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A Cdk7-Cdk4 T-Loop Phosphorylation Cascade Promotes G1 Progression

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Summary

Eukaryotic cell division is controlled by cyclin-dependent kinases (CDKs), which require phosphorylation by a CDK-activating kinase (CAK) for full activity. Chemical genetics uncovered requirements for the metazoan CAK Cdk7 in determining cyclin-specificity and activation order of Cdk2 and Cdk1 during S and G2 phases. It was unknown if Cdk7 also activates Cdk4 and Cdk6 to promote passage of the Restriction (R) Point, when continued cell-cycle progression becomes mitogen-independent; or if CDK-activating phosphorylation regulates G1 progression. Here we show that Cdk7 is a Cdk4- and Cdk6-activating kinase in human cells, required to maintain activity, not just to establish the active state, as is the case for Cdk1 and Cdk2. Activating phosphorylation of Cdk7 rises concurrently with that of Cdk4 as cells exit quiescence, and accelerates Cdk4 activation in vitro. Therefore, mitogen signaling drives a CDK-activation cascade during G1 progression, and CAK might be rate-limiting for R-point passage.

Introduction

Faithful cell division depends on strict alternation between genome duplication and segregation. This orderly progression is controlled in large part by cyclin-dependent kinases (CDKs) [reviewed in (Morgan, 2007)]. CDK activation minimally depends on two events: binding to a cyclin and phosphorylation of a conserved Thr residue in the activation (T) loop. Cyclins are expressed at different times in response to extracellular, mitogenic signals or internal, cell-autonomous cues. Phosphorylation of the CDK T loop is catalyzed by a CDK-activating kinase (CAK), and is required for full activity and biological function, but CAK has never been shown to fluctuate during the cell cycle or to play a rate-limiting role in regulating cell-cycle transitions [reviewed in (Fisher, 2005)].

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The sole CAK identified in metazoans is itself a CDK, Cdk7, which forms trimeric complexes with cyclin H and the RING-finger protein Mat1. In addition to its role in activating CDKs, Cdk7 phosphorylates the carboxyl-terminal domain (CTD) of RNA polymerase II (Pol II) and other proteins, as part of the general transcription factor TFIIH (Fisher, 2005). We recently showed that Cdk7 also executes a CDK-activating function within the transcription cycle, by phosphorylating Cdk9, the catalytic subunit of positive transcription elongation factor b (P-TEFb), to promote elongation and processing of Pol II transcripts (Larochelle et al., 2012). Although T-loop phosphorylation of Cdk1 and Cdk2 has been detected when certain transgenes are expressed in mouse cells deprived of Cdk7 by conditional gene deletion (Ganuza et al., 2012), no metazoan CAK other than Cdk7 has been identified, and immunodepletion or selective inhibition of Cdk7 reliably abolishes detectable CAK activity in metazoan cell extracts (Matsuoka et al., 1994; Larochelle et al., 1998; Wohlbold et al., 2006; Larochelle et al., 2012).

Expression and activity of the three-subunit CAK complex are roughly constant throughout the cell cycle in continuously dividing cells [(Fisher, 2005) and references therein]. In part to investigate how a seemingly constitutive activity contributes to cell-cycle regulation, we constructed human colon carcinoma HCT116 cells in which wild-type Cdk7 was replaced by an analog-sensitive (AS) version that can be inhibited by allele-specific, bulky adenine analogs (Larochelle et al., 2007). Inhibition of $Cdk7^{as}$ in these cells revealed that CAK helps determine cyclin-pairing rules and thus the order of Cdk2 and Cdk1 activation (Merrick et al., 2008). Cdk2 is the exclusive partner of cyclin E in G1 and the preferred partner of cyclin A until mid-S phase. Cdk1 also binds cyclin A, but only after Cdk2 appears to be saturated. Cdk7 helps to enforce this order by preferentially phosphorylating the Cdk2 monomer, which then binds cyclin to become active. Cdk1 cannot be phosphorylated in the absence of cyclin, and cannot bind stably to cyclin in the absence of T-loop phosphorylation, implying that the two events must occur in concert (Larochelle et al., 2007). Mathematical modeling indicated that a distinct kinetic path to activation would suffice to give Cdk2 priority in cyclin-binding, and account for the observed activation of Cdk2/cyclin A before Cdk1/cyclin A (Merrick et al., 2011). Cdk1 then binds cyclin B in G2 phase, when there is little free Cdk2 available. Nonetheless, inhibition of Cdk7—which impairs Cdk1/cyclin B assembly (Larochelle et al., 2007)—liberates cyclin B to bind Cdk2, forming small amounts of a complex not detected in unperturbed cells (Merrick et al., 2008). Although this analysis showed how Cdk7 helps order cell-cycle events, it did not provide evidence for a rate-limiting function.

Cdk4 and Cdk6 are active in G1 prior to the Restriction (R) Point—when further cell-cycle progression becomes mitogen-independent until the next G1 (Pardee, 1974)—and respond directly to mitogenic signals that induce expression of, and promote assembly with, D-type cyclins (Matsushime et al., 1992; Matsushime et al., 1994; Meyerson and Harlow, 1994). Cdk4 T-loop phosphorylation also increases when quiescent cells are stimulated to enter the cell cycle (Bockstaele et al., 2006). In addition, growth factors stimulate Tyr phosphorylation of p27, converting it from a CDK inhibitor (CKI) to a non-inhibitory scaffold or assembly factor (Chu et al., 2007; Grimmler et al., 2007; James et al., 2008). Cdk4 and Cdk6 phosphorylate the retinoblastoma tumor suppressor protein Rb, which in its unphosphorylated state binds and inhibits E2F family transcriptional activators. Phosphorylation by cyclin D-dependent kinases allows transcription of some E2F targets, including *Cyclin E*. Cyclin E activates Cdk2, which phosphorylates Rb further, triggering its dissociation from E2F and the transcription of genes needed for S phase (Lundberg and Weinberg, 1998; Rubin et al., 2005; Burke et al., 2010).

Rb, E2F and their upstream regulators are frequently deregulated in cancer cells (Weinberg, 1995; Sherr, 1996) and by DNA tumor viruses (Nevins, 1994), presumably because they

control commitment to division and impose restraints on tumor-cell proliferation and viral replication. Understanding how growth-factor signals are transduced to activate Cdk4 and Cdk6, the effector kinases that initiate inactivation of Rb, is therefore of both biological and medical importance. Key aspects of that regulation remain unknown, including the identity of a physiologic Cdk4- or Cdk6-activating kinase.

Cdk7 can activate Cdk4 and Cdk6 in vitro (Matsuoka et al., 1994; Aprelikova et al., 1995), and purified Cdk7^{as} phosphorylated endogenous Cdk4 selectively in crude extracts of human cells (Larochelle et al., 2006). In mouse fibroblasts lacking Cdk7 due to gene disruption, Cdk4-associated kinase activity was reduced, although the mechanism was not investigated (Ganuza et al., 2012). It is also not clear if CAK activity towards Cdk4 or Cdk6 *needs* to be regulated in vivo. When G1 progression was blocked by cyclic AMP (cAMP) in mouse macrophages treated with colony-stimulating factor 1, activating phosphorylation of Cdk4/cyclin D was prevented by its association with p27, while cellular CAK activity appeared unchanged (Kato et al., 1994). More recent studies suggested that cAMP interferes with Cdk4 activation through other mechanisms, but did not detect an effect on CAK itself (Rocha et al., 2008). However, both primary and tertiary structural differences between Cdk4 and Cdk2 support the idea that T-loop phosphorylation might be regulated differently for the two CDKs (Matsuoka et al., 1994; Day et al., 2009; Takaki et al., 2009), and were invoked to suggest that Cdk4 might be activated by a CAK other than Cdk7 in metazoan cells (Bockstaele et al., 2009).

