Current Biology, Vol. 14, R778–R786, September 21, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.09.019

Regulation of Early Events in Chromosome Replication

Review

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Eukaryotic genomes are replicated from large numbers of replication origins distributed on multiple chromosomes. The activity of these origins must be coordinated so that the entire genome is efficiently and accurately replicated yet no region of the genome is ever replicated more than once. The past decade has seen significant advances in understanding how the initiation of DNA replication is regulated by key cell-cycle regulators, including the cyclin dependent kinases (CDKs) and the anaphase promoting complex/cyclosome (APC/C). The assembly of essential prereplicative complexes (pre-RCs) at origins only occurs when CDK activity is low and APC/C activity is high. Origin firing, however, can only occur when the APC/C is inactivated and CDKs become active. This two step mechanism ensures that no origin can fire more than once in a cell cycle. In all eukaryotes tested, CDKs can contribute to the inhibition of pre-RC assembly. This inhibition is characterised both by high degrees of redundancy and evolutionary plasticity. Geminin plays a crucial role in inhibiting licensing in metazoans and, like cyclins, is inactivated by the APC/C. Strategies involved in preventing re-replication in different organisms will be discussed.

Introduction

In all cell types, from the simplest bacteria to the most complex multicellular organisms, precise coordination of DNA replication with chromosome segregation during the cell division cycle is required to ensure that both daughter cells inherit a complete and intact complement of genetic material. The relatively large genomes of eukaryotic cells are replicated from multiple replication origins on multiple chromosomes. The evolution of a system for controlling multiple replication origins so they are all activated precisely once during each S phase was undoubtedly an important step in the evolution of the modern eukaryotic cell, because it removed a major constraint on genome size — the amount of DNA that can be replicated from a single replication origin. In this review, the regulation of replication origin licensing in a number of organisms will be discussed. Differences in regulation as well as some unifying ideas will be presented.

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CDKs and APC/C: Opposing Forces in the Cell Cycle

The eukaryotic cell cycle is driven by the periodic accumulation and destruction of cyclins. In Figure 1, the temporal regulation of cyclin levels as well as some of the essential regulators of CDK activity relevant to this review are shown. Mitotic (M-)CDKs, which are essential for entering mitosis, activate an E3 ubiquitin ligase called the anaphase promoting complex or cyclosome (APC/C). The first form of APC/C to act is regulated by a WD-40 repeat protein called Cdc20 (Figure 1A). APC/C^{Cdc20} is required for sister chromatid separation and begins the process of cyclin proteolysis. Because Cdc20 can only activate APC/C that has been hyperphosphorylated by M-CDK, APC/C^{Cdc20} is believed to promote its own inactivation (Figure 1B) by reducing cyclin levels (Figure 1C). In many cell types, a second form of APC/C, regulated by a related WD-40 protein, Cdh1, becomes active after Cdc20-dependent CDK inactivation, because Cdh1 is inhibited by direct CDK phosphorylation (Figure 1D). APC/C^{Cdh1} not only contributes to cyclin proteolysis, but also targets Cdc20 for proteolysis (Figure 1E,F), completing the inactivation of APC/C^{Cdc20}.

APC/C activity during mitosis is regulated by several networks (not shown in Figure 1), including the spindle assembly checkpoint, the FEAR network (Cdc fourteen early anaphase release) and MEN (mitotic exit network). The latter two involve the activation of Cdc14, a protein phosphatase which dephosphorylates a number of CDK substrates. During G1 phase, CDKs are kept inactive by both APC/C^{Cdh1} and cyclin dependent kinase inhibitors (CKIs) (Figure 1H). G1 cyclins are generally not APC/C substrates. G1–CDK activation at a point known as 'START' in yeast — the 'restriction point' in mammalian cells — is required to inactivate APC/C^{Cdh1} (Figure 1I) and to target CKIs for ubiquitin-mediated degradation by a second E3 ubiquitin ligase called SCF (Figure 1J). Together, these processes promote activation of the S phase (S-)CDKs (Figure 1K). Later in the cell cycle, M-CDKs are activated and inhibit G1 cyclin transcription (Figure 1L).

DNA replication is limited to once per cell cycle in eukaryotic cells because the assembly of essential pre-replication complexes (pre-RCs) at replication origins can only occur in a window of time during the low CDK, high APC/C period from late mitosis through early G1 phase. Origin firing can only occur after the APC/C has been inactivated and CDKs reaccumulate. Thus, pre-RCs cannot assemble at origins during S, G2 and M phases. The pre-RC assembly reaction, known as licensing, involves the loading of the presumptive replicative helicase, the Mcm2–7 complex, in an ATP-dependent reaction that requires the origin recognition complex (ORC) and two essential factors, Cdc6 and Cdt1. For a detailed discussion of the licensing reaction see [1]. In the next section, pre-RC regulation by CDKs and APC/C in several different organisms will be considered.

