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Regulating DNA Replication in Eukarya

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DNA replication is tightly controlled in eukaryotic cells to ensure that an exact copy of the genetic material is inherited by both daughter cells. Oscillating waves of cyclin-dependent kinase (CDK) and anaphase-promoting complex/cyclosome (APC/C) activities provide a binary switch that permits the replication of each chromosome exactly once per cell cycle. Work from several organisms has revealed a conserved strategy whereby inactive replication complexes are assembled onto DNA during periods of low CDK and high APC activity but are competent to execute genome duplication only when these activities are reversed. Periods of high CDK and low APC/C serve an essential function by blocking reassembly of replication complexes, thereby preventing rereplication. Higher eukaryotes have evolved additional CDK-independent mechanisms for preventing rereplication.

The *Eukarya* include a wide spectrum of organisms, with genome sizes ranging from $\sim 10^7$ bp in yeasts to $\sim 10^{12}$ bp in protozoa. Rapid duplication of large genomes is achieved by distribution of the genetic material across several chromosomes. Each of these chromosomes initiates replication from sites called replication origins, which must fire no more than once per cell cycle to ensure a single error-free copy of the genome. Generating replication forks from an origin more than once leads to rereplication, an event that creates multiple copies of a single genomic region within a single cell. This leads to gene amplification and promotes genome instability (Green et al. 2010), a phenomenon observed in many human cancers (Lengauer et al. 1998). The process of genome duplication is therefore under stringent control to ensure that few, if any, defects are transmitted from one generation to the next.

GENERAL STRATEGY FOR INITIATION OF EUKARYOTIC DNA REPLICATION

Origin Licensing: Loading of the Replicative Helicase

Eukaryotic cells initiate DNA replication in two discrete steps. First, an inactive form of the replicative helicase is assembled onto double-stranded DNA (dsDNA) in a process called origin licensing. This occurs during late mitosis and G_1 phase of the cell cycle. The six-subunit origin-recognition complex (ORC) binds to DNA sequences called origins of replication and recruits the Cdc6 and Cdt1 proteins. Together these three **licensing factors** direct the loading of the helicase, the minichromosome maintenance (MCM) complex, around dsDNA. The MCM complex thus loaded is topologically linked to DNA and forms a double hexamer (Donovan et al. 1997; Rowles et al. 1999; Seki

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and Diffley 2000; Evrin et al. 2009; Remus et al. 2009; Gambus et al. 2011). This form of the inactive helicase is also referred to as the pre-replicative complex (pre-RC).

Origin Firing: Activation of the Replicative Helicase

During S phase, the inactive pre-RC is converted into an active helicase that unwinds dsDNA, thus allowing DNA polymerases to access and copy the two template strands. This second step of origin firing involves the formation of the CMG complex, named after its components: Cdc45, the MCM proteins, and the GINS complex (Moyer et al. 2006; Aparicio et al. 2009). The active CMG helicase is then coupled to a DNA polymerase, either Pol ε for the leading strand or Pol δ for the lagging strand (Kunkel and Burgers 2008). This process requires the activity of the Sld2, Sld3, Sld7, and Dpb11 proteins as well as the two protein kinases cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) (Bousset and Diffley 1998; Kamimura et al. 1998; Zou and Stillman 1998, 2000; Kamimura et al. 2001; Masumoto et al. 2002; Tanaka et al. 2007; Zegerman and Diffley 2007; Tanaka et al. 2011b). These six **firing factors** are essential for initiating DNA synthesis from licensed origins.

CELL-CYCLE CONTROL OF THE INITIATION OF DNA REPLICATION

Control of Initiation of Replication during an Unperturbed Cell Cycle

The two steps of initiation described above are isolated from each other in different stages of the cell cycle. No origin firing can be allowed in G_1 while pre-RC complexes are assembled, lest there be regions of the genome that have not been properly licensed. Conversely, no origin licensing can be permitted during S phase while origin activation is triggered (Fig. 1). This ensures that multiple replication forks do not initiate from the same origin, thus preventing re-replication. Insulation of these two steps is achieved by the concerted action of two enzyme complexes: the CDK and the anaphase-promoting complex/cyclosome (APC/C).

Eukaryotes express different cyclin proteins during different stages of the cell cycle, leading to cell cycle stage-specific cyclin–CDK complexes