Here we analyze activation of Cdk4 and Cdk6 in *Cdk7^{as}* cells, and show that Cdk7 is a physiologic CAK for both cyclin D-dependent kinases. Whereas Cdk2 and Cdk1 complexes remained phosphorylated for hours after Cdk7 activity was shut off, T-loop phosphorylation of Cdk4 and Cdk6 was rapidly lost. Therefore, Cdk7 activity is required to maintain the active state of Cdk4 and Cdk6, not merely to establish it as in the case of Cdk1 and Cdk2. This indicates that phosphatases work in opposition to Cdk7 in vivo, to dampen activation of Cdk4 and Cdk6 and delay G1 progression. Perhaps to balance that antagonism, CAK activity towards Cdk4 can be regulated by T-loop phosphorylation of Cdk7 itself. That modification—previously shown to accelerate phosphorylation of the Pol II CTD but not of Cdk2/cyclin A (Larochelle et al., 2001)—stimulated Cdk4 activation in vitro. In human cells entering the division cycle after quiescence, Cdk7 and Cdk4 T-loop phosphorylation increased with similar kinetics, suggesting that mitogen signaling triggers a cascade of CDK activation leading to Rb phosphorylation and R-point passage. These results reveal cell-cycle regulation of CAK activity, and a potentially rate-limiting role of Cdk4 T-loop phosphorylation in human cells.

Results

A stringent requirement for Cdk7 activity at S-phase entry

We showed previously that Cdk2 activity is rate-limiting for passage of the R point and entry to S phase (Merrick et al., 2011). Despite Cdk2 inhibition, however, a fraction of cells was able to continue into S phase, and the inhibitor-resistant population increased with increasing concentrations of serum added to the culture medium (Figure S1A). We sought to test if the dependence on Cdk7 activity was also contingent on the strength of mitogenic signaling. To do so, we compared effects of the allele-specific inhibitor 3-MB-PP1 on *Cdk7$^{as/as}$* HCT116 cells at different concentrations of serum. Cells were released from serum starvation into media with various amounts of serum and either DMSO or 10 μM 3-MB-PP1, collected 24 hr after release and analyzed for DNA content by flow cytometry. At serum concentrations above 1.25%, G1/S progression was slowed but not blocked by Cdk7 inhibition, consistent with previous results (Larochelle et al., 2007). At 1.25% serum or below, however, virtually no cells progressed through S phase when we added 3-MB-PP1

(Figure 1A), suggesting a more stringent dependence on Cdk7 when growth factors are limiting, consistent with the increased requirement for total CDK activity (Yao et al., 2008). These data also indicate a stricter need for Cdk7 than for Cdk2 in G1 progression, possibly because Cdk7 has additional targets needed to promote passage of the R point and entry to S phase.

To ask if the greater efficacy of Cdk7 inhibition reflected a broader impact on G1 CDK activity, we analyzed Rb phosphorylation on Cdk2- and Cdk4/6-specific sites (Zarkowska and Mittnacht, 1997). We showed previously that selective inhibition of Cdk2 diminished phosphorylation of Cdk2-specific sites Thr821 and -826 and, to a lesser extent, of Ser795, a target of both Cdk2 and Cdk4. However, Cdk2 inhibition had no effect on the Cdk4/6-specific sites, Ser807 and -811 (Merrick et al., 2011). To ask whether Cdk7 inhibition prevented phosphorylation of Cdk2-specific sites, Cdk4/6-specific sites or both, we arrested wild-type or *Cdk7*$^{as/as}$ cells in G0/G1 by serum starvation, released them into media containing DMSO or 3-MB-PP1 and monitored Rb phosphorylation for 12 hr after release. Phosphorylation of all sites—Cdk2-specific, Cdk4/6-specific and shared—was reduced by selective inhibition of Cdk7 (Figures 1B, S1B), suggesting that Cdk7 acts generally to support CDK activity and promote Rb phosphorylation and G1 progression.

Epistasis between Cdk7 and Cdk4/6

Based on this model, we hypothesized that combined inhibition of Cdk4/6 and Cdk2 would mimic inhibition of Cdk7 and have synthetic effects on proliferation, whereas simultaneous inhibition of Cdk7 and Cdk4/6 would have less-than-additive, “epistatic” effects. To test this, we treated wild-type, *Cdk7*$^{as/as}$ or *Cdk2*$^{as/as}$ cells with a Cdk4/6 inhibitor, PD0332991 (Fry et al., 2004), with or without 3-MB-PP1, and measured proliferation by crystal violet staining (Figures 1C, S1C). In all three lines, proliferation was reduced ~60% by saturating doses of PD0332991 alone. By itself, inhibition of Cdk2^{as} by 10 μM 3-MB-PP1 caused a similar reduction. *Cdk7*^{as} cells were more sensitive to 3-MB-PP1; to allow comparison with *Cdk2*^{as} cells, we chose a concentration, 50 nM, which likewise inhibited proliferation by ~60%. Addition of a saturating dose of PD0332991 to *Cdk7*^{as} cells reduced proliferation only by an additional ~2-fold. In contrast, when Cdk4/6 and Cdk2 inhibition were combined, proliferation was nearly abolished (Figure 1C). From this analysis, we surmised that Cdk4/6 and Cdk2 act in parallel, whereas Cdk7 might work upstream of Cdk2, Cdk4 and Cdk6.

A G1 execution point for Cdk7

The strict Cdk7 activity requirement for mitotic entry allowed us to determine an execution point—after which Cdk7 activity was no longer needed—even in cells grown in saturating serum concentrations (Larochelle et al., 2007). The heightened requirement for Cdk7 at S-phase entry when growth factors were limiting allowed us to make a similar determination in G1. To do so, we synchronized *Cdk7*$^{as/as}$ or wild-type cells by serum starvation and released them into medium containing 1% serum, to which we added 3-MB-PP1 at various times. We collected cells 24 hr after release and analyzed whether they had entered S phase by flow cytometry (Figures 2A, S2) and BrdU incorporation (Figure 2B). When Cdk7 was inhibited starting at the time of release or 3 hr later, the majority of cells did not progress into S phase. However, when inhibition was initiated ≥6 hr after serum addition, >50% of cells entered S phase. Therefore, a G1 execution point for Cdk7 function occurs 3–6 hr after release from serum starvation.

We next asked if there was a correlation between timing of the Cdk7 execution point and the Cdk7-dependence of various Rb phosphorylation marks, in *Cdk7*$^{as/as}$ cells subjected to the same synchronization and drug-treatment regimen and harvested 12 hr after release. When

3-MB-PP1 was added prior to the execution point, phosphorylations on both Cdk4/6- and Cdk2-specific sites were diminished. Passage through the execution point correlated with the loss of 3-MB-PP1-sensitivity of Rb phosphorylation at the Cdk4/6-dependent sites Ser807/811 and Ser795, whereas the Cdk2-specific sites at Thr821/826 remained sensitive for at least 9 hr after release (Figure 2C). This indicated a temporal correlation between the requirement for Cdk7 and a specific readout of Cdk4/6 activity, and suggested that the stronger G1 block due to inhibition of Cdk7, relative to Cdk2, might be due to the additional loss of Cdk4/6 activation.