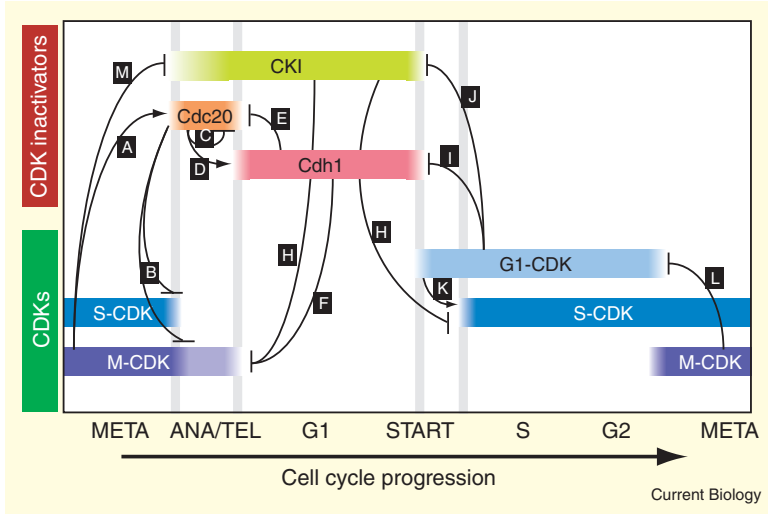

Figure 1. Cell cycle regulation.

The main cell cycle regulators involved in DNA replication control are shown. Details of the individual steps are indicated by letters and are described in the text.

Budding Yeast

The cell cycle of the budding yeast *Saccharomyces cerevisiae* is characterised by a relatively long and variable G1 phase and a very short G2 phase. Transcription and protein synthesis are required for transit through G1 phase, and it is during G1 phase that growth control occurs. Mating pheromone induces a G1 arrest by preventing activation of G1-CDKs, and cells can exit the cell cycle from G1 phase when deprived of nutrients.

Pre-RCs can only assemble during a window of opportunity from late mitosis through G1 phase, because CDKs inhibit pre-RC assembly [2–4]. Inactivation of CDKs in either S- or G2/M-arrested cells, by overexpression of the CKI Sic1 or by using a temperature sensitive *cdk1* (*cdc28*) mutant, drives the reassembly of pre-RCs [3,5]. If CDKs are subsequently reactivated in these G2-arrested cells, a complete extra round of DNA replication occurs. CDK inactivation in G2/M can induce pre-RC assembly even when the APC/C and MEN are inactive [5], arguing that the only essential role for the APC/C and MEN in pre-RC assembly is to inactivate M-CDKs.

CDKs inhibit pre-RC assembly by directly inhibiting each pre-RC component. The Cdc6 protein accumulates during early G1 phase, but is not present from late G1 phase until the following mitosis [6,7]. Both regulated transcription and regulated proteolysis contribute to the periodic accumulation of Cdc6 protein. Phosphorylation of Cdc6 by CDKs targets the protein for ubiquitin-mediated proteolysis by SCF^{CDC4} [6–11]. SCF^{CDC4}-dependent proteolysis of Cdc6 is especially rapid from late G1 phase through S phase. Later in the cell cycle, Cdc6 becomes partially stabilized but remains inactive for pre-RC assembly [12] (S. Mimura, T. Seki, S. Tanaka and J.D., unpublished data).

The Mcm2–7 complex enters the nucleus at the end of mitosis, remains nuclear during G1 phase and becomes primarily cytoplasmic later in the cell cycle. This regulated accumulation of Mcm2–7 in the nucleus does not require Cdc6, so is independent of the loading of Mcm2–7 onto chromatin [13,14]. Inactivation of CDKs in nocodazole-arrested cells can induce the reaccumulation of Mcm2–7 in the nucleus, and ectopic expression of stable Clb2 (M-CDK) in G1-arrested cells causes redistribution of free Mcm2–7 to the cytoplasm [14,15], indicating that the nuclear localisation of Mcm2–7 is regulated by CDKs.

