration FPLC were performed on lysates from insulin-treated KB cells (see Supplementary data). Fractions were assayed for kinase activity and products were subjected to SDS-PAGE/western blotting with indicated antisera (F/T, flowthrough).

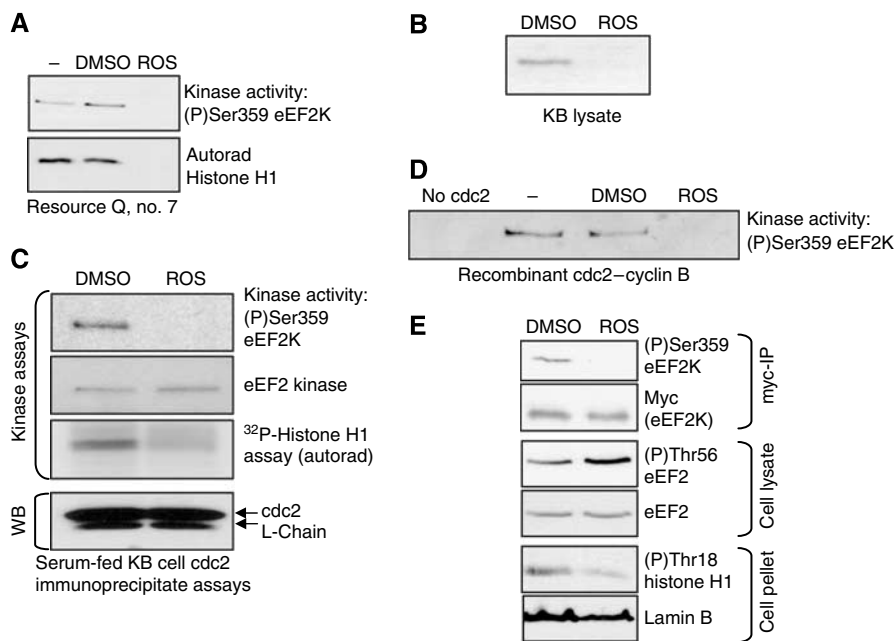


Figure 4 Ser359 kinase activity is inhibited by roscovitine. (A) Fraction 7 of Resource Q FPLC (Figure 3A) was assayed for kinase activity against eEF2K and histone H1 in the presence of 10 μ M roscovitine or DMSO. Assay products were analysed by western blotting or autoradiography as indicated. (B) KB cell lysates or (C) cdc2 immunoprecipitates were assayed for activity towards Ser359 in eEF2K in the presence or absence of roscovitine (10 μ M). Assay products were subjected to SDS-PAGE/western blot using the indicated antibodies. In (C), immunoprecipitates were also assayed against histone H1: in this case the figure is an autoradiograph of the stained gel. (D) Recombinant cdc2-cyclin B complexes were pre-incubated at room temperature for 20 min with 10 μ M roscovitine (or DMSO as control). cdc2-cyclin B complexes were then incubated with 2 μ g of GST-eEF2 kinase and ATP. Assay products were analysed by western blotting. (E) HEK293 cells were transfected with myc-eEF2K. 40 h later, cells were treated with DMSO or 50 μ M roscovitine for 1 h prior to lysis. SDS-PAGE/western blotting was performed on myc immunoprecipitates, cell lysates or cell pellets as indicated.

insufficiently sensitive to detect endogenous eEF2K (data not shown), we overexpressed (myc)-eEF2K, using HEK293 cells (because they can be transfected more efficiently than KB

cells). After immunoprecipitation, a signal for phosphorylated Ser359 in eEF2K was clearly seen in control cells (Figure 4E). This signal was completely lost if cells were

pretreated with roscovitine (for only 1 h, to avoid affecting cell cycle progression), consistent with the conclusion that cdc2 is the only kinase that phosphorylates this site within cells. Furthermore, roscovitine treatment increased the phosphorylation of eEF2 (Figure 4E). These data provide strong evidence that Ser359, an inhibitory site in eEF2K, is regulated by cdc2 *in vivo* and that this is important for the control of eEF2K activity and eEF2 phosphorylation. Analysis of the phosphorylation of endogenous histone H1 (a cdc2 substrate) confirmed the efficacy of roscovitine (Figure 4E).

Regulation of eEF2K and eEF2 phosphorylation during the cell cycle

There is substantial evidence that calcium transients have an important function during mitosis in many types of cells,

including mammalian cells (FitzHarris *et al*, 2005; Whitaker, 2006). Indeed, calcium (through its binding to CaM) apparently has an important function in mitotic progression (Rasmussen and Means, 1989; Baitinger *et al*, 1990; Torok *et al*, 1998). Phosphorylation of eEF2K at Ser359 strongly inhibits its activity even at high (supraphysiological) concentrations of Ca²⁺ (10 μM) (Knebel *et al*, 2001).