Cdk4 and Cdk6 depend on Cdk7 for activation in vivo

To test this model, we asked if Cdk7 is a physiologic CAK for Cdk4 and Cdk6 in human cells, by treating *Cdk7*$^{as/as}$ cells (grown in 10% serum here and in all subsequent experiments) with DMSO or 10 μM 3-MB-PP1 for 8 hr, immunoprecipitating Cdk4 or Cdk6 complexes and measuring their ability to phosphorylate an Rb protein fragment in vitro. Cdk4 and Cdk6 recovered from DMSO-treated cells—or from 3-MB-PP1-treated wild-type cells—were active, whereas 3-MB-PP1 treatment of *Cdk7*$^{as/as}$ cells nearly abolished Rb kinase activity of both CDKs (Figure 3A, B). The loss of activity was not due to decreased cyclin D expression or complex assembly, because similar amounts of cyclin D co-precipitated with Cdk4 and Cdk6 in DMSO- or 3-MB-PP1-treated cells. Similarly, we saw no effects of acute Cdk7 inhibition on abundance of the CKIs p21 and p27 in complexes with cyclin D (Figure S3A).

To ask if the decrease in Cdk4 activity was in fact due to CAK deficiency, we measured Cdk4 T-loop phosphorylation directly, with an antibody previously shown to recognize this modification specifically (Bockstaele et al., 2006). The Cdk4-Thr172 phosphorylation signal (T172-P) was diminished by 3-MB-PP1 treatment in both extracts and anti-Cdk4 immunoprecipitates from *Cdk7*$^{as/as}$ cells, whereas the drug had no effect on T172-P in wild-type cells (Figures 3C, S3B). In the immunoprecipitated sample, a residual T-172P signal appeared even after Cdk7 inhibition, indicating either 1) cross-reactivity of the antibody, e.g. with unphosphorylated Cdk4; 2) residual Cdk4 T-loop phosphorylation due to another, 3-MB-PP1-resistant CAK; or 3) a population of Cdk4 protected from dephosphorylation, analogous to cyclin-bound, phosphatase-resistant populations of Cdk1 and Cdk2 (Larochelle et al., 2007; Merrick et al., 2008). In contrast to 3-MB-PP1-resistant Cdk2 complexes, however, any Cdk4 that remained phosphorylated after Cdk7 inhibition was inactive (Figure 3A). The antibody did not recognize Cdk6, so to test if diminished Cdk6 activity was due to loss of T-loop phosphorylation, we treated Cdk6 complexes recovered from DMSO- or 3-MB-PP1-treated cells with purified Cdk7. This treatment restored PD0332991-sensitive Rb kinase activity to Cdk6 immunoprecipitates (Figures 3D, S3C), suggesting that inhibition of Cdk7 in vivo led to the accumulation of activation-competent Cdk6 complexes lacking only T-loop phosphorylation. CAK treatment in vitro also stimulated Cdk6 recovered from cells in which Cdk7 was not inhibited, suggesting that Cdk6/cyclin D complexes normally exist in equilibrium between unphosphorylated and phosphorylated forms (see Discussion). We conclude that Cdk7 activity is required for normal levels of T-loop phosphorylation of Cdk4 and Cdk6 in vivo. Together with previous results showing Cdk7-dependence of Cdk1 and Cdk2, this suggests that Cdk7 is a universal CAK for CDKs involved in human cell division.

A Cdk4/6-specific activation pathway

The conformational changes that accompany full activation of Cdk2 lead to coordination of the phosphorylated T-loop within the Cdk2/cyclin A complex (Russo et al., 1996). Consistent with a protected T-loop conformation, the phosphorylated Thr160 residues of Cdk2/cyclin complexes were resistant to phosphatases in vitro (Poon and Hunter, 1995; Cheng et al., 2000), and cyclin-bound Cdk2 remained phosphorylated for up to 12 hr in the

absence of Cdk7 activity in vivo (Merrick et al., 2008). In contrast, phosphorylated, monomeric Cdk2—which is susceptible to phosphatase attack in vitro—was completely dephosphorylated by 2–3 hr after CAK shutoff in vivo. From these results we deduced that the primary pathway of Cdk2 activation in human cells is T-loop phosphorylation of monomeric Cdk2 first, followed by cyclin-binding.

To ask which pathway(s) Cdk4 and Cdk6 follow in vivo, we measured the kinetics of inactivation of Cdk4 and Cdk6 after CAK shutoff. We treated *Cdk7*$^{as/as}$ cells with DMSO or 3-MB-PP1 for various times, immunoprecipitated Cdk4, Cdk6 or Cdk2, and measured associated kinase activity. By two different treatment protocols, we observed a monotonic increase in both Cdk4- and Cdk6-associated activity between the 15-min and 3-hr time points, which did not occur in the case of Cdk2 (Figure 4A–C and data not shown). We suspect this is due to an intrinsic lability of cyclin D-dependent kinases, such that any manipulation of the cells is sufficient to cause a transient loss of activity. Nevertheless, 3-MB-PP1 treatment of *Cdk7*$^{as/as}$ cells caused a >50% decrease in Cdk4 and Cdk6 activity, relative to the matched, DMSO-treated control, within 30 min, whereas Cdk2 retained the majority of its activity after 3 hr (Figure 4A–C). We also looked directly at T-loop phosphorylation with phosphospecific antibodies. Cdk4-Thr172 phosphorylation decreased within 15 min of Cdk7 inhibition, but 3-MB-PP1 treatment for >3 hr was needed to cause a decrease in phosphorylation of Cdk2-Thr160 or Cdk1-Thr161 (Figure 4D). There was no effect of a 24-hr 3-MB-PP1 treatment on Cdk1, Cdk2 or Cdk4 T-loop phosphorylation in wild-type HCT116 cells. We conclude that Cdk2 and Cdk1 require Cdk7 for their initial activation, but then remain active because the phosphorylated T loop is protected within the CDK/cyclin complex. In contrast, Cdk4 and Cdk6 require Cdk7 activity to maintain the activated state because endogenous phosphatases are able to work on their T loops, which remain exposed after cyclin-binding and phosphorylation (Day et al., 2009; Takaki et al., 2009).

Cdk7 T-loop phosphorylation enhances Cdk4 activation

We reasoned that, to overcome competition by phosphatases, Cdk7 might need to be in its most active form. The CAK complex can exist in at least three different, active states: Cdk7 in ternary complexes with cyclin H and Mat1, with or without T-loop phosphorylation; or in a binary complex with cyclin H alone, a form that is only stable when the Cdk7 T loop is phosphorylated. Of these forms, the phosphorylated trimer is the most efficient at phosphorylating the Pol II CTD because of an ~20-fold higher rate of catalysis, but no differences among the different forms were detected with Cdk2/cyclin A as a substrate (Larochelle et al., 2001). We tested the three different CAK isoforms (dimer, trimer and phospho-trimer) for their ability to activate Cdk4/cyclin D1, which was purified as a complex from insect cells expressing both subunits. This results in an active complex, refractory to activation by Cdk7 (Figure S4A), presumably because it is phosphorylated on the T loop. Consistent with a previous report (Takaki et al., 2009), the Cdk4/cyclin D complex could be inactivated by treatment with λ-phosphatase and reactivated by subsequent incubation with CAK in vitro. In this assay, the Cdk7 phospho-trimer was ~3-fold more active towards Cdk4 than were the other two isoforms (Figure 5A). In keeping with our previous results, all three forms were roughly equivalent in their ability to activate Cdk2/cyclin A in parallel assays (Figure 5B).