The regulation of Cdt1 in *S. cerevisiae* is intimately connected to that of Mcm2–7. Cdt1 protein levels are approximately constant throughout the budding yeast cell cycle. Like Mcm2–7, however, the subcellular localisation of Cdt1 is regulated so that it is nuclear in G1 and cytoplasmic later in the cell cycle [16]. Free Mcm2–7 is bound to Cdt1 and, in the absence of Cdt1, Mcm2–7 does not accumulate in the nucleus during G1 phase. Conversely, Cdt1 does not accumulate in the nucleus in the absence of Mcm2–7. Moreover, addition of the SV40 TAg NLS onto Mcm7 (Mcm7–SV40 NLS) causes constitutive nuclear localisation of both Mcm2–7 [14] and Cdt1 [16].

The phosphorylation of two ORC subunits, Orc2 and Orc6, also contributes to preventing re-replication. The mechanism of ORC inactivation by CDK phosphorylation is presently not known, but presumably does not affect its DNA binding activity as ORC is bound at origins *in vivo* throughout the cell cycle [17–19]. Recently it has been shown that the S-CDK, Clb5, is specifically recruited to ORC at origins by virtue of interaction with an ‘RXL’ motif within Orc6 [20], where it contributes to preventing re-replication.

Re-replication is induced in G2/M-arrested cells expressing a stabilized version of Cdc6 together with Mcm7–SV40 NLS and mutant forms of Orc2 and Orc6 that lack CDK consensus sites [21]. All three proteins — Cdc6, Mcm2-7 and ORC — must be de-regulated in order to induce substantial re-replication. Thus, the regulation of Cdc6, Mcm2-7 (with Cdt1) and ORC all contribute to preventing re-replication.

Fission Yeast

The cell cycle of the fission yeast *Schizosaccharomyces pombe* is characterised by a short G1 phase and a long G2 phase, during which most growth control occurs. G1 phase is so short that, when grown in rich medium, DNA replication begins prior to cytokinesis. Nonetheless, this

G1 phase has more similarities to the longer G1 phases of budding yeast and metazoan somatic cells than to the short G1 phases of metazoan early embryos. Transcription and protein synthesis are required for G1 transit, and cells can be arrested during G1 phase by mating pheromone and nutrient starvation.

The first experiments indicating that CDKs have a role in preventing re-replication in any eukaryotic organism came from experiments in fission yeast. Conditions which cause depletion of M-CDK, including deletion of the mitotic cyclin, cdc13$^+$ [22], overexpression of the CKI rum1$^+$ [23] or heat shock of a *cdk1* (cdc2$^+$) temperature-sensitive mutant [24], all induce re-replication.

Early efforts to understand once-per-cell-cycle replication focused on the Cdc6 ortholog (cdc18$^+$) because its overexpression induces re-replication [25,26]. CDKs were implicated in this control because cdc18$^+$ mutants lacking CDK consensus sites were more efficient than wild-type cdc18$^+$ at inducing re-replication [27,28]. Like budding yeast Cdc6, *S. pombe* cdc18$^+$ is an unstable protein and proteolysis of cdc18$^+$ is directed by two Cdc4 homologs, pop1$^+$ and pop2$^+$/sud1$^+$ [29–32]. CDK phosphorylation is required for interaction of pop1 and pop2 with cdc18$^+$ [32], and CDK phosphorylation site mutants of cdc18$^+$ are more stable than wild-type cdc18$^+$ [28], indicating that, as in budding yeast, phosphorylation of cdc18$^+$ is required for recognition by SCF.

Because overexpression of Cdc6 in budding yeast does not induce re-replication, these results initially made the block to re-replication in fission yeast seem 'simpler', as perhaps cdc18$^+$ was the main target of CDK inhibition. Subsequent analysis, however, has uncovered a similar complexity in replication control in fission yeast to that in budding yeast, which was probably masked in some way by the overexpression of cdc18$^+$ [33,34].

Whereas Cdt1 is co-regulated with Mcm2–7 in budding yeast, in fission yeast, cdt1$^+$ is co-regulated with cdc18$^+$. Like cdc18$^+$, cdt1$^+$ protein levels fluctuate during the cell cycle, peaking in late mitosis and gradually disappearing during S phase [34]. This is partly because cdc18$^+$ and cdt1$^+$ are both targets of the cell-cycle transcription factor cdc10$^+$, responsible for M/G1 transcription [35,36]. The mechanism of Cdt1 proteolysis has not yet been investigated.