The finding that cdc2 phosphorylates eEF2K at Ser359 could therefore be important for the control of eEF2K, eEF2 and translation elongation during M-phase. To study this further, HeLa cells were blocked in early S-phase using the DNA polymerase inhibitor aphidicolin (Oguro *et al*, 1979). At various times after release from this block, progress through the cell cycle was monitored by flow cytometry (Figure 5A). Cells were also lysed and samples were analysed for kinase

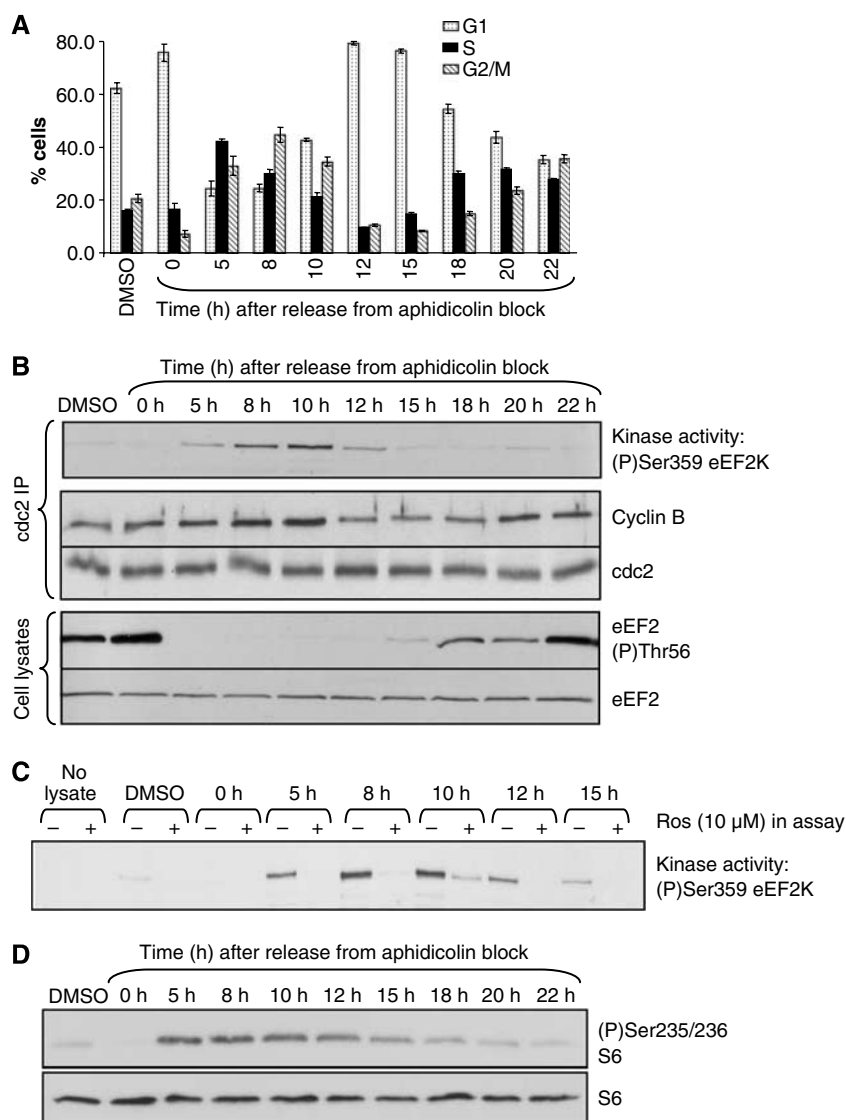


Figure 5 Regulation of eEF2K Ser359 kinase activity during the cell cycle. HeLa cells were synchronized in late G1-S-phase by a 16 h aphidicolin block (or DMSO as control) and then released into fresh medium for various times. (A) Cell cycle progression was monitored by flow cytometry of propidium iodide-stained cells at the indicated intervals. Series indicate G1 (2N), S and G2/M (4N) DNA content. Results show mean ± s.d. for three independent experiments. (B) Cell extracts were prepared from synchronized HeLa cells at the indicated times after release. *In vitro* kinase assays versus recombinant GST-eEF2K were performed on cdc2 immunoprecipitates from cell lysates. Assay products were subjected to SDS-PAGE and blotted with indicated antisera. Cell lysates were also subjected to SDS-PAGE and probed for eEF2 (P)Thr56, total eEF2. (C) Cell lysates from each time point after aphidicolin release were incubated with GST-eEF2K and ATP, ± 10 μM roscovitine. SDS-PAGE/western blot analysis of assay products was performed using the (P)Ser359 eEF2K antibody. (D) As (B) but cell extracts were subjected to SDS-PAGE/western blot for S6 (P)235/236 and total S6.

activity against Ser359 in eEF2K or by SDS-PAGE/western blot for other proteins (Figure 5B).