We next compared kinetics of Cdk4/cyclin D and Cdk2/cyclin A activation by two different Cdk7 isoforms: the phosphorylated dimer and the phosphorylated trimer reconstituted by adding purified Mat1 to the dimer (Figure 5C). It was initially suggested that Mat1 “switched” the substrate specificity of Cdk7/cyclin H from CDK to the Pol II CTD (Adamczewski et al., 1996; Yankulov and Bentley, 1997); our subsequent analysis revealed that T-loop phosphorylation (which was not manipulated in earlier studies), was required for

Mat1-dependent stimulation of CTD kinase activity (Figure S4B), which occurred without any loss of CAK activity (Larochelle et al., 2001). Assembly of Mat1 with the dimer thus mimics effects on substrate selection due to T-loop phosphorylation of the trimer (Larochelle et al., 2006), and is easier to control, because it allows comparisons between the same enzyme preparation (phosphoCdk7/cyclin H) with or without Mat1. In this analysis, addition of Mat1 accelerated Cdk4 activation compared to the mock-treated dimer, but had little effect on the rate of Cdk2/cyclin A activation measured in parallel (Figure 5C). We conclude that, in contrast to Cdk2/cyclin A activation, Cdk4 T-loop phosphorylation is enhanced by the same factors that stimulate Cdk7 activity towards the Pol II CTD (Larochelle et al., 2001; Larochelle et al., 2006).

CAK regulation at cell-cycle entry: a CDK activation cascade in G1

The stimulatory effect of Cdk7 T-loop phosphorylation on Cdk4 activation suggested a means to boost Cdk4 activity in response to growth factors—a distinctive feature of Cdk4 regulation in metazoan cells (Matsushime et al., 1991). Previous studies detected no change in Cdk7 phosphorylation, measured by electrophoretic mobility shifts, in continuously cycling HeLa cells or in *Drosophila* embryos at different stages of the early division cycles (Garrett et al., 2001; Larochelle et al., 2001). We used a Cdk7-Thr170 phosphorylation-specific antibody (Larochelle et al., 2012) to monitor the activation state of Cdk7 in HCT116 cells or telomerase-expressing human retinal pigment epithelial cells (RPE-hTERT) cells—a non-transformed line that enters G0 in response to contact inhibition (Merrick et al., 2011). When HCT116 cells were released from serum starvation (Figure 6A), or RPE-hTERT cells grown to confluence were released into G1 by dilution in fresh medium (Figure 6B), Cdk7 phosphorylation increased, reaching a peak 8–12 hr after release, whereas levels of total Cdk7, cyclin H and Mat1 remained roughly constant. Although Cdk7 T-loop phosphorylation increased in both cell lines, we consistently detected this change only in lysates prepared under harsh extraction conditions (Figure 6 and data not shown), suggesting that Cdk7-activating phosphorylation might occur in a relatively insoluble cellular compartment (e.g. chromatin), and possibly explaining why this phenomenon had previously gone undetected.

We attempted to detect a change in activity of Cdk7, correlated with the increase in T-loop phosphorylation, as HCT116 cells were released from serum starvation. In anti-Cdk7 immunoprecipitates, we recovered similar amounts of phosphorylated Cdk7—and, predictably, CTD kinase activity—from extracts of serum-starved or –stimulated cells (Figure S5A). We also attempted to perform this comparison on Cdk7 coimmunoprecipitated with Mat1; although the complexes from serum-stimulated cells were ~2-fold more active than those from starved cells, we were again unable to recover the phosphorylated and unphosphorylated forms in the relative proportions in which they were present in the extracts (Figure S5B). Although this precluded meaningful comparisons between the extracts, it offers another potential explanation of why no fluctuations in Cdk7 activity have previously been detected. It also suggests that the Cdk7 complex undergoes additional changes during G1 progression that affect its recognition by antibodies, and might influence its functions in the cell.

In both HCT116 and RPE-hTERT cells, increases in Cdk4-Thr172 phosphorylation correlated with the increase in Cdk7-Thr170 phosphorylation and the accumulation of cyclin D (Figure 6A, B). Cyclin D-associated kinase activity also increased during G1 progression, as expected, in extracts of HCT116 cells (Figure 6A). Therefore, the two modifications minimally required for Cdk4 activity—association with cyclin and T-loop phosphorylation by CAK—might both be directly responsive to mitogenic stimulation. Growth factor-dependent signaling also neutralizes inhibitory effects of p27, in part by allowing T-loop phosphorylation to occur within ternary Cdk4/cyclin D/p27 complexes (Chu et al., 2007;

Grimmler et al., 2007; James et al., 2008; Ray et al., 2009). Taken together with those previous studies, our data suggest that Cdk4 activity is tuned to mitogenic signals through multiple, convergent pathways.

Discussion

Cdk7: A Universal CAK

By chemical genetics we have shown that Cdk4 and Cdk6 depend on Cdk7 for activation in vivo, suggesting that Cdk7 is a universal CAK for all cell-cycle CDKs. It was proposed that Cdk4 might have its own unique CAK, due to divergence of the Cdk4 T-loop sequence—in which a Pro residue occupies the +1 position following the phosphorylated Thr—from that of other CDKs (Bockstaele et al., 2009). Cdk7 *is* a Pro-directed kinase, however, which phosphorylates the Pol II CTD at Ser5 of the sequence $Y_1S_2P_3T_4S_5P_6S_7$, but expands its substrate repertoire to phosphorylate other CDKs (Larochelle et al., 2006). Therefore neither the Cdk4 T loop containing a +1 Pro, nor the Cdk6 T loop lacking one, poses a recognition problem for Cdk7 a priori. It remains possible that another CAK might exist in mammalian cells, as was recently suggested (Ganuza et al., 2012), but if so, it is unable to sustain measurable activity of Cdk4 or Cdk6 (this report), or to activate Cdk1 or Cdk2 de novo (Larochelle et al., 2007), after chemical inhibition of Cdk7 in HCT116 cells.

Although CDKs share a common activating kinase, each appears to have a distinct activation *mechanism*, due to the enzymatic versatility of Cdk7. The sequence initially proposed for CDK activation placed cyclin binding before T-loop phosphorylation (Jeffrey et al., 1995; Russo et al., 1996). Subsequent studies showed that, in vivo, phosphorylation precedes cyclin binding by Cdk2 (Merrick et al., 2008), and occurs in concert with cyclin binding by Cdk1 (Larochelle et al., 2007). Cdk4 and Cdk6 appear to follow the canonical pathway in which T-loop phosphorylation occurs after cyclin-binding.