The *S. pombe* ortholog of Orc2 (orp2$^+$) is phosphorylated by CDKs *in vivo*, and *orp2* mutants lacking CDK consensus sites allow more re-replication to occur in cells overexpressing cdc18$^+$ [37]. The mitotic cyclin cdc13$^+$ has been localized to origins by chromatin immunoprecipitation, and cells expressing a tagged orp2$^+$ were partially defective in cdc13$^+$ origin binding and hypersensitive to cdc18$^+$-induced re-replication [38]. Thus, ORC, cdt1$^+$ and cdc18$^+$ all appear to contribute to preventing re-replication in *S. pombe*.

Xenopus

The early embryonic divisions in the frog *Xenopus laevis* are somewhat simpler than the yeast cell cycles described above: there is no discrete G1 phase, the cell cycle is uncoupled from growth and various cell cycle 'checkpoints' are absent. APC/C^{Cdc20} is the dominant form of the APC/C — the Cdh1 ortholog appears to be absent in these early cell cycles [39]. Additionally, although a *Xenopus* CKI has been identified [40,41], its levels in early embryos are too low to be effective in inhibiting CDKs [40].

McGarry and Kirschner [42] identified geminin in a screen for APC/C substrates in *Xenopus*. Like cyclins, geminin has a destruction box which is required for its ubiquitylation and degradation *in vitro* by APC/C. Addition of recombinant geminin blocked DNA replication by preventing licensing. Specifically, the loading of ORC and Cdc6 proceeded normally in the presence of stable geminin, but the Mcm2–7 complex was not loaded [42]. Subsequent work has shown that geminin inhibits licensing by binding to and inactivating Cdt1 [43,44]. These experiments established geminin as both an APC/C substrate and a licensing inhibitor. In contrast to exogenously added recombinant geminin, endogenous geminin is not degraded efficiently at the end of mitosis [45,46]. Instead, APC/C-dependent ubiquitylation appears sufficient to inhibit geminin function [45,47]. The small molecule CDK inhibitor 6-dimethyl aminopurine (6-DMAP) blocks licensing when added to metaphase-arrested *Xenopus* extracts [48]. Because Cdc20 can only activate the form of APC/C that is hyper-phosphorylated by M-CDK (Figure 1), 6-DMAP inhibits licensing by preventing APC/C^{Cdc20} activation and consequent geminin inactivation [47].

APC/C-dependent inhibition of geminin is not the only mechanism for preventing re-replication in *Xenopus* egg extracts, because immunodepletion of geminin does not induce extensive re-replication [42]. Depletion of geminin in mitotic extracts only partially restores licensing function. Treatment of this geminin-depleted extract with 6-DMAP or roscovitine results in full restoration of licensing activity [44], suggesting that, in addition to promoting licensing via geminin degradation, CDKs also inhibit licensing by an additional mechanism. Addition of excess Cdt1 to geminin-depleted extracts also restored full licensing activity suggesting that this additional CDK-dependent mechanism may work through Cdt1 [44].

Cdc6 is displaced from nuclei by CDKs; however, Cdc6 mutants in which all CDK consensus sites have been eliminated are not exported yet still support once-per-cell-cycle replication [49]. It has recently been shown that there are two isoforms of Cdc6 in *Xenopus*: one, Xcdc6A, is embryonic, disappearing after the mid-blastula transition (MBT); the other, Xcdc6B, is present but non-functional in embryos, but essential in somatic cells [50]. It will be interesting to see if the somatic Cdc6 is regulated differently from the embryonic form.

Drosophila

The fruitfly *Drosophila* and distantly related dipteran *Sciara coprophila* have been especially useful in understanding replication control in cells that disobey the 'once per cell cycle' paradigm as part of their developmental program. These include cells undergoing multiple rounds of endoreduplication — for

example, nurse cells, follicle cells and cells in a variety of larval tissues — as well as cells undergoing localized, programmed gene amplification — for example, the chorion gene cluster in follicle cells. These localized amplification events occur from discrete origins [51–53], require specific DNA sequences [54] and the same replication components which are involved in normal chromosomal DNA replication [53,55–59].

A *Drosophila* geminin homolog interacts with Cdt1, the product of the *dup* locus [60]. Overexpression of this geminin homolog inhibits replication, while its depletion induces some over-replication in certain endoreduplicating cells and induces prolonged periods of DNA synthesis in follicle cells, presumably reflecting overamplification of the chorion genes [60]. Reduction of geminin by siRNA in a *Drosophila* embryonic cell line also resulted in an increase in ploidy [61].

The APC/C plays an additional and somewhat confusing role in regulating DNA replication in *Drosophila* by targeting the largest subunit of ORC, Orc1 for degradation [62]. The apparently contradictory activities of the APC/C in eliminating inhibitors of licensing (geminin and cyclins) and activators of licensing (Orc1) are also seen in mammalian cells (see below).