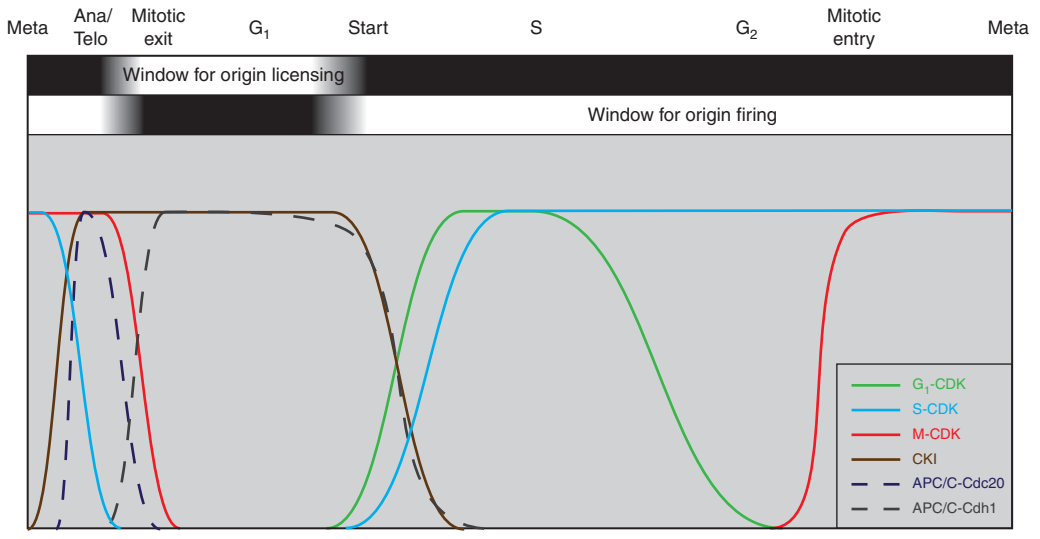

Figure 1. Regulation of DNA replication in the cell cycle. A generic diagram summarizing the oscillation of CDK activity in the cell cycle in response to the fluctuation of APC/C activity and the presence of CDK inhibitor (CKI). The details of regulation in different organisms are described in the corresponding sections of the article.

(Table 1). The cyclin subunit of the CDK contributes to determining substrate specificity, thus resulting in cell cycle stage-dependent phosphorylation of different target proteins. G_1-phase cyclin–CDKs (G_1-CDKs) phosphorylate proteins to promote S-phase entry, S-phase cyclin–CDKs (S-CDKs) are required to activate DNA replication, and mitotic cyclin–CDKs (M-CDKs) regulate accurate chromosome segregation through mitosis. Although different cyclins can confer some substrate specificity, experiments in fission yeast have shown that a single cyclin–CDK fusion can support a near-normal cell cycle (Coudreuse and Nurse 2010).

The APC/C is a multisubunit E3 ubiquitin ligase that polyubiquitinates different proteins targeted to it by a substrate adaptor (e.g., Cdc20 or Cdh1) (Peters 2006). The resulting protein–ubiquitin conjugates are then degraded by the proteasome. The S-phase and G_2/M-phase cyclins are targeted for APC/C-mediated degradation. G_1-phase cyclins are resistant to such regulation. Instead, they are processed for degradation during S phase by a different E3 ligase, the Skp1–Cul1–F-box protein (SCF) complex (Ang and Wade Harper 2005).

M-CDK-dependent phosphorylation of the APC/C results in activation of this E3 ligase (Rudner and Murray 2000; Kraft et al. 2003). This results in ubiquitination of S-phase and G_2/M-phase cyclins, leading to their degradation and a subsequent loss of associated S-CDK and M-CDK activity. Mitosis ensues and is followed by a period of high APC/C-Cdh1 activity that defines the G_1 phase in cycling cells and the G_0 phase in differentiated/quiescent cells. In actively proliferating cells, growth factor stimulation induces transcription of G_1-phase cyclins, resulting in an accumulation of G_1-CDK activity. Phosphorylation of the APC/C adaptor Cdh1 by G_1-CDK prevents it from binding to and activating the APC/C, effectively inhibiting its function and allowing S-CDK activity to accumulate (Zachariae et al. 1998; Jaspersen et al. 1999). S-CDK then inhibits and switches off the APC/C during S and G_2 phases. Hence, CDK and APC/C enzymes regulate each other, and their peak activity times are mutually exclusive in the cell cycle, setting up biphasic oscillations (Fig. 1). Vertebrate cells have additionally evolved proteins that inhibit APC/C function during S phase, such as Emi1 and Emi2 (Reimann et al. 2001; Tung et al. 2005). During prometaphase, Cdc2 and Plk1 cooperate to phosphorylate these inhibitors, presenting them for $SCF^{\beta\text{-}TrCP}$-mediated degradation, thus relieving the inhibition of the APC/C and promoting mitosis (Nakayama and Nakayama 2006).

Origin licensing occurs exclusively during late mitosis and G_1, when APC/C activity is high and S-CDK activity is low. S-CDK phosphorylation inhibits pre-RC assembly during S, G_2, and M phases, and thus all origins must be licensed before cells can enter S phase, because high CDK activity in the rest of the cell cycle would prevent any further licensing. If large regions of a chromosome are left unlicensed, it is possible that adjacent replication forks are unable to travel far enough to fuse into each other, leaving unreplicated stretches of DNA in between (Blow et al. 2011). Hence, several origins are licensed but only subsets are used to generate replication forks in every cell cycle, leaving clusters of dormant origins as a backup system. This ensures that every last base of the parental DNA gets copied. Pre-RCs that do not fire normally get displaced by passing replication forks, thus marking chromosome regions that have already been duplicated. Because many more origins need to be licensed than are used per cell divi-

Table 1. Regulators of cyclin-dependent kinase (CDK) in different organisms

	Saccharomyces cerevisiae	*Schizosaccharomyces pombe*	Metazoans
G_1-CDK	Cln1, Cln2, Cln3-Cdc28	Cig1, Puc1-Cdc2	Cyclin D–CDK4, cyclin E–CDK2
S-CDK	Clb5, Clb6-Cdc28	Cig2-Cdc2	Cyclin A–CDK2
M-CDK	Clb1, Clb2, Clb3, Clb4-Cdc28	Cig2, Cdc13-Cdc2	Cyclin A–CDK1, cyclin B–CDK1
CKI	Sic1	Rum1	p21, p27

CKI, CDK inhibitor.

sion, it is proposed that cells are prevented from entering S phase until complete assembly of replication complexes (Shreeram et al. 2002) and that conditions that decrease the total number of active replication complexes may result in genome instability (Kawabata et al. 2011).