Therefore, Cdk4 and Cdk6, which act prior to the R point, might be regulated by competition between an activating kinase (Cdk7) and a T-loop phosphatase, so that the population of cyclin D-bound CDK is a variable mixture of phosphorylated and unphosphorylated forms. This conclusion is supported by our ability to “superactivate” Cdk6 complexes recovered from unperturbed cells by treatment with recombinant Cdk7 (Figures 3D, S3C). In contrast, most or all cyclin-bound Cdk2 is phosphorylated (Merrick et al., 2008), and T-loop phosphorylation is *required* to form stable complexes of Cdk1 and cyclin (Larochelle et al., 2007). We propose a model in which CDK activation progresses from an “analog” mode—acutely responsive to fluctuations in mitogenic signal strength—to more switch-like, binary mechanisms as cells make the commitment to divide, and as different CDKs are channeled into kinetically distinct pathways (Figure 7).

Divergent regulatory mechanisms are likely to reflect structural differences between Cdk2 and Cdk4 (Day et al., 2009; Takaki et al., 2009). Even after Cdk4 is bound to cyclin D and phosphorylated at Thr172, it requires additional conformational changes, perhaps due to binding of a substrate or an assembly factor, to become competent for catalysis. Most Cdk4/cyclin D in the cell appears to be bound to p27, which can act as an inhibitor or as an accessory factor permissive for activity, depending on its own phosphorylation state (Chu et al., 2007; Grimmler et al., 2007; James et al., 2008). In addition, the T loop of Cdk4 remains fully accessible to solvent despite phosphorylation and cyclin binding (Day et al., 2009; Takaki et al., 2009), in contrast to that of Cdk2 (Russo et al., 1996). Cdk4 thus resembles monomeric Cdk2, in which the phosphorylated T loop is also accessible. Both Cdk4/cyclin D and Cdk2 monomer rapidly lose phosphorylation after inhibition of Cdk7 in vivo [(Merrick et al., 2008) and this report]. Cdk7 phosphorylates Cdk2 monomers and Cdk2/cyclin A complexes with similar catalytic efficiencies (k_{cat}/K_m) but at a ~20-fold faster rate

with the monomeric substrate (Merrick et al., 2008). This raises the possibility that activation of Cdk2—which is required prior to R-point passage (Merrick et al., 2011)—might also be regulated by changes in Cdk7 enzymatic properties that have so far escaped detection. (It also raises a question: Do the same phosphatases target Cdk4/cyclin D and monomeric Cdk2 in vivo?) The T loop of Cdk2 becomes protected after phosphorylation and cyclin-binding, however, unlike that of Cdk4. Cdk7 is therefore required to *maintain* Cdk4 and Cdk6 activity, but only to *establish* the active state of Cdk2.

A Cdk7–Cdk4 Activation Cascade Responsive to Mitogens

Cdk4 and Cdk6 execute their known function upstream of the R point, when further cell-cycle progression becomes mitogen-independent. Accordingly, their activity level is coupled to growth-factor signaling through multiple mechanisms. Cyclin D expression depends on mitogens (Matsushime et al., 1991), as does phosphorylation of p27. Both p21 and p27 can promote activation of Cdk4/cyclin D, by assisting with complex assembly and translocation to the nucleus (LaBaer et al., 1997; Cheng et al., 1999); Association of Cdk4 with p27 depends on phosphorylation of the latter by growth factor-dependent kinases (Larrea et al., 2008). Moreover, phosphorylation of p27 by mitogen-stimulated Tyr kinases neutralizes its inhibitory effect on Cdk4 activity (Chu et al., 2007; Grimmler et al., 2007; James et al., 2008). Interestingly, Tyr phosphorylation of p27 might also promote Cdk4 *activation* by giving Cdk7 access to the T loop in the ternary Cdk4/cyclin D/p27 complex, whereas unphosphorylated p27 blocks that access (Ray et al., 2009).

Previously, no fluctuations in Cdk7 expression or phosphorylation state, or CAK activity, had been detected in cycling cells, implying that T-loop phosphorylation of Cdk4 would occur constitutively as cyclin D was expressed and inhibition by p27 was relieved. Here, we showed that T-loop phosphorylation of Cdk7 itself is induced in human cells stimulated to re-enter the cell cycle, and that this modification is capable of stimulating Cdk7 activity towards Cdk4. This suggests a cascade of CDK T-loop phosphorylation during G0–G1 progression, in which mitogenic signaling leads to activation in turn of Cdk7 and Cdk4. An important goal for the future is to identify the kinase(s) responsible for Cdk7 activation in early G1. Previously, we showed that, in vitro, Cdk7 is a target for reciprocal activation by Cdk1 and Cdk2 but *not* by Cdk4 (Garrett et al., 2001). Because Cdk1 and Cdk2 are presumably inactive during early G1, our results imply the existence of another Cdk7-activating kinase—possibly but not necessarily another CDK—that is active during this interval.

Rate-limiting functions for Cdk7?

Enzymologic and chemical-genetic analyses showed that Cdk7 is specifically adapted to perform different roles throughout the cell cycle and in non-cycling cells. Cdk7 has two distinct substrate recognition modes to accomplish its dual functions in CDK activation and gene expression (Larochelle et al., 2006), and can support different activation pathways for its closely related targets, Cdk1 and Cdk2 (Larochelle et al., 2007; Merrick et al., 2008). Finally, Cdk7 can act as both an effector kinase and a CAK within the transcription cycle (Larochelle et al., 2012). Nonetheless, the prevailing view of CAK has been that of a constitutive activator, rather than a true regulator of the cell-cycle machinery (Harper and Elledge, 1998).

Here we establish the mechanistic basis for a rate-limiting function of Cdk7 in G0–G1 progression, which is rooted in 1) the structure of Cdk4/cyclin D complexes, in which the phosphorylated T loop remains exposed, ensuring that steady-state activity levels are determined by a balance of CAK and phosphatase activities; 2) the ability of Cdk7 to accelerate phosphorylation of certain substrates, including Cdk4, upon phosphorylation of

its own T loop; and 3) the increase of Cdk7 T-loop phosphorylation during synchronous G1 progression stimulated by mitogens. We recently discovered another context in which Cdk7 might be regulated: on chromatin, T-loop phosphorylation was low in the population of Cdk7 crosslinked in promoter regions, and increased progressively towards 3' ends of transcribed genes (Larochelle et al., 2012). Therefore, T-loop phosphorylation is also likely to stimulate functions of Cdk7 in transcript elongation and co-transcriptional RNA processing, and might serve to coordinate the activation of G1 CDKs with the wholesale induction of gene expression as cells enter the division cycle in response to growth factors (Donner et al., 2010).

Experimental Procedures

Immunological methods

To analyze protein levels, phosphorylation, complex formation and activity, cells were lysed in 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 % (v/v) Triton-X 100, 1 mM DTT, 50 mM NaF, 80 mM β-glycerophosphate, 0.2 mM Na_3VO_4, and protease inhibitors; or, in Figure 6, in 50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 1.0 % NP-40, 0.5 % deoxycholate, 0.1 % SDS, 50 mM NaF, 80 mM β-glycerophosphate, 0.2 mM Na_3VO_4, and protease inhibitors (RIPA). Immunoprecipitation was performed by incubating 300–500 μg of extract protein for 3 hr at 4°C with Protein A-Sepharose beads, containing antibodies to Cdk4 (06-139) (Upstate Biotechnologies), Cdk6 (C-21), Cdk2 (M2), Mat1 (sc-13142) (Santa Cruz); Cdk7 (MO1.1, Sigma); or cyclin D1 (dcs-11, Biomarkers). Immunoblotting was performed with antibodies to Cdk4 (C-22), Cdk6 (C-21), Cdk2 (M2), Rb (sc-102), phospho-Rb T821/826 (sc-16669) and Mat1 (sc-13142) (Santa Cruz); cyclin D (554203, BD Pharmingen); phospho-Cdk1-Thr161, -Cdk2-Thr160, -Rb S807/811 and -Rb S795 (Cell Signaling); cyclin H (ab10542, Abcam); Cdk7 (MO1.1) (Sigma); phospho-Cdk4 (gift of P. Roger); and phospho-Cdk7 (Larochelle et al., 2012).