Flow cytometry revealed that the proportion of G2/M cells was maximal 8 h after release. The proportion of G1 cells increased at 10–12 h (indicating exit from mitosis) and cells began to re-enter S-phase at 18 h. Ser359 kinase activity in cdc2 immunoprecipitates was undetectable immediately after release but then rose, peaking at 8–10 h, when cyclin B levels were highest (Figure 5B). Total cdc2 levels were constant throughout (Figure 5B). The Ser359 kinase activity appears to ‘lag behind’ the proportion of ‘G2 + M’ cells, presumably because cdc2 is only activated at late times when more cells are in M-phase and not during the preceding G2-phase. At all time points, Ser359 kinase activity (measured in cell lysates) was blocked by roscovitine (Figure 5C).

The phosphorylation of endogenous eEF2 was high immediately after removing the block but low through G2 and mitosis, only rising again well after cells had reached G1 and began to re-enter S-phase (Figure 5B). Phosphorylation of S6, another target for mTORC1 signalling, remained high in G2/M and fell as cells re-entered G1 and progressed into S-phase (Figure 5D).

To further substantiate the control of eEF2 and eEF2K Ser359 kinase activity during the cell cycle, we considered it important to employ a second, different, way to synchronize cells, blocking with thymidine. The data were very similar to those from the aphidicolin block (Supplementary Figure S4A; see also Figure 5B): Ser359 kinase activity was again maximal 8–9 h after release, when the proportion of cells in mitosis appears highest (Supplementary Figure S4B), as is kinase activity against histone H1. The data also confirm that eEF2 phosphorylation is very low during mitosis (Supplementary Figure S4A).

Importantly, these data demonstrate that (i) eEF2K Ser359 kinase activity is greatest when one would expect cdc2 activity to be highest and (ii) in accordance with the fact that phosphorylation at Ser359 inhibits eEF2K activity even at high Ca^{2+} concentrations, eEF2 phosphorylation is low in mitotic cells. Phosphorylation of any of three sites in eEF2K can inactivate it, that is, Ser78 (Browne and Proud, 2004), Ser359 (Knebel *et al*, 2001) or Ser366 ((Wang *et al*, 2001) albeit only at low Ca^{2+} concentrations). We therefore measured kinase activity against these specific sites in eEF2K in lysates of cells sampled at different points in the cell cycle. Activity against Ser78 and Ser366 did not vary, that against Ser78 being low at all times tested (Supplementary Figure S4C). Ser366 is a substrate for S6 kinases and S6 phosphorylation was also similar at all times after release from thymidine block. In contrast, activity against Ser359 did change, being low early after release and highest at the times when the proportion of G2 + M cells was highest (5 and 9 h). eEF2 phosphorylation was lowest at these times, although it did also decrease at 3 h (both after release from thymidine (Supplementary Figure S4A and C) or aphidicolin (data not shown). At this time point, only a small activation of Ser359 kinase was evident. eEF2K is subject to an array of regulatory inputs and additional factors may explain this decrease.

Regulation of the Ser359 kinase activity of cdc2 by amino acids

Previous work has shown that amino-acid starvation and rapamycin each decrease the phosphorylation of Ser359 in

eEF2K (Knebel *et al*, 2001; Browne and Proud, 2004), implying that they may affect the activity of the relevant kinase, which we have identified as cdc2. As the above data indicate that the Ser359 kinase activity that can be detected in cell lysates is due to cdc2, we examined the effects of amino-acid starvation on this activity. When KB cells were starved of amino acids, Ser359 kinase activity declined slightly by 1.5 h, markedly by 3 h and activity was almost completely lost by 5 h (Figure 6A). This decrease could be due to inactivation of the kinase or to its degradation. To study this, we used the proteasome inhibitor MG132. As expected, MG132 increased levels of c-myc, which is degraded by the proteasome (Figure 6A). However, MG132 actually enhanced the decrease in Ser359 kinase activity seen in amino acid-starved cells. The decline in Ser359 kinase activity is therefore not due to its proteasomal degradation, but to other mechanisms. Proteasome activity may help maintain intracellular amino-acid levels and thus mTORC1 signalling: indeed, MG132 exacerbates the effect of amino-acid starvation (Vabulas and Hartl, 2005).

Amino-acid starvation also decreased the eEF2K Ser359 kinase activity measured in cdc2 immunoprecipitates (Figure 6B), whereas treatment of KB cells with rapamycin did not significantly change the Ser359 kinase activity detected in cell lysates or cdc2 immunoprecipitates (Figure 6B–D). Importantly, these data further demonstrate that, like Ser359 phosphorylation itself (Browne and Proud, 2004), Ser359 kinase activity is regulated by amino acids (Figure 6C and D). This implies that amino acids may regulate the activity of cdc2 and we wanted to investigate this further.

We wished to ask how amino acids regulate the activity of cdc2 against eEF2K. However, the control of CDKs is complex, involving both activating and inhibitory phosphorylation sites in the CDK, and interactions with cyclins, inhibitory proteins (CKIs: see Kaldis and Aleem (2005)) and other regulators (Kaldis and Aleem, 2005; Nebreda, 2006). Amino-acid starvation did not change the levels of cdc2 itself, whereas those of cyclin B, if anything, increased (Figure 6B). There were no apparent changes in levels of p21 or p27, two CDK inhibitors (not shown). The phosphorylation of both Thr161 (activating site) and Tyr15 (inhibitory) was higher in the amino acid-starved cells (Figure 6B). Although Tyr15 phosphorylation could have an important function in the inactivation of cdc2 caused by amino-acid starvation, other regulatory inputs are almost certainly involved, as rapamycin also appears to increase Tyr15 phosphorylation but does not affect cdc2 activity against eEF2K. Given the complexity of the control of cdc2, further work beyond the scope of this study is required to identify how amino acids regulate cdc2.