Control of DNA Replication on Exposure to Genotoxic Stress

Cells have evolved checkpoint pathways to respond to external genotoxic insults by arresting the cell cycle, so that the cell can pause replication and correct mutations and lesions rather than segregating chromosomes with errors to daughter cells (Harper and Elledge 2007). The S-phase checkpoint acts to prevent licensed origins from firing and also stabilizes ongoing replication forks so that polymerases may resume activity once the damage has been corrected; these details are discussed in a separate article.

REGULATION OF REPLICATION INITIATION IN *Saccharomyces cerevisiae*

Cell-Cycle Regulation of Pre-RC Assembly

On exit from mitosis, CDK activity is lowered in two ways: by ubiquitin-mediated degradation of mitotic cyclin Clb2 by the 26S proteasome (Schwab et al. 1997; Visintin et al. 1997) and by Sic1 inhibition of G_1-CDK activity (Nugroho and Mendenhall 1994; Schwob et al. 1994). During this period, the Cdc14 phosphatase also promotes pre-RC assembly. First, it dephosphorylates Cdh1, thereby promoting its association with APC/C (Visintin et al. 1998; Zachariae et al. 1998; Jaspersen et al. 1999). Cdc14 also dephosphorylates the transcription factor Swi5, promoting its nuclear localization to activate transcription of Sic1 and Cdc6 (Knapp et al. 1996; Visintin et al. 1998). Finally, Cdc14 dephosphorylates Sic1, stabilizing it from SCF^{Cdc4}-mediated degradation (Visintin et al. 1998). Cdc6 also acts as an M-CDK inhibitor by direct binding to Clb2 (Elsasser et al. 1996; Calzada et al. 2001).

During G_1 phase a cell begins the next complete round of cell cycle on reaching a sufficiently large cell size (Skotheim et al. 2008). Passage through the point of no return, or "Start," is initiated by the G_1-CDK Cln-Cdc28 (Tyers et al. 1993; Dirick et al. 1995; Stuart and Wittenberg 1995). Two transcription factor complexes, SBF (Swi4–Swi6) and MBF (Mbp1–Swi6), are activated by phosphorylation of their allosteric inhibitor Whi5 (Costanzo et al. 2004; de Bruin et al. 2004). Whereas SBF activates transcription of Cln cyclins (Nasmyth and Dirick 1991; Spellman et al. 1998; Eser et al. 2011), MBF promotes transcription of Clb5 along with other replication genes (Lowndes et al. 1992; Koch et al. 1993; Spellman et al. 1998; Eser et al. 2011).

A key barrier to origin firing during G_1 phase is the CDK inhibitor Sic1, which must be degraded before cells can initiate DNA synthesis (Donovan et al. 1994; Schwob et al. 1994; Schneider et al. 1996). Simultaneous phosphorylation on multiple CDK consensus sites by Cln-Cdc28 and Clb-Cdc28 targets Sic1 for SCF-mediated polyubiquitination and proteolysis (Feldman et al. 1997; Verma et al. 1997a,b; Koivomagi et al. 2011a).

Another barrier to origin firing in G_1 phase is APC/C-Cdh1 activity, which actively degrades Clb cyclins. During late G_1 phase Cln2-Cdc28 and Clb5-Cdc28 phosphorylate Cdh1 and prevent its association with APC/C (Zachariae et al. 1998; Jaspersen et al. 1999). This allows accumulation of Clb5-Cdc28 activity essential for origin firing.

CDK Control of Origin Licensing

CDK phosphorylates several initiation proteins to inhibit pre-RC assembly. Clb-Cdc28 is recruited to ORC in an RxL-dependent manner (Weinreich et al. 2001; Wilmes et al. 2004) and phosphorylates Orc2 and Orc6 (Nguyen et al. 2001). These mechanisms inhibit interaction between ORC and Cdt1 (Chen et al. 2007), impeding loading of MCM complexes onto DNA (Chen and Bell 2011).

Cln-Cdc28 and Clb-Cdc28 phosphorylate Cdc6 to promote its subsequent degradation by the SCF^{Cdc4} complex (Drury et al. 1997, 2000; Elsasser et al. 1999; Perkins et al. 2001). During mitosis, binding of Clb2 to phosphorylated Cdc6 not only protects itself from SCF-

mediated degradation but also prevents Cdc6 from interacting with ORC, rendering Cdc6 inactive for pre-RC assembly (Mimura et al. 2004). On mitotic exit, degradation of Clb2 by the APC/C releases Cdc6 to promote pre-RC assembly in the subsequent G_1 phase.