Kinase Assays

Immunoprecipitates were incubated in 10 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 5 μCi γ-[^{32}P]-ATP, with 3 μg of an 86 kDa fragment of Rb (gift of S.Blain) for 1.5 hr (Cdk4), 1 hr (Cdk6) or 10 min (Cdk2) at room temperature. Reactions were stopped by sample buffer addition and analyzed by SDS-PAGE and autoradiography and phosphorimaging. We activated recombinant Cdk4/cyclin D and Cdk2/cyclin A with different Cdk7 isoforms generated as previously described (Larochelle et al., 2006). Prior to activation, 400 ng of CDK/cyclin complexes were treated with 1 unit λ phosphatase (New England BioLabs) in 50 mM HEPES pH 7.4, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, 1 mM $MnCl_2$, for 30 min at 30°C. The reaction was stopped by addition of NaF and Na_3VO_4 to final concentrations of 15 and 10 mM, respectively. We used 150 ng of Cdk4 or Cdk2 per reaction; and 50 or 6 ng of Cdk7 to activate Cdk4 or Cdk2, respectively. CTD kinase activity of Cdk7 was tested as previously described (Larochelle et al., 2001).

Cdk4/Cyclin D Purification

Full-length human Cdk4 (residues 1–303) containing an amino-terminal GST tag followed by a TEV protease cleavage site, and truncated cyclin D1 (residues 19–267) were cloned in a modified pFastBacDual vector (Invitrogen). Hi5 cells were infected with recombinant baculovirus, harvested and lysed by passage through an EmulsiFlex-C3 (Avestin). Lysate was centrifuged and passed over Glutathione Sepharose 4B (GE Healthcare). Proteins were eluted and passed over a Source 15Q (GE Healthcare) column to remove glutathione and eluted. After overnight digestion at 10°C with GST-tagged TEV protease (1% by mass), the protease, tag and uncleaved complex were removed by passage over Glutathione Sepharose

4B. Proteins were concentrated and run over a Superdex 200 column equilibrated in 25 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM DTT.

Cell Synchronization, Cell-cycle analysis, and Proliferation Assays

Wild-type, *Cdk2*$^{as/as}$ or *Cdk7*$^{as/as}$ HCT116 cells were synchronized by incubation in serum-free medium for 72 hr and released into media containing different concentrations of fetal calf serum. Analysis of cell cycle distribution was by propidium iodide staining and flow cytometry to measure DNA contact, and by BrdU incorporation, as previously described (Merrick et al., 2008; Merrick et al., 2011). Cell proliferation was measured by crystal violet staining, as previously described (Merrick et al., 2011) and quantified by spectrophotometric detection of solubilized crystal violet at 595 nm (Kueng et al., 1989).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Chemical genetics reveals that Cdk7 is the CAK for Cdk4 and Cdk6 in human cells.
- Cdk4 and Cdk6 are acutely sensitive to CAK shutoff in vivo, unlike Cdk1 or Cdk2.
- Cdk4 activation is stimulated by Cdk7 T-loop phosphorylation in vitro.
- Cdk7 T-loop phosphorylation is induced by mitogenic stimulation in G1.

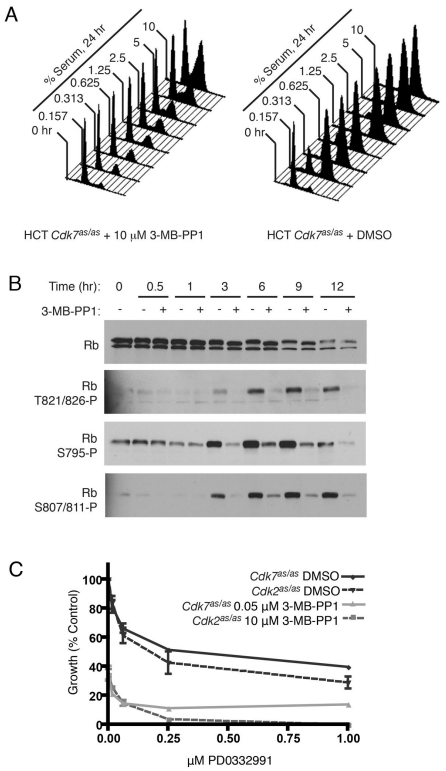

Figure 1. Chemical genetics uncovers a Cdk7 requirement in G1/S progression

A. *Cdk7*$^{as/as}$ HCT116 cells were synchronized in G0/G1 by serum starvation and released into media containing increasing concentrations of serum (as indicated), with DMSO or 10 μM 3-MB-PP1 (as indicated), and nocodazole (added to trap cells in mitosis). Cells were collected 24 hr after release and cell-cycle distribution was analyzed by flow cytometry to determine DNA content.

B. *Cdk7*$^{as/as}$ cells were synchronized as in (A) and released into 1% serum-containing media with DMSO or 10 μM 3-MB-PP1. Cells were collected at indicated times and Rb phosphorylation was analyzed with indicated phospho-specific antibodies.

C. *Cdk2*$^{as/as}$ and *Cdk7*$^{as/as}$ HCT116 cells were grown for 5 d in DMSO or the indicated concentration of 3-MB-PP1 and various concentrations of PD0332991. Cells were fixed and stained with crystal violet solution, and proliferation was quantified by solubilization and spectrophotometric measurement of dye-binding and expressed as a percentage of growth in the DMSO-treated controls. Each data point is the mean ± SEM of 3 replicates.

See also Figure S1.

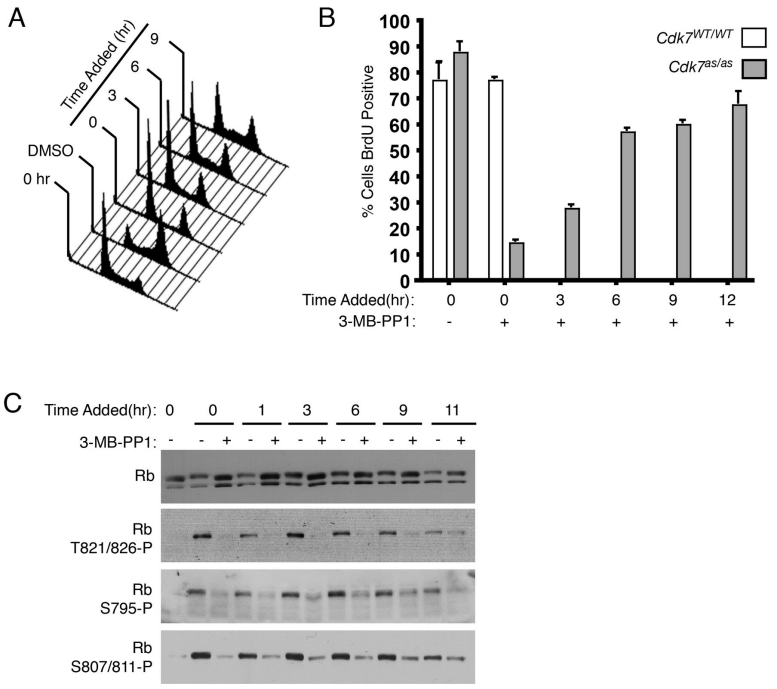

Figure 2. Cdk7 execution point correlates with timing of Rb phosphorylation by Cdk4/6

A. *Cdk7*$^{as/as}$ cells were synchronized as in Figure 1 and released into 1% serum-containing medium (with nocodazole), to which DMSO was added at time 0 or 10 μM 3-MB-PP1 was added at the indicated times. Cells were collected 24 hr after release and cell-cycle distribution was analyzed by flow cytometry.