During *Drosophila* development, cells exit the cell cycle after mitosis 16 (M16) and remain quiescent for long periods of time. Unlike earlier cell cycles, Mcm2–7 does not associate with chromatin after M16 [63], suggesting that these cells prepare for quiescence prior to mitosis. This differs from budding yeast and mammalian tissue culture cells which, at least under certain conditions, assemble pre-RCs at the end of mitosis and disassemble them upon entry into quiescence [17,64–66].

The *Drosophila* CKI encoded by the *dacapo* (*dap*) gene is expressed just prior to M16 and is required for the withdrawal of cells from the cell cycle after M16 [67]. Consistent with this, Mcm2–7 becomes associated with chromosomes after M16 in *dap* mutants and these mutants then undergo an additional round of replication [63]. Dap is an inhibitor of cyclin E–cdk2, and ectopic expression of cyclin E in *Drosophila* endocycles can induce Mcm chromatin association [68]. These results indicate that cyclin E has a positive role in licensing. It has also been shown, however, that continuous expression of cyclin E prevents endoreduplication [69,70], consistent with the idea that cyclin E can also inhibit licensing. The role of cyclin E in regulating licensing is discussed below.

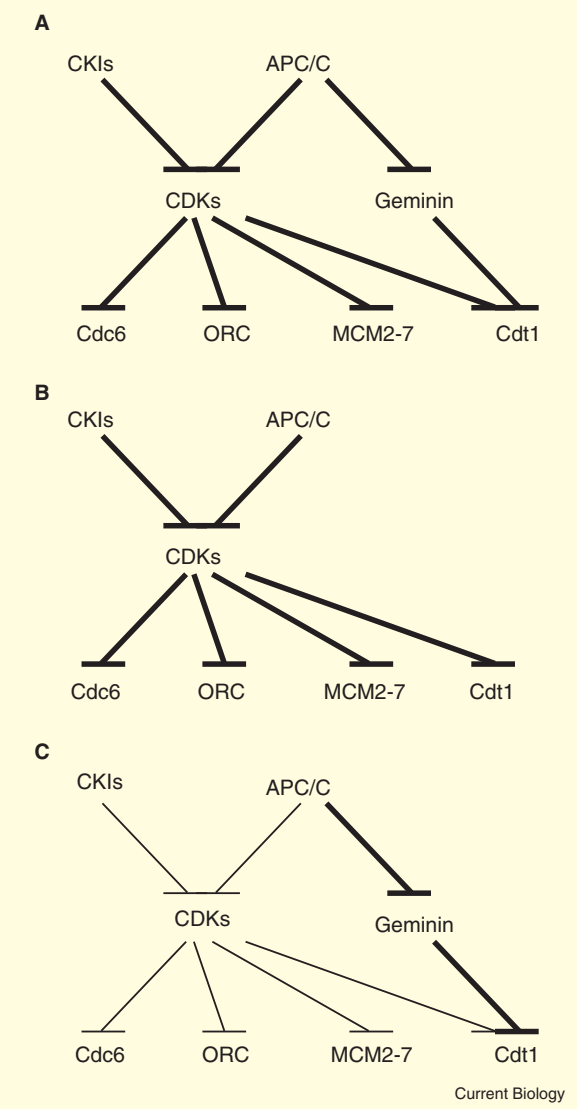

Figure 2. A general view of pre-RC regulation.

(A) The APC/C acts to remove inhibitors of pre-RC assembly, including cyclins and geminin. CKIs can also contribute to CDK inhibition. CDKs generally inhibit most or all pre-RC components, while geminin is a Cdt1 inhibitor specifically found in metazoans. (B) In yeast, geminin is absent and regulation occurs entirely through CDKs. (C) In metazoans, Geminin is especially important, while CDKs may play a lesser role, at least in some cell types.

Mammals

Geminin levels fluctuate during the cell cycle in HeLa cells: geminin is absent during G1 phase and accumulates during S, G2 and M phases, consistent with the idea that it is an APC/C substrate [42]. Depletion of geminin by siRNA in human tissue culture cells induces some re-replication [71], indicating that geminin plays a critical role in replication control.

Similar to the situation in *Drosophila*, in mammals the APC/C plays an additional and somewhat contradictory role in regulating pre-RC assembly. Cdc6 is absent from early G1 and quiescent cells in many (but not all) cell lines [64,72,73]. Overexpression of Cdh1 is sufficient to induce Cdc6 degradation in human cells [72], suggesting that APC/C^{Cdh1} is most important in regulating Cdc6 proteolysis. It is presently unclear how the APC/C-dependent degradation of a licensing inhibitor (geminin) and a licensing activator (Cdc6) are coordinated to ensure efficient pre-RC assembly.