As a result of a stable interaction with the MCM complex, Cdt1 and MCM are regulated as a single unit in budding yeasts (Tanaka and Diffley 2002). Phosphorylation of CDK consensus sites in Mcm3 results in soluble Cdt1/Mcm2–7 being transported out of the nucleus (Labib et al. 1999; Nguyen et al. 2000; Liku et al. 2005), where they are retained during G_2. Interestingly, every cell cycle requires a new round of MCM gene transcription, and "old" MCM proteins are degraded by ubiquitin-mediated proteolysis at the end of mitosis (Cheng et al. 2002; Braun and Breeden 2007).

CDK phosphorylation of pre-RC components is the primary barrier to rereplication in budding yeasts. The inhibition of ORC, Cdc6, and Cdt1 creates redundant pathways that must be simultaneously deregulated to result in significant overreplication of DNA in a single cell (Nguyen et al. 2001). Yeast strains that are mutated in either one or two of these modules show undetectable or mild rereplication phenotypes (Green et al. 2006), suggesting that CDK-mediated prevention mechanisms work together to ensure that rereplication becomes an extremely rare event (Diffley 2011).

CDK Control of Origin Firing

Phosphorylation of Sld2 and Sld3 by S-CDK promotes interaction of these proteins with the amino-terminal and carboxy-terminal BRCT domains of Dpb11, respectively (Tanaka et al. 2007; Zegerman and Diffley 2007; Muramatsu et al. 2010). Cln-Cdc28 cannot phosphorylate Sld2 and Sld3 during G_1 phase, possibly because of substrate specificity conferred by cyclin (Koivomagi et al. 2011b).

Yeast strains expressing an Sld3–Dpb11 fusion protein in combination with a phosphomimetic mutant of Sld2 can promote CDK-independent DNA synthesis in G_1-arrested cells, thereby bypassing the requirement for S-CDKs (Zegerman and Diffley 2007). This indicates that Sld2 and Sld3 are the minimal set of CDK substrates required for DNA replication. Interestingly, the *Jet1* allele of Cdc45 bypasses the Sld3 phosphorylation requirement for cell survival, thus implicating Cdc45 in the interaction between Sld3 and Dpb11 (Tanaka et al. 2007).

DDK Control of Origin Firing

The first evidence that DDK promotes initiation via Mcm2–7 came from the isolation of a mutant allele of Mcm5 (*mcm5-bob1*), which bypassed the requirement for DDK (Hardy et al. 1997). Purified DDK phosphorylates Mcm2, Mcm4, and Mcm6 subunits within double hexamers bound to DNA and has weak or no activity toward subunits within soluble hexamers (Francis et al. 2009). DDK phosphorylation of an amino-terminal region in Mcm4 facilitates Mcm–Cdc45 complex formation during S phase (Sheu and Stillman 2006). Mutational analysis of Mcm4 reveals that the unstructured amino terminus of this protein contains an inhibitory activity that is alleviated on DDK phosphorylation. Accordingly, deletion of this region results in an Mcm4 protein that promotes DDK-independent DNA synthesis in cells arrested at G_1 phase and rescues the viability of strains lacking functional DDK (Sheu and Stillman 2010). These data suggest that Mcm2, -4, and -6 are essential substrates for DDK in vivo. The exact mechanism by which DDK promotes origin firing is currently unclear and may involve the recruitment of firing factors such as Sld3, Sld7, and Cdc45 to origins (Tanaka et al. 2011a) perhaps via a DDK-phosphorylated MCM complex.

Activity of DDK is restricted to S phase as a result of APC/C-mediated degradation of the regulatory Dbf4 subunit (Oshiro et al. 1999; Weinreich and Stillman 1999; Ferreira et al. 2000). This has been proposed to prevent premature firing of origins during G_1 phase. Although expression of a nondegradable Dbf4 mutant does not induce significant rereplication (Ferreira et al. 2000), overproduction of Dbf4 in the CDK bypass yeast strain (see above) is lethal (Zegerman and Diffley 2007). This

observation highlights the importance of regulated origin firing to prevent premature initiation events during G_1 phase.

REGULATION OF INITIATION IN *Schizosaccharomyces pombe*

Origin Licensing in Fission Yeasts

In *Schizosaccharomyces pombe* origin licensing requires the combined actions of ORC (Orp1 to -6), Cdc18, Cdt1, and the MCM complex (Hofmann and Beach 1994; Nishitani and Nurse 1995; Grallert and Nurse 1996; Maiorano et al. 1996; Muzi Falconi et al. 1996; Ogawa et al. 1999; Nishitani et al. 2000). No consensus autonomously replicating sequence has been defined for this species, and ORC binds to AT-rich sequences in the genome by virtue of nine AT-hook motifs on the amino terminus of Orp4 (Chuang and Kelly 1999; Kong and DePamphilis 2001; Lee et al. 2001; Hayashi et al. 2007). Accordingly, the binding to AT-rich sequences by Orp4 is ATP-independent (Chuang and Kelly 1999), whereas ORC in budding yeasts binds to origin DNA in the ATP-bound state (Bell and Stillman 1992; Klemm and Bell 2001). However, the evolutionary conservation of the ATP-binding sites in ORC suggests an essential role of the ATPase activity in licensing, most likely during the loading of the MCM helicase.