Rapamycin treatment of amino acid-fed cells did not affect the Ser359 kinase activity in cdc2 immunoprecipitates (Figure 6B and D), although it did abolish S6 phosphorylation (Figure 6C and D), which we studied to demonstrate the efficacy of the rapamycin. Treatment of cells with insulin also did not alter Ser359 kinase activity in cell lysates or anti-cdc2 immunoprecipitates (Figure 6D). As discussed above, this is strongly reminiscent of the situation for Thr37/46 in 4E-BP1 (Figure 6D), which are also quite insensitive to rapamycin and insulin (Figure 6D), but which do appear to be controlled through mTORC1, based on a range of criteria (Wang *et al*, 2005; Fonseca *et al*, 2007). This contrasts with S6 phosphorylation, which is increased by insulin and blocked by

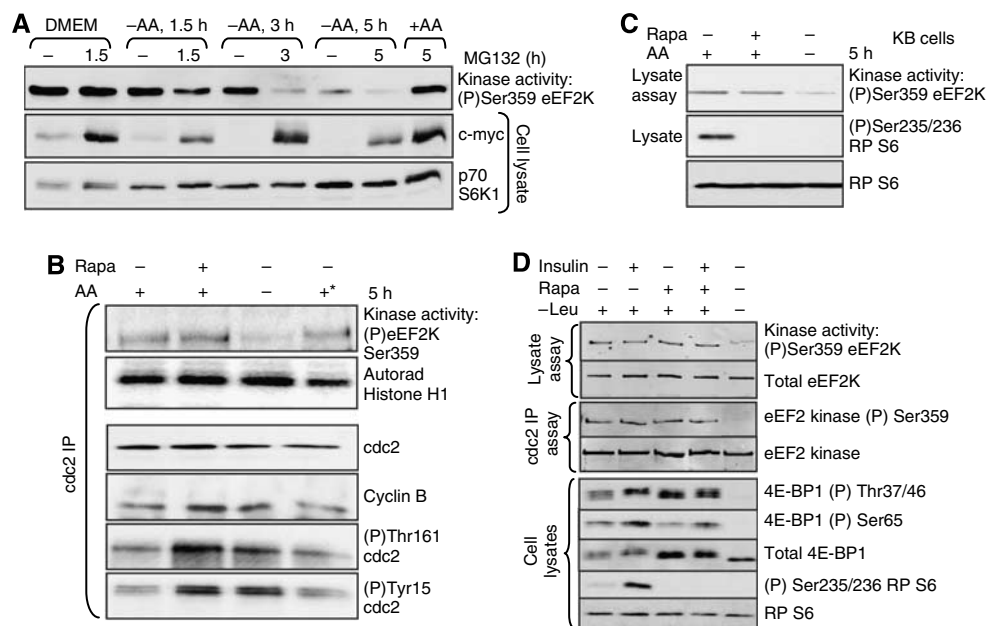


Figure 6 Effect of amino-acid deprivation or TSC2 status on *cdc2* activity. (A) KB cells were starved of serum for 12 h. Then the medium was changed to EBSS lacking amino acids ('-AA') or containing amino acids ('+AA') for the times indicated. The proteasome inhibitor MG132 (20 μ M) or DMSO (-) was present for the duration of the starvation. Cells were lysed and Ser359 kinase activity was assayed using recombinant GST-eEF2K as substrate. Assay products and cell lysates were analysed by SDS-PAGE/western blotting using the indicated antisera. (B, C) KB cells were starved of serum for 14 h and subsequently of amino acids (where indicated) for 5 h (*amino acids were present in the EBSS medium) or treated with 100 nM rapamycin for 1 h. Cells were lysed. In (B), *cdc2* was immunoprecipitated from lysates and subsequent immunoprecipitates were subjected to kinase assay using recombinant GST-eEF2K, SDS-PAGE and blotted with indicated antisera. In (C), kinase assays versus GST-eEF2K were performed on lysates. Lysates and assay products were subjected to SDS-PAGE/western blotting with indicated antisera. (D) KB cells were serum starved for 14 h and subsequently starved of leucine (where indicated -Leu) for 5 h, or treated with 100 nM rapamycin for 1 h and/or 100 nM insulin for 25 min where indicated. Cells were lysed. Where indicated *cdc2* was immunoprecipitated from lysates and subsequent immunoprecipitates were subjected to kinase assay using recombinant GST-eEF2K, followed by SDS-PAGE and western blot.

rapamycin (Figure 6D). In contrast, TSC1/TSC2, which negatively regulate mTORC1 signalling (Manning and Cantley, 2003), do regulate these events (Wang *et al.*, 2005).