CDKs also appear to play a role in inhibiting inappropriate pre-RC assembly. Elimination of M-CDK activity by deleting the *Cdc2* (CDK1) gene induces multiple rounds of endoreduplication [74]. Moreover,

when CDKs are inhibited in G2, either by treatment of cells with 6-DMAP or genetic ablation of CDK1, Mcm2-7 rebinds and chromatin is re-licensed [75–77].

It is interesting that CDK inhibition in G2 HeLa cells (as well as yeast) promotes licensing, while CDK inhibition in *Xenopus* egg extracts inhibits licensing. Although it is unlikely, geminin may already be inactive in these G2 HeLa cells. A more interesting possibility is that CDK inhibition may inactivate geminin by promoting activation of APC/C^{Cdh1}. As Cdh1 appears to be absent from egg extracts, 6-DMAP could not activate APC/C^{Cdh1} in this situation and geminin might be stabilized.

Several potential CDK targets have been identified in mammalian cells. In addition to being an APC/C substrate, Cdc6 can also be targeted for proteolysis by cyclin A–CDK2 [78]. This proteolysis only affects soluble forms of Cdc6, while forms that are bound to chromatin persist through S and G2 phases [64,73,78,79]. CDKs can also regulate the nuclear localisation of ectopically expressed Cdc6 [80–83], which is nuclear in G1 phase but redistributes to the cytoplasm in S and G2 phases. This relocalisation requires CDK sites in the amino terminus of Cdc6. Expression of cyclin A (but not cyclin E) induces relocalisation. Thus, the pool of Cdc6 which is not bound to chromatin may be targeted for proteolysis and/or nuclear export by CDKs.

CDKs regulate Cdt1 via two distinct mechanisms. First, CDKs target Cdt1 for proteolysis via SCF^{Skp2} [84–86] and second, CDKs inhibit a novel DNA binding activity of Cdt1 [85]. Neither of these mechanisms require geminin. The binding of geminin to Cdt1 has been reported to inhibit Cdt1's DNA binding activity [87], though in *Xenopus* geminin does not interfere with Cdt1 chromatin association and is actually recruited to chromatin by Cdt1 [46,88]. CDK inactivation in G2 promotes the re-accumulation of Cdt1 on chromatin [85] and overexpression of Cdt1 together with Cdc6 induces re-replication [89].

ORC may also be regulated by CDKs [90]. While Orc2–6 (together with Cdc6) remain bound to chromatin during G1, S and G2 phases, the Orc1 subunit is released from chromatin during S phase [91–93]. In one study [92] examining ORC in human cells, Orc1 was degraded in a SCF^{Skp2}-dependent manner which presumably requires CDK phosphorylation of ORC. In a second study [93] using a hamster cell line, Orc1 was not degraded, but instead was released from chromatin during S phase when it accumulated as either a monoubiquitinylated or diubiquitinylated form. Yet another study [65] suggested that Orc1 remains chromatin bound throughout the cell cycle. Whether these differences are related to differences in experimental design or reflect cell line differences has not been resolved. Finally, overexpression of cyclin A or K cyclin from Kaposi sarcoma-associated herpesvirus induces the relocalisation of Orc1 to the cytoplasm [94]. So, perhaps like Cdc6, free Orc1 can be either degraded or exported (or both) in a CDK-dependent manner.

As in *Drophila*, cyclin E plays an enigmatic role in regulating DNA replication in mammalian cells. Neither cyclin E nor CDK2 are essential for mouse development — apart from being required for development of extraembryonic tissues — arguing against any essential role for cyclin E in regulating replication in the mitotic cell cycle [95–97]. However, cyclin E deficiency causes two specific DNA replication defects; cells re-entering the cell cycle from quiescence fail to load Mcm2-7 and endoreplication in trophoblast giant cells and megakaryocytes is impaired [96,97].

These results indicate that cyclin E plays a positive role in licensing in specific situations, consistent with *in vitro* studies [98]. One potential role for cyclin E in licensing is probably activation of the E2F transcription factor. Several pre-RC components including Cdc6, Cdt1 and Orc1 as well as the inhibitor geminin are E2F targets [99–102]. Of these, Cdc6 appears to be most important since forced Cdc6 expression in quiescent cells is sufficient to drive Mcm2–7 loading [103].