Origin Firing in Fission Yeasts

In fission yeasts Hsk1 activity is required for Sld3 recruitment to origins during G_1 phase (Nakajima and Masukata 2002), but Sld3 associates with origins independently of Sna41 (Yamada et al. 2004). This is followed by the sequential recruitment of Cut5, GINS, and Sna41 (Dolan et al. 2004; Yabuuchi et al. 2006). Cdc23 is also required for Sna41 association within the preinitiation complex (pre-IC) (Gregan et al. 2003). However, the details of how the replicative helicase is activated currently remain unclear.

CDK Control of Origin Licensing

Orp2 is phosphorylated by Cdc2 in vivo, and a fission yeast strain expressing a nonphosphorylatable Orp2 protein is sensitized to rereplication (Vas et al. 2001). Overexpression of Cdc18 in such a strain results in more rereplication than that observed on overexpression in a wild-type strain. This suggests that CDK phosphorylation of Orp2 is redundant with Cdc18 regulation in rereplication control in fission yeasts. The mitotic cyclin Cdc13 localizes to replication origins in an ORC-dependent manner during G_2 phase and early mitosis. A yeast strain expressing a tagged Orp2 that reduces Cdc13 origin association shows hypersensitivity to endoreduplication, suggesting a role for Orp2–Cdc13 association in rereplication control (Wuarin et al. 2002). The mechanism behind this regulation is currently unknown, but it is possible that direct binding of Cdc13 to Orp2 somehow reduces the accessibility of other pre-RC assembly factors to ORC. This scenario is similar to the Clb5-Orc6 and Clb2-Cdc6 interactions in budding yeasts, where direct binding may play a role in inhibiting factors involved in pre-RC assembly (see above).

Cdc18 is an unstable protein, and its levels are regulated throughout the cell cycle. Cdc18 accumulation begins in late mitosis and decreases during S phase. Cdc10 controls the transcription of Cdc18, which accumulates during G_1 phase (Kelly et al. 1993; Nishitani and Nurse 1995; Muzi Falconi et al. 1996; Baum et al. 1998). On S-phase entry, Cig2-Cdc2 phosphorylates Cdc18 and targets it for polyubiquitination by the SCF complex and degradation by the proteasome (Jallepalli et al. 1997, 1998; Kominami and Toda 1997; Kominami et al. 1998; Lopez-Girona et al. 1998; Wolf et al. 1999). Overproduction of wild-type Cdc18 alone induces rereplication (Nishitani and Nurse 1995; Muzi Falconi et al. 1996), and Cdc18 mutants lacking CDK consensus sites promote rereplication even more efficiently than the wild-type proteins (Jallepalli et al. 1997; Lopez-Girona et al. 1998). Expression of either of these mutants at a low level, however, is not sufficient to induce rereplication.

Cdt1 levels peak during late M phase as a consequence of Cdc10-mediated transcriptional control, and protein levels decline during S phase as a result of proteolysis (Hofmann and

Beach 1994; Nishitani et al. 2000). Cdt1 degradation is mediated by CRL4-Cdt2 in a proliferating cell nuclear antigen (PCNA)-dependent manner (Hu and Xiong 2006; Ralph et al. 2006; Guarino et al. 2011), and chronic, low-level expression of both Cdt1 and Cdc18 is required to induce rereplication, suggesting that these two proteins are redundantly regulated (Gopalakrishnan et al. 2001).

Although fission yeast MCMs are constitutively nuclear (Yanow et al. 2001), their association with chromatin is cell-cycle-regulated. MCM complex assembly may also be regulated by the action of Mcb1 (MCM-binding protein) during the cell cycle (Ding and Forsburg 2011).

CDK Control of Origin Firing

Fission yeast Drc1 and Sld3 are phosphorylated by S-phase CDK to promote formation of a ternary complex with Cut5 (Nakajima and Masukata 2002; Noguchi et al. 2002; Fukuura et al. 2011). The interaction recruits the complex to chromatin and may generate a platform for the formation of CMG complex. An Sld7 homolog in fission yeast has not been identified, and it is not clear if pre-IC factors are limiting for replication initiation in fission yeast or if they are targeted during checkpoint-dependent inhibition of late origin firing.

REGULATION OF REPLICATION INITIATION IN METAZOANS

Current knowledge on DNA replication in metazoans is based on studies performed primarily using three model systems: *Xenopus* egg extracts, *Drosophila* embryos and cell lines, and immortalized or cancerous mammalian cell lines. Advances in RNA interference (RNAi) and transgenesis have enabled genetic studies in cell culture or whole animals to elucidate regulation of these pathways. The details of origin recognition, origin licensing, and origin firing in these systems are summarized below. Plant DNA replication is discussed in detail elsewhere and is not included here.