B. *Cdk7*$^{WT/WT}$ or *Cdk7*$^{as/as}$ cells were synchronized as in (A) and released into 1% serum-containing medium. DMSO or 10 μM 3-MB-PP1 was added at the indicated times, together with bromodeoxyuridine (BrdU) to detect DNA synthesis occurring after drug treatment, and nocodazole to prevent additional rounds of DNA synthesis. Cells were fixed and stained for BrdU incorporation 24 hr after release. Each data point is the mean +/− SEM of three fields of cells.

C. *Cdk7*$^{as/as}$ cells were synchronized and released as in (A). The first lane contains extract from cells prior to serum addition; subsequent lanes contain extracts from cells to which DMSO or 10 μM 3-MB-PP1 was added at indicated times, collected 12 hr after release. Rb phosphorylation was analyzed with indicated phosphospecific antibodies.

See also Figure S2.

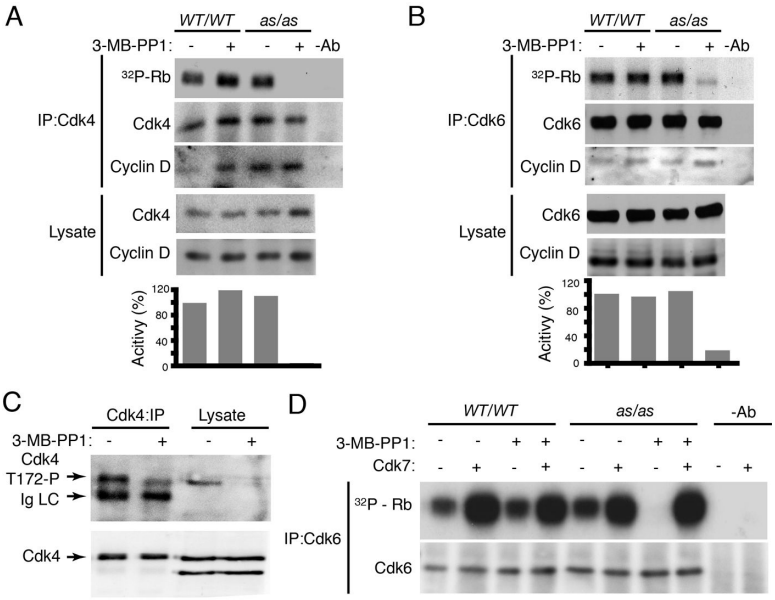

Figure 3. Cdk4 and Cdk6 depend on Cdk7 activity for activation in vivo

A. Wild-type (*WT/WT*) or *Cdk7*$^{as/as}$ cells were treated with DMSO or 10 μM 3-MB-PP1 for 8 hr, collected and lysed. Extracts were immunoprecipitated with anti-Cdk4 antibody, immunoblotted for Cdk4 and cyclin D, and tested for Rb kinase activity. Incorporation was quantified by Phosphorimager.

B. Same as (A) except that immunoprecipitation was with anti-Cdk6 antibody.

C. *Cdk7*$^{as/as}$ cells were treated with DMSO or 10 μM 3-MB-PP1 for 8 hr, lysed and extracts were immunoprecipitated with anti-Cdk4 antibody. The immunoprecipitates and whole-cell extract were immunoblotted with antibodies specific for phosphorylated Cdk4-Thr172 (T172-P) and total Cdk4 (Ig LC: immunoglobulin light chain).

D. Extracts were immunoprecipitated with anti-Cdk6 antibody as in (B) and treated with purified recombinant Cdk7/cyclin H/Mat1, immunoblotted for Cdk6 and tested for Rb kinase activity. Incorporation was quantified by Phosphorimager.

See also Figure S3.

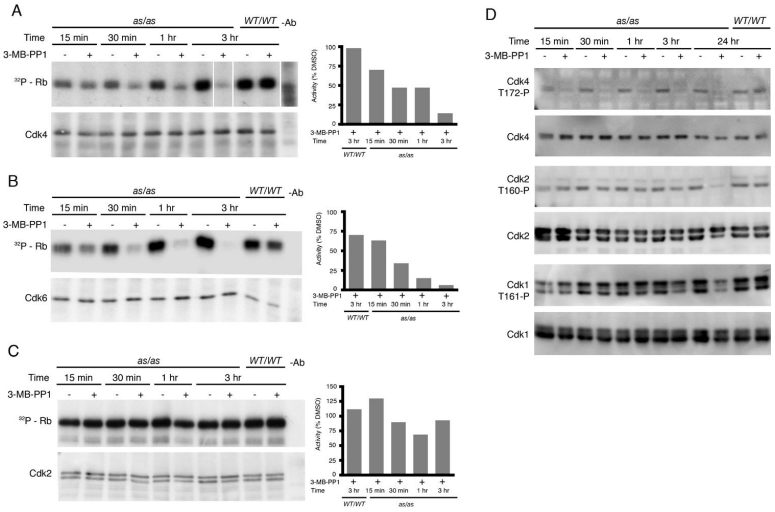

Figure 4. Cdk7 is required to maintain Cdk4 and Cdk6 activity, but only to establish active state of Cdk1 and Cdk2

A. Wild-type or *Cdk7*$^{as/as}$ cells were treated with DMSO or 10 μM 3-MB-PP1 for the indicated times and collected immediately after treatment for extract preparation. Extracts were immunoprecipitated with anti-Cdk4 antibody, immunoblotted for Cdk4 (bottom) and tested for Rb kinase activity (top). (Note: Sample corresponding to the 3-hr 3-MB-PP1 treatment of *Cdk7*$^{as/as}$ cells was loaded out of order and repositioned to conform to the immunoblot, but all data come from the same autoradiographic exposure.) Incorporation was quantified by Phosphorimager; each 3-MB-PP1-treated sample was normalized to corresponding DMSO-treated sample.

B. Same as in (A) except that immunoprecipitation was with anti-Cdk6 antibody.

C. Same as in (A) except that immunoprecipitation was with anti-Cdk2 antibody.

D. Cells were treated as in (A) and whole-cell extracts were immunoblotted for phosphorylated and total Cdk4, Cdk2 and Cdk1, as indicated.