Redundancy and Rapid Evolution: Hallmarks of Pre-RC Regulation

Three general principles can be gleaned from the discussion above. First, the assembly of pre-RCs is restricted to a period of high APC/C and low CDK activity. APC/C promotes pre-RC assembly by eliminating cyclins and, in metazoans, geminin (Figure 2). Second, pre-RC assembly is generally inhibited by multiple mechanisms. Virtually every conceivable way of inhibiting pre-RC components is used: proteolysis, nuclear export and inhibitor binding. And third, mechanisms by which individual pre-RC components are inhibited are quite different in different organisms, indicating a high degree of evolutionary plasticity.

One of the main reasons for employing multiple strategies to inhibit licensing is probably that preventing re-initiation is crucial for maintaining genome integrity. If just one out of 50,000 potential origins in a human cell were to re-fire during a single cell cycle, this could have grave consequences for a cell. Consequently, being 99.998% efficient at preventing inappropriate pre-RC assembly is not good enough! If this were the only reason, however, one might expect organisms with larger genomes and larger numbers of origins to have additional levels of regulation. Instead, the block to re-replication appears to be as complicated in yeast as it is in human cells, suggesting that there may be additional reasons for some of the complexity. In budding yeast, pre-RCs can only assemble during G1 phase because CDKs are active during the remainder of the cell cycle, when they inhibit Cdc6, Mcm2-7 and ORC; CDK activation then promotes origin firing.

This idea that replication is regulated by oscillation between two mutually exclusive states is diagrammed in Figure 3A. Although elegant in its simplicity and fundamentally correct, this scheme has an inherent design weakness. During the transitions between high and low CDK activity there is potential for misregulation at intermediate CDK levels. For example, as cells activate CDKs at the end of G1 phase, there is a risk that an origin might fire at a CDK level too low to prevent re-licensing. Such an origin might, therefore, re-licence and fire for a second time in one S phase.

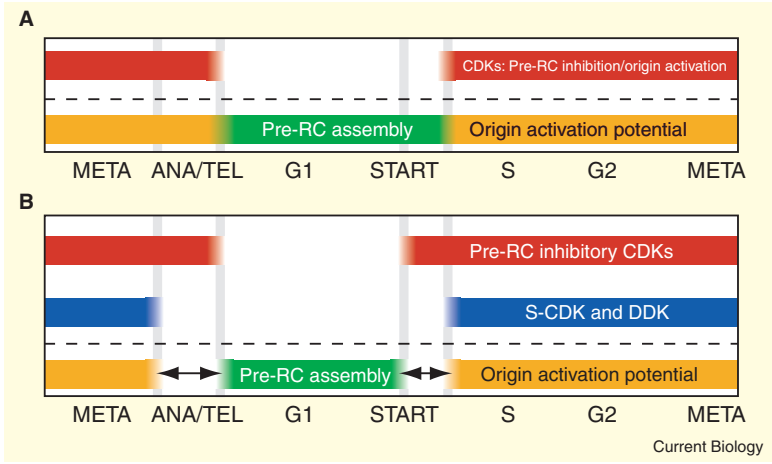

Figure 3. Pre-RC regulation by CDKs.

(A) A hypothetical model for pre-RC regulation. In this simplest view, CDKs can both inhibit assembly of new pre-RCs and activate replication origins with existing pre-RCs. Drawbacks of this two state model are described in the text.

(B) Regulation of replication in budding yeast. Pre-RC assembly is kept separate from origin activation by the G1-CDKs and M-CDKs which can prevent pre-RC assembly but cannot trigger origin firing (see text for details).

Likewise, as CDKs are inactivated at the end of mitosis, there is a risk that at some intermediate CDK level, some pre-RCs may assemble. If a small number of pre-RCs were to assemble in the presence of CDK activity, there would be a risk of initiating replication from these pre-RCs before mitosis ends. There would then be nothing to prevent this origin from being re-licensed during the following low CDK G1 phase and re-firing during S phase.

Closer examination of the way pre-RC components are regulated suggests how this problem is avoided in yeast (Figure 3B). Firstly, two key S phase promoting factors, Clb5 (the S phase cyclin) and Dbf4 (the regulatory subunit of the Cdc7 protein kinase), are both APC/C substrates. Both are degraded at the metaphase–anaphase transition and both are absent from cells arrested in late mitosis by mutation in the MEN. This is because Clb5, and presumably Dbf4, are APC/C^{Cdc20} targets [104–107]. However, pre-RCs have not yet assembled at origins in cells arrested with any MEN mutants [17], because the M-CDK, Clb2–CDK1, is still present and active and is not degraded until APC/C^{Cdh1} is activated. Although Clb2 can prevent pre-RC assembly, it is very poor at activating origin firing [108]. Thus, during mitosis, S phase promoting factors are degraded before, not concomitant with, pre-RC assembly (Figure 3B).