Loss of TSC2 leads to activation of *cdc2*

In view of the ability of amino acids to maintain the activity of *cdc2* against eEF2K, we wished to assess whether mTORC1 signalling, a major pathway that is positively regulated by amino acids, has a function in controlling *cdc2* activity. The loss of either TSC1 or TSC2 activates mTORC1 signalling. We therefore used TSC2^{-/-} mouse embryonic fibroblasts (MEFs) to study whether the Ser359 kinase activity (due to *cdc2*) was controlled by TSC1/TSC2. As expected, loss of TSC2 increased phosphorylation of S6, a substrate for the S6 kinases that are activated by signalling through mTORC1 (Figure 7A) (Zhang *et al.*, 2003). Loss of TSC2 also significantly increased Ser359 kinase activity, 3.5-fold in lysates (Figure 7A) and 3.4-fold in *cdc2* IPs (Figure 7B) ($P < 0.02$, $n = 3$ in each case), indicating that it may be positively regulated by mTORC1. Consistent with this, levels of eEF2 phosphorylation were much lower in TSC2^{-/-} cells than in the controls (Figure 7A). However, the levels of *cdc2*-cyclin B complexes in TSC2^{+/+} and TSC2^{-/-} cells were indistinguishable (Figure 7B). FACS analysis revealed only a small increase in the percentage of cells in G2 plus M for TSC2^{-/-} versus TSC2^{+/+} MEFs (Figure 7C). Again, kinase activity in lysates of both cell lines was completely inhibited by the CDK inhibitor roscovitine (Figure 7D), consistent with it being entirely due to *cdc2*. Amino-acid deprivation decreased Ser359 kinase

activity in both TSC2^{+/+} and TSC2^{-/-} cells (data not shown), consistent with earlier data showing that TSC2 is not required for the control of mTORC1 signalling by amino acids (Smith *et al.*, 2005).

Leucine starvation decreases *cdc2* kinase activity and retards the G2-M transition

Leucine is the amino acid that most strongly promotes mTORC1 signalling, and thus starvation of cells for leucine alone inhibits it (Kimball and Jefferson, 2006). Deprivation of KB cells of leucine, in the presence of the other 14 amino acids present in DMEM (which include all the other essential amino acids), resulted in a marked decrease in the activity of the eEF2K Ser359 kinase measured either in cell lysates or anti-*cdc2* immunoprecipitates (Figure 6D). This is consistent with a role for mTORC1 in the control of Ser359 kinase activity.

We also examined the effect of leucine starvation on Ser359 kinase activity and mitotic entry (Figure 8A and B) in synchronized HeLa cells. Thus, all cells were studied 9 h after release allowing direct comparisons to be made (9 h being a time at which the proportion of mitotic cells is high, as judged by the fact that this is late in the period when the number of G2 + M cells is highest (Supplementary Figure S4A and B)). Longer times of leucine starvation led to larger decreases in the activity of *cdc2* immunoprecipitates against eEF2K or histone H1 (Figure 8A).

As *cdc2* activity is essential for M-phase entry, we tested the effect of leucine starvation on the progression of cells into