Regulation of Origin Recognition in Metazoans

The replicon model proposed that initiation of DNA replication is determined by the binding of initiator proteins to a specific sequence of DNA at the origin of replication, termed the replicator (Jacob and Brenner 1963). Although this model holds true for prokaryotes and certain animal viruses, replicators in eukaryotes do not share a consensus sequence. Budding yeast ORC is the only known eukaryotic initiator that displays sequence specificity and binds to an 11-bp autonomously replicating sequence element in vivo. However, in vitro, ORC from all species has intrinsic nonspecific DNA-binding activity and is capable of assembling pre-RCs onto diverse DNA sequences. Recruitment of a Gal4–ORC fusion protein to a plasmid in cultured human cells converts the sequence adjacent to the Gal4-binding sites into an origin of replication and confers on it the property of once-per-cell-cycle replication (Takeda et al. 2005b). Thus, ORC binding to DNA is a primary requirement for any sequence to function as an origin. In vivo, ORC can be recruited by different sequence-specific binding proteins to chromosomal loci, for example, to telomeres by interaction with telomeric repeat-binding factor 2 (TRF2) (Tatsumi et al. 2008) or to the chorion gene cluster in *Drosophila* by Myb (Beall et al. 2002). Such a mechanism may also be used by viral genomes, for example, Epstein–Barr virus, that can recruit ORC to *oriP* in an Epstein–Barr nuclear antigen 1 (EBNA1)-dependent process (Dhar et al. 2001). There is increasing evidence that CpG islands and G-rich elements that can form G-quadruplexes influence origin recognition in vivo, and these are discussed in greater detail in a separate article (see article by Leonard and Méchali 2013, and references therein).

ORC binds to nucleosome-free regions of DNA in vivo (Sequeira-Mendes et al. 2009; Karnani et al. 2010; MacAlpine et al. 2010), and the nature of the chromatin around chromosomal ORC-binding sites influences origin licensing. Recruitment of ORC to different regions of the genome is necessary but not sufficient for pre-RC formation in vivo. For example, ORC

binding to HP1 protein has no apparent role in origin determination; rather, it has been implicated in gene silencing and heterochromatin formation in many species (Pak et al. 1997; Shareef et al. 2001; Prasanth et al. 2010).

The retinoblastoma protein interacts with ORC in human and fly cells and negatively regulates DNA replication (Bosco et al. 2001; Mendoza-Maldonado et al. 2010), likely by recruiting histone deacetylase (HDAC) activities that generate repressive chromatin marks (Brehm et al. 1998). In support of this idea, stage 10 mosaic embryos derived from flies with mutations in Rpd3/Hdac1 are capable of replication across the entire genome in follicle cells, whereas this is restricted to the chorion genes in wild type (Aggarwal and Calvi 2004). Hdac11 has also been implicated as an inhibitor of origin licensing in mammalian cells (Wong et al. 2010).

To counter these deacetylating activities, ORC and Cdt1 recruit Hbo1, a histone acetyltransferase that binds to origins and acetylates surrounding chromatin during G_1 to promote MCM loading (Miotto and Struhl 2008, 2010). Reduction of Hbo1 levels by small interfering RNA (siRNA) treatment results in defective licensing and cell-cycle arrest. Increased acetylation and open chromatin is thus a common feature of most origins in both flies and human cells, as revealed by genome-wide mapping of origins in fly and human cell lines (MacAlpine et al. 2010; Mesner et al. 2011).

The Set8 histone methyltransferase also regulates origin licensing in human cells (Tardat et al. 2010). Targeting of Gal4–Set8 fusion protein to Gal4-binding sites on plasmid DNA in cultured cells is sufficient to promote MCM loading on adjacent sequences. Set8 is a substrate of the CRL4-Cdt2-dependent degradation pathway in S phase (see below), and expression of a nondegradable Set8 protein results in rereplication in cultured human cells.

In rapidly dividing *Xenopus* embryos, DNA synthesis initiates at intervals of ~10 kb in specific clusters (Blow et al. 2001). This allows a large amount of DNA to be replicated in a relatively short phase of 20 min. Although exogenous AT-rich asymmetric sequences can outcompete replication complexes (Stanojcic et al. 2008), the nature of the DNA sequences at the initiation sites and the composition of histone modifications around the chromosomal initiation sites are unknown.

Regulation of Origin Licensing in Metazoans

ORC, Cdc6, and Cdt1 are essential for origin licensing in *Xenopus* egg extracts, *Drosophila* embryos, and cultured mammalian cells (Whittaker et al. 2000; Gillespie et al. 2001; Rialland et al. 2002; Mailand and Diffley 2005; Svitin and Chesnokov 2010; Gambus et al. 2011). Whereas fly ORC contains six subunits, similar to yeast, ORC in both vertebrates exists as a stable Orc1–Orc5 assembly, with little or no Orc6 protein associated. This five-subunit complex is functional for MCM loading and sequence-independent replication of DNA substrates in a reconstituted system (Gillespie et al. 2001; Vashee et al. 2003). The absence of stoichiometric amounts of Orc6 subunit suggests that the mechanism of MCM loading in higher eukaryotes may be different from that in budding yeasts, where Orc6–Cdt1 interactions are critical for licensing (Chen et al. 2007; Chen and Bell 2011). Despite significant homology between metazoan Orc6 proteins (Dhar and Dutta 2000), human Orc6 interacts weakly with ORC, compared with its fly counterpart (Chesnokov et al. 1999; Vashee et al. 2003; Siddiqui and Stillman 2007). However, it is still required for DNA synthesis and may have evolved to perform other roles in origin recognition (Prasanth et al. 2002; Balasov et al. 2007; Thomae et al. 2008, 2011).