Similarly, at the end of G1 phase, pre-RC components are inactivated by the G1 cyclins, Cln1/Cln2 [9,15]. Again, this occurs before, not concomitant with, the activation of the S-phase-promoting factors Dbf4 and Clb5. Consequently, in budding yeast, pre-RC assembly is kept completely separate from origin firing because Clb2 and Cln1/Cln2, which can both inhibit licensing but cannot promote S phase, provide ‘insulation’ at both ends of G1. The G1 cyclins only inhibit Cdc6 and Mcm2–7, not ORC, so only a subset of the pathways are involved in providing this insulation at the end of G1 phase.

Because preventing re-replication is so fundamental to genome stability and cell cycle control, one might have expected that mechanisms involved in regulating pre-RC assembly would have arisen once at an early stage in eukaryotic cell evolution and then been conserved from that point on. At the upper-most level of regulation this is true — in all organisms that have been studied, pre-RC assembly is limited to a period of high APC/C and low CDK activity, and CDKs contribute to the inhibition of pre-RC assembly. It has, however, been surprising to see that pre-RC components are regulated so differently in different organisms, implying rapid evolution of the regulatory pathways downstream of APC/C and CDKs [109].

The significant overlap of pathways in preventing pre-RC assembly probably provides the means for rapid evolution. No single pathway in budding yeast — for example cell-cycle-regulated transcription of pre-RC components, Cdc6 stabilization, Mcm2–7 nuclear localisation, ORC phosphorylation — is essential for viability, and so any one might easily be lost in evolution. Presumably, at some point, further loss of pathways would be detrimental and may provide the drive to establish new pathways.

While redundancy may provide the means for change, what is less clear is whether the different regulation seen in different cells has any deeper significance. It is likely that differences in cell cycles may require different types of regulation. For example, in early metazoan embryos, the first divisions occur without transcription, eliminating one level of potential regulation. Additionally, differences in the regulation of quiescence in different cell types may be important in understanding differences in pre-RC regulation. It may be more important for metazoans to ensure that pre-RCs do not assemble at origins in cells during quiescence, as the quiescent state may need to be maintained for many years. Whereas reassembly of pre-RCs upon re-entry into the cell cycle appears to be straightforward in budding yeast, because Cdc6 and Mcm2–7 are re-synthesized prior to the synthesis of the G1 cyclins [110], the same does not appear to be true in mammalian cells, where licensing in mitosis differs from that in cells re-entering the cell cycle from quiescence in the requirement for cyclin E (see above).

Another factor that may contribute to differences in regulation relates to the fact that many pre-RC proteins also play roles outside of DNA replication. The nature of these additional functions may place restrictions on how they can be regulated. For example, the Orc1 subunit plays a central role in transcriptional silencing

of the mating type genes in budding yeast [111,112]. Although it has not been tested directly, Orc1 may need to remain bound at silencers throughout the entire cell cycle to execute its silencing function which would be incompatible with the regulation of Orc1 by displacement from the ORC complex, as seen in mammalian cells.

Several replication proteins have been shown to have roles in mitosis. The Orc6 subunit is involved in cytokinesis in both *Drosophila* and mammalian cells [113,114] and the fission yeast Cdc7 homolog, hsk1$^+$, is required for centromeric cohesion [115]. Additionally, Mcm2–7 has been implicated in transcriptional control [116–120], Cdc6 has been implicated in checkpoint regulation [121–123] and geminin has roles during development that appear to be separable from its role in regulating replication [60,124–126]. Each of these non-replication functions may place constraints on when and where active protein must be present and, consequently, may limit the way in which they can be inhibited.

Finally, a great deal of the discussion above has emphasized differences in pre-RC regulation between different organisms. However, it should be emphasized that even within a single organism, different mechanisms of regulation act in different cell types. Examples include the lack of APC/C^{Cdh1} in early embryos, the two isoforms of *Xenopus* Cdc6 expressed during development and the role of cyclin E in a subset of cell types. It is likely that the next few years will see many more examples like these uncovered. Exploitation of such differences may provide some novel approaches to specifically inhibit proliferation in cancer cells.

Acknowledgments

I am grateful to all members of my laboratory for helpful discussions and especially to Lucy Drury, Niels Mailand, Catherine Merrick and Satoru Mimura for critical reading of the manuscript.

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