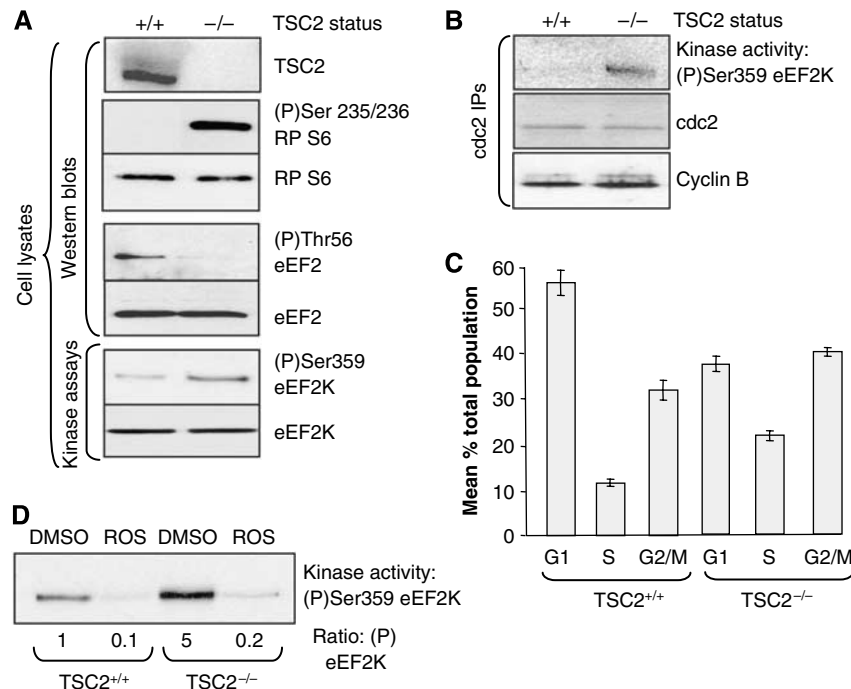


Figure 7 (A–C) TSC2^{+/+} or TSC2^{-/-} MEFs were starved of serum for 16 h. In (A), cell lysates and kinase assays versus GST–eEF2K were analysed by SDS–PAGE/western blotting using the indicated antisera. Quantification was performed using the ImageJ software. In (B), lysates from serum-fed TSC2^{+/+} or TSC2^{-/-} cells were used for cdc2 immunoprecipitation and subsequent kinase assays. Reaction products were subjected to SDS–PAGE and blotted with indicated antisera. In (C), FACS analysis was performed to determine proportions of TSC2^{+/+} and TSC2^{-/-} MEF cells at different stages of the cell cycle by flow cytometry of propidium iodide-stained cells. Chart indicates percentage of cells with G1 (2N), S or G2/M (4N) DNA content (means \pm s.d.; $n = 3$). **(D)** TSC2^{+/+} and TSC2^{-/-} MEF lysates were assayed for activity towards Ser359 in eEF2K in the presence or absence of roscovitine (10 μ M). Assay products were subjected to SDS–PAGE/western blot using the indicated antibody.

mitosis. As FACS analysis cannot distinguish whether cells are in G2- or M-phase, we used DAPI staining to examine the proportion of cells containing condensed DNA (characteristic of mitotic cells). As shown in Figure 8B, when cells are blocked in G1/S, no cells showed condensed DNA. However, when cells were released for 9 h (when the proportion of mitotic cells is high) in DMEM or medium plus leucine, about 8% of cells showed condensed DNA (Figure 8B). When cells were starved of leucine for the last 3 h of this release period, condensed DNA was only observed in 0.4% and when cells are starved of leucine for the entire release period or for the last 5 h, no cells showed condensed DNA. This indicates that leucine is required for cell cycle progression at the G2/M boundary.

Discussion

This study identifies cdc2 as providing a novel regulatory input to eEF2K and the translation elongation machinery. By inactivating eEF2K, this would keep the elongation machinery active during mitosis to allow the synthesis of those proteins that are made in mitotic cells (for example, p58 PITSLRE) (Wilker *et al*, 2007) (Figure 9). Several complementary lines of evidence support the identification of cdc2 as a Ser359 kinase and the conclusion that it phosphorylates this site in amino acid-fed cells: (i) the co-purification of cdc2–cyclin B with Ser359 kinase activity; (ii) the ability of cdc2 immunoprecipitates (from cell lysates) to phosphorylate eEF2K at Ser359; (iii) the fact that recombinant cdc2–cyclin B phosphorylates eEF2K at Ser359; (iv) the finding that the

CDK inhibitor roscovitine inhibits the activity of the purified Ser359 kinase and Ser359 kinase activity in cell lysates; (v) the observation that treatment of cells with roscovitine inhibits the phosphorylation of eEF2K at Ser359 (an inhibitory site) and increases the phosphorylation of its substrate eEF2 and (vi) that Ser359 kinase activity peaks during mitosis, where cdc2–cyclin B is most active. Our data show that cdc2 represents the principal, and probably only, eEF2K Ser359 kinase activity in KB cells under amino acid-fed conditions and, importantly, that cdc2 contributes to the control of both eEF2K and eEF2. In lysates from other types of cells, for example, MEFs and HeLa cells, we also show that Ser359 kinase activity is blocked by roscovitine, consistent with it being due to cdc2.

Early work showed that in mitotic cells protein synthesis is inhibited at the level of initiation, not elongation (Fan and Penman, 1970). This is consistent with our finding that eEF2 is dephosphorylated and thus active during mitosis. During mitosis, translation initiation appears to be altered such that general cap-dependent translation is impaired, whereas cap-independent mRNAs (driven by internal ribosome entry segments, IRESs (Cornelis *et al*, 2000; Sachs, 2000; Pyronnet *et al*, 2001; Wilker *et al*, 2007) can continue. For example, a recent study (Wilker *et al*, 2007) showed that 14-3-3 σ has an important function in regulating translation during mitosis, by binding to translation factors including eIF4B. However, irrespective of the mode of initiation, mRNA translation requires the same elongation machinery. The phosphorylation of eEF2K by the mitotic kinase cdc2 may serve to ensure that eEF2K is inactive during mitosis, even