The function of ORC is restricted to G_1 phase by regulated ORC–DNA interactions across the cell cycle. *Xenopus* ORC is released from chromatin on licensing (Sun et al. 2002) and is only weakly associated with chromatin later in G_2/M (Rowles et al. 1999). Mammalian ORC undergoes complex disassembly during S phase as a consequence of ubiquitination of the Orc1 subunit. Ubiquitination results in Orc1 degradation in human cells (Mendez et al. 2002; Tatsumi et al. 2003) but, interestingly, not in hamster nuclei (Natale et al. 2000; Li and DePamphilis 2002). Human Orc1 ubiquitination

is mediated by the SCF^{Skp2} ubiquitin ligase, which is known to act on phosphorylated substrates. Hence, it has been suggested that Orc1 degradation in human cells may be promoted by CDK phosphorylation, representing a CDK-dependent mechanism to prevent relicensing. *Drosophila* cells uniquely regulate ORC and the APC/C ubiquitinates Orc1 for degradation after mitosis, in a situation similar to human Cdc6 (see below).

In metazoan cells ORC exhibits cell-cycle-dependent complex assembly. Human Orc4 protein requires an intact ATP-binding site for complex assembly in vitro, and this subunit does not associate stably with ORC across the cell cycle in vivo (Ranjan and Gossen 2006; Siddiqui and Stillman 2007). Immunofluorescence studies show that as cells proceed from G_1 to G_2/M, the chromatin-bound fraction of Orc2 and Orc3 decreases significantly (Prasanth et al. 2004; Siddiqui and Stillman 2007), and chromatin immunoprecipitation reveals a loss of ORC subunits at origins across the cell cycle (Gerhardt et al. 2006). This may also be promoted via CDK phosphorylation of Orc2, because a nonphosphorylatable Orc2 protein is found associated with origins during G_2/M (Lee et al. 2012). It is therefore possible that assembly of ORC itself may be one mechanism to prevent unscheduled licensing.

Both *Xenopus* and human Cdc6 are phosphorylated by S-CDK, and ectopically expressed protein, on phosphorylation, is transported out of the nucleus into the cytoplasm (Petersen et al. 1999; Coverley et al. 2000; Pelizon et al. 2000; Delmolino et al. 2001). This is postulated to be a CDK-dependent control to prevent relicensing and may be redundant with other mechanisms, as expression of a nonphosphorylatable mutant of Cdc6 alone does not result in significant rereplication. It has been reported, however, that a significant fraction of native phosphorylated Cdc6 is retained on chromatin across S and G_2/M in human cells (Coverley et al. 2000; Mendez and Stillman 2000; Alexandrow and Hamlin 2004) and may regulate entry into mitosis (Clay-Farrace et al. 2003; Lau et al. 2006). At the end of mitosis, human Cdc6 is targeted for APC/C-Cdh1-mediated degradation (Petersen et al. 2000) and degraded in early G_1 phase. The consequence of having undegraded Cdc6 in cycling human cells is, at present, unknown. However, APC/C-mediated degradation may be a way of preventing unscheduled licensing in quiescent G_0-phase cells. On cell-cycle reentry in cultured human cells, cyclin E–CDK2-mediated phosphorylation of Cdc6 blocks Cdh1 binding and stabilizes Cdc6 earlier than geminin, generating a window of opportunity to license origins (Duursma and Agami 2005; Mailand and Diffley 2005). We note that the APC/C-dependent degradation sequence in *Drosophila* Orc1 is bounded by a consensus CDK site, similar to the situation in human Cdc6. It is possible that the cyclin E-dependent association of *Drosophila* MCMs with chromatin may be via Orc1 stabilization (Su and O'Farrell 1997, 1998).

Metazoan Cdt1 is regulated by multiple pathways. Chromatin-bound Cdt1 is ubiquitinated during S phase by CRL4-Cdt2 ubiquitin ligase and targeted for degradation (Arias and Walter 2005; Li and Blow 2005). This pathway is dependent on its interaction with PCNA and is essential to prevent rereplication during S phase (Arias and Walter 2006; Senga et al. 2006).

Additionally, a second SCF^{Skp2}-dependent pathway also promotes Cdt1 degradation throughout the cell cycle (Li et al. 2003; Nishitani et al. 2006). Although Cdt1 binds cyclin A in an RxL-dependent manner and is phosphorylated at consensus CDK sites (Li et al. 2003; Sugimoto et al. 2004), mutations of these motifs do not result in significant rereplication (Takeda et al. 2005a; Nishitani et al. 2006), implying that the SCF^{Skp2}-dependent proteolysis is a minor pathway. The CRL4-Cdt2 pathway is essential for Cdt1 degradation from fission yeasts to metazoans (Jin et al. 2006; Guarino et al. 2011). However, a role for the SCF^{Skp2} in Cdt1 degradation has only been shown in human cells, and this pathway may have arisen recently in evolution (Kim and Kipreos 2007).