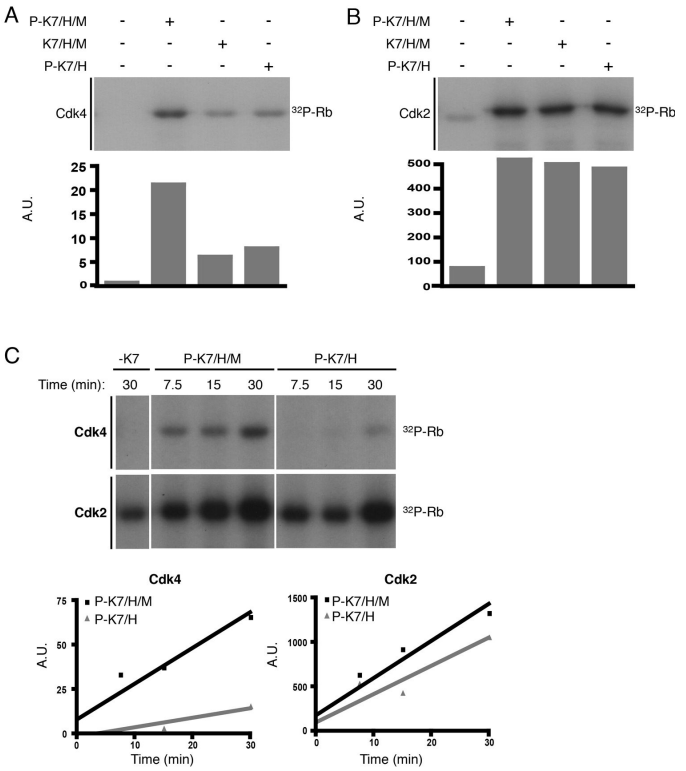

Figure 5. Cdk7 T-loop phosphorylation stimulates activation of Cdk4

A. Purified Cdk4/cyclin D was treated with T-loop phosphorylated Cdk7/cyclin H/Mat1 (P-K7/H/M), unphosphorylated Cdk7/cyclin H/Mat1 (K7/H/M), or phosphorylated Cdk7/cyclin H (P-K7/H) for 30 min and tested for Rb kinase activity. Incorporation was quantified in arbitrary units (A.U.) by Phosphorimager.

B. Same as (A) except Cdk2/cyclin A was activated by Cdk7 isoforms.

C. Purified Cdk4/cyclin D or Cdk2/cyclin A was treated with 50 or 6.25 ng, respectively, of phosphorylated Cdk7/cyclin H/Mat1 (P-K7/H/M) or phosphorylated Cdk7/cyclin H (P-K7/H), as indicated, for the indicated times and tested for Rb kinase activity. Incorporation was quantified by Phosphorimager.

See also Figure S4.

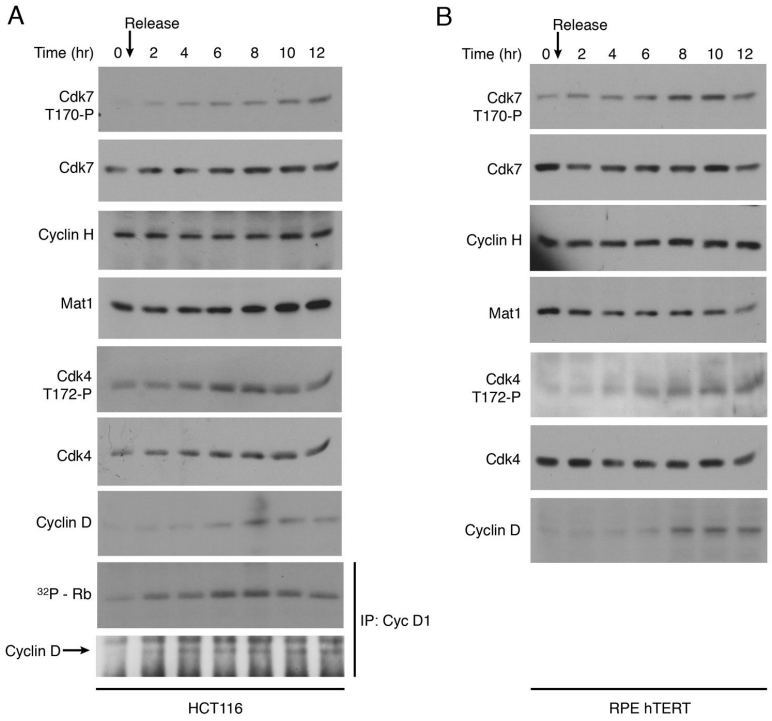

Figure 6. Cdk7 and Cdk4 T-loop phosphorylation occur with similar kinetics during cell cycle reentry

A. HCT116 cells were synchronized by serum starvation and released into fresh medium containing 10% serum. Extracts were prepared in RIPA buffer at indicated times after release, and immunoblotted for Cdk7 phosphorylated at Thr170 (T170-P), total Cdk7, cyclin H, Mat1, Cdk4 phosphorylated at Thr172 (T172 -P), total Cdk4 and cyclin D. Rb kinase activity (^{32}P-Rb) and cyclin D recovery were measured in cyclin D1 immunoprecipitates from the same extracts.

B. RPE-hTERT cells were synchronized in G0 by contact inhibition and released into fresh, 10% serum-containing medium. Extracts were prepared in RIPA buffer at the indicated times and immunoblotted for the same proteins measured in (A).

See also Figure S5.

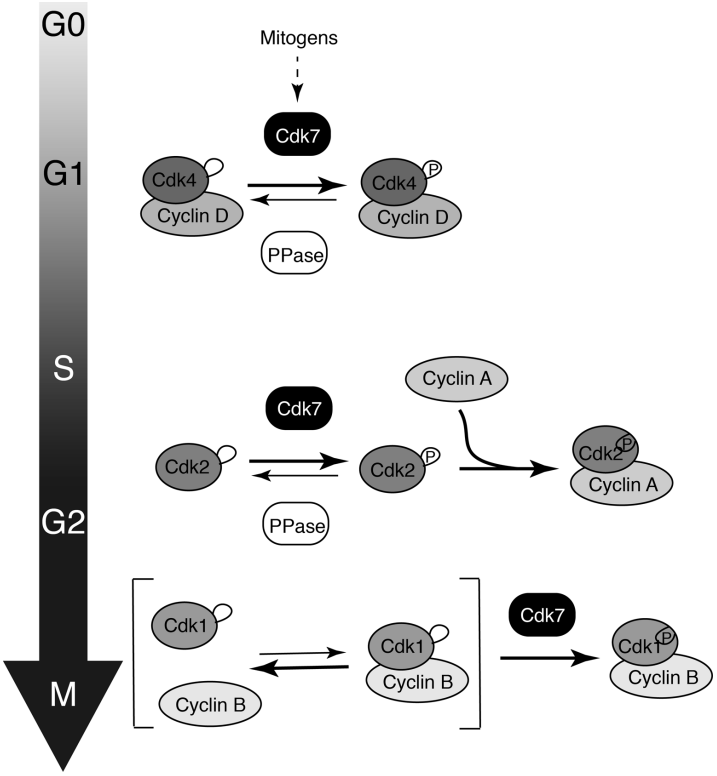

Figure 7. Changing modes of CDK activation during cell-cycle progression: from analog sensor to binary switch?

A CDK T-loop phosphorylation cascade operates during G1. Maximal rates of Cdk4 T-loop phosphorylation require the phosphorylated form of Cdk7, which is induced by mitogens. We propose that the increased activity of Cdk7 promotes activation of Cdk4 in the face of an opposing phosphatase. The Cdk2 T loop is exposed to phosphatases only prior to cyclin-binding. Assembly and T-loop phosphorylation of Cdk1 are coupled, and apparently unopposed by cellular phosphatases.