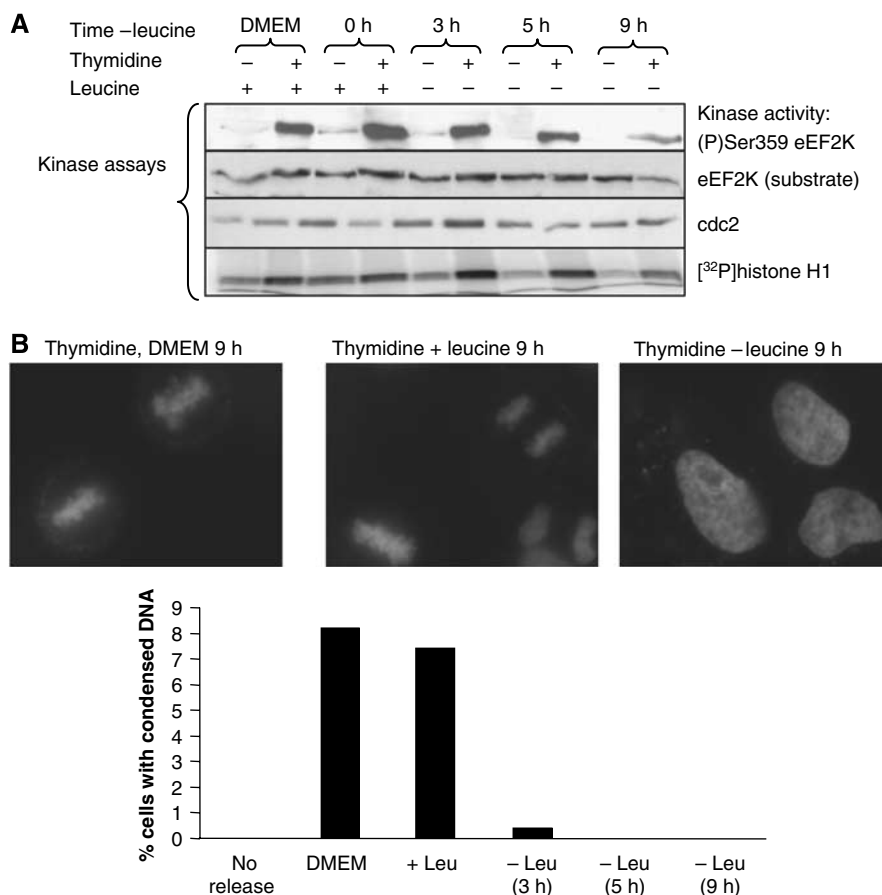


Figure 8 Effect of leucine starvation on *cdc2* kinase activity and cell cycle progression. **(A)** HeLa cells were subjected to ‘double thymidine’ block where indicated. Cells were released from the block and allowed to continue for 9 h. Leucine was removed for the final 3, 5 or 9 h where indicated. Cells were lysed and anti-*cdc2* immunoprecipitates were assayed for kinase activity against recombinant GST-eEF2K (using unlabelled ATP) or histone H1 ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$). Western blotting was performed on immunoprecipitates using the indicated antisera. For histone H1 kinase activity, an autoradiograph of the dried gel is shown. **(B)** HeLa cells were synchronized in late G1-S-phase by double thymidine block and then released for the times indicated. DAPI-stained cells were analysed by fluorescence microscopy and images were obtained from randomly selected fields, as detailed in Supplementary data. Data are represented as percentage of nuclei with condensed chromosomes. A minimum of 470 cells per treatment was counted.

though Ca^{2+} levels rise, and that eEF2 remains functional to permit IRES-driven translation to continue. Phosphorylation of eEF2 caused by rising Ca^{2+} levels would impair IRES-mediated as well as cap-dependent translation. Although it would be informative to test whether *cdc2*-mediated phosphorylation of eEF2K during mitosis affects protein synthesis, this is not feasible, as the only available approach is to use a *cdc2* inhibitor such as roscovitine, which would itself prevent M-phase entry.

An early study reported that eEF2 is substantially phosphorylated in mitotic cells (Celis *et al.*, 1990). The approaches used differed both in terms of obtaining mitotic cells (release from aphidicolin- or thymidine-induced S-phase block versus mitotic shake-off) and the procedure used to study eEF2 phosphorylation (analysis with a phospho-specific antibody versus 2D gel electrophoresis, as the phosphorylation site in eEF2 (Thr56) had not been identified at that time). While this paper was under review, it was reported (Sivan *et al.*, 2007) that eEF2 phosphorylation was elevated in cells in M-phase, although it was not explained how the synthesis of specific polypeptides could continue in mitotic cells if elongation is inhibited. Our data do provide an explanation for this, by showing that eEF2 is unphosphorylated in mitotic cells.

Our data show that amino-acid availability positively regulates the activity of *cdc2* towards eEF2K, consistent with earlier data that amino-acid deprivation leads to dephosphorylation of Ser359 (Knebel *et al.*, 2001; Browne and Proud, 2004). mTORC1 mediates effects of amino acids, especially leucine: two main lines of evidence indicate mTOR may be involved in the control of *cdc2* by amino acids (see Figure 9 for a summary of this). First, loss of TSC2, a negative regulator of mTORC1, leads to enhanced, roscovitine-sensitive, Ser359 kinase activity. Earlier studies showed that loss of TSC2 results in downregulation of $\text{p}27^{\text{kip1}}$ and enhanced CDK2 activity (Soucek *et al.*, 1998; Rosner *et al.*, 2007), but this is the first indication that *cdc2* is regulated by TSC2. Second, *cdc2* kinase activity was markedly decreased when cells were starved of leucine or of all amino acids. *cdc2* has not previously been recognized as a target for control by mTOR signalling. This is likely because, although it is affected by amino-acid status, it is rather resistant to rapamycin. Further analysis of this interesting point is required.