Cdt1 activity is also regulated by its interaction with geminin (Wohlschlegel et al. 2000; Tada et al. 2001), a protein discovered as an inhibitor of DNA replication in *Xenopus* (McGarry and Kirschner 1998). Geminin is targeted for degradation by APC/C-Cdh1 and is hence

absent from late mitosis until the end of G_1, when licensing occurs (Nishitani et al. 2001). In *Drosophila* and mammalian cells, a major CDK-independent block to rereplication appears to require geminin, and reduction of protein levels by RNAi is sufficient to promote rereplication in many different cell lines (Quinn et al. 2001; Mihaylov et al. 2002; Melixetian et al. 2004; Zhu et al. 2004). Reduction of geminin levels by immunodepletion from *Xenopus* egg extracts or by injecting antisense oligonucleotides in embryos does not induce rereplication (McGarry and Kirschner 1998; McGarry 2002). However, expression of nondegradable Cdt1 promotes more rereplication in a geminin-depleted extract, suggesting that both these pathways may be important for rereplication control in this species (Kerns et al. 2007).

Similar to yeast and *Xenopus*, mammalian MCMs are loaded onto the chromatin at the end of mitosis and are removed from chromatin as the cells pass through S phase (Mendez and Stillman 2000). The MCM-BP protein is imported into the nucleus late in S phase and interacts strongly with Mcm7 and may promote disassembly of the CMG complex (Nishiyama et al. 2011). Treatment of HeLa cells with siRNAs against MCM-BP results in G_2/M-arrested cells with MCMs persisting on chromatin for longer periods of time. The interaction between MCM-BP and MCMs appears to be replication-dependent, as nuclear MCM-BP does not stimulate disassembly of MCMs in aphidicolin-arrested cells.

Regulation of Origin Firing in Metazoans

Homologs of essential firing factors are known in metazoans, and CDK-dependent activation of replication origins has been verified, with roles for cyclin E in initiation of S phase and cyclin A during the elongation phase (Strausfeld et al. 1996; Mahbubani et al. 1997; Coverley et al. 2002). Cyclin E is essential only in quiescent mouse fibroblasts that are reentering the cell cycle on growth factor stimulation (Geng et al. 2003) and may be redundant with cyclin A in most other cases (Kalaszczynska et al. 2009). DDK activity is similarly required for G_1/S transition and DNA synthesis in mammalian cells and in *Xenopus* (Strausfeld et al. 1994; Jackson et al. 1995; Jiang et al. 1999; Walter 2000; Jares et al. 2004)

Despite highly divergent sequences to the budding yeast counterparts, the vertebrate homologs of Sld2 (RecQL4) and Sld3 (Treslin/Ticrr) have been identified and are essential for replication initiation (Sangrithi et al. 2005; Kumagai et al. 2010; Sanchez-Pulido et al. 2010; Sansam et al. 2010; Boos et al. 2011). Analysis of TopBP1 (Dpb11 homolog) has revealed that it contains nine BRCT domains, referred to as BRCT0 to BRCTVIII (Makiniemi et al. 2001; Huo et al. 2010). Treslin binds to BRCTI/II domains of TopB1 in a CDK-dependent manner (Boos et al. 2011; Kumagai et al. 2011), similar to budding yeast Sld3–Dpb11 interaction. Based on homology between BRCTIII/IV of Dpb11 and BRCTIV/V of TopBP1, RecQL4 is expected to interact with TopBP1 via this domain. In contrast to the yeast proteins, the RecQL4–TopBP1 interaction is CDK-independent (Matsuno et al. 2006). Also, an amino-terminal fragment of TopBP1 containing BRCTI-III repeats is necessary and sufficient for Treslin function in *Xenopus* extracts (Kumagai et al. 2010). It is presently unclear if RecQL4 can interact with this amino-terminal fragment, perhaps via BRCTIII or other proteins that may be essential for initiation. Recently, GEMC1 and DUE-B have been identified as proteins that are phosphorylated in vivo that interact with Cdc45 and TopBP1 and have essential roles in vertebrate DNA synthesis (Balestrini et al. 2010; Chowdhury et al. 2010). It is unknown if these interactions are regulated across the cell cycle and what their specific roles are in activating the replicative helicase. RecQL4 may be a part of the active helicase in mammals, owing to its stable interaction with the CMG complex during S phase (Xu et al. 2009). These observations suggest significant differences in the regulation of yeast and metazoan DNA replication, and further work is required to elucidate these mechanisms.

The Mcm10 protein is essential during early steps of DNA synthesis. Recombinant Mcm10 interacts with single-stranded DNA (ssDNA)

via an evolutionarily conserved oligonucleotide/oligosaccharide binding (OB)-fold domain. Mcm10 also recruits polymerase α–primase complex (Zhu et al. 2007), which is required to initiate de novo replication. This recruitment is dependent on the And-1/Ctf4 protein. Based on its known interaction partners, it is possible that Mcm10 either mediates the initial melting reaction or stabilizes ssDNA generated during the initial unwinding reaction. If this is the case, then the activity of Mcm10 must be regulated because it is reported to bind chromatin independently of CDK or Cdc7 activities. (Van Hatten et al. 2002; Wohlschlegel et al. 2002). Mcm10 appears to be important for CMG complex assembly (Im et al. 2009) and may couple helicase to polymerase in replisomes in a Ctf4-dependent process (Zhu