There is evidence that the mTOR orthologue, *tor1*⁺, regulates mitotic entry in *Schizosaccharomyces pombe* (Weisman and Choder, 2001) but recent data show that nutrient starvation actually advances M-phase entry in this

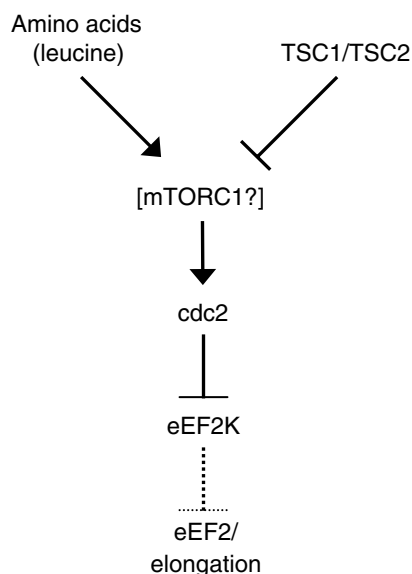


Figure 9 Schematic representation of the proposed role cdc2 has in regulating eEF2K. The data are consistent with mTORC1 positively regulating cdc2 (see Discussion) although this is not yet proven. cdc2-mediated inactivation of eEF2K allows translation to proceed during mitosis.

organism (Petersen and Nurse, 2007), indicating (not surprisingly) that nutrient control of mTOR differs between mammals and fission yeast.

The finding that amino acids regulate cdc2's activity against eEF2K raises the possibility that they, and perhaps mTORC1 signalling, also regulate its activity against other substrates and control other events at the G₂/M transition of the cell cycle, in addition to the well-known role of mTORC1 in regulation at G₁/S (Reiling and Sabatini, 2006). Interestingly, cdc2 has been previously shown to phosphorylate 4E-BP1 at Thr70 (another target for mTORC1) and this residue is hyperphosphorylated during mitosis (Heesom *et al*, 2001). It clearly makes physiological sense that cells take account of the availability of essential amino acids before commitment to this key step of cell division.

Materials and methods

Chemicals, biochemicals and other experimental details are given in the Supplementary data.

Cell synchronization

HeLa cells were synchronized using aphidicolin at a concentration of 1 µg/ml (made up in DMSO) or DMSO control for 16 h. Cells were

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released from the block by washing five times with sterile PBS. The medium was replaced with fresh complete DMEM and cells were lysed or analysed as detailed below at various time points after release. Where indicated HeLa cells were synchronized using a double thymidine block—HeLa cells were grown to a confluency of 30% and the medium was changed to fresh complete DMEM containing 2 mM thymidine or control medium (complete DMEM). Cells were incubated for 17 h and were released from the block by washing four times with sterile PBS. Again, the medium was replaced with complete DMEM and cells were incubated for 9 h. The medium was replaced with fresh complete DMEM containing 2 mM thymidine or control medium as above. Cells were incubated for a further 16 h and the medium was aspirated, cells were washed four times with sterile PBS and the medium was replaced with a medium containing leucine, unless otherwise indicated (in figure legends). The leucine-free medium constituents were as follows: DME/F-12 base medium (Sigma) supplemented with 0.1545 g/l calcium chloride, 0.365 g/l L-glutamine, 0.09125 g/l L-lysine, 0.0612 g/l magnesium chloride, 0.04884 g/l MgSO₄, 0.0172 g/l L-methionine and 1.2 g/l sodium bicarbonate. The medium was then filtered through a 0.45 µm filter and then supplemented with 10% (v/v) dialysed bovine serum. To prepare the 'plus leucine' medium, the above medium was supplemented with 0.059 g/l L-leucine. Plates were starved of leucine for the times indicated.

Assays of Ser359 kinase activity

Kinase activity was assayed by incubating 2 µg of bacterially expressed GST-eEF2K with 50 µM cold ATP (GST-eEF2K and ATP were diluted in kinase assay buffer: 50 mM MOPS pH 7.0, 5 mM magnesium chloride) and 20 µl of each FPLC fraction in a final volume of 60 µl. Reactions were incubated for 1 h at 30°C with shaking at 1000 r.p.m., and were stopped by adding 15 µl of 5 × SDS-PAGE sample buffer. Samples were then heated at 95°C for 5 min and subjected to SDS-PAGE and western blotting with anti-phospho Ser359 antibody.

cdc2 activity assays

cdc2 kinase activity was analysed by incubating 2 µg of bacterially expressed GST-eEF2K with 50 µM cold ATP (as above) and 20 µl of immunoprecipitated cdc2 in a final volume of 60 µl. Assays were incubated and stopped as described above. Samples were subjected to SDS-PAGE and western blotting to detect the phosphorylation of eEF2K at Ser359.

Statistical analysis

All data are from at least three independent experiments. Statistical analysis was performed using Student's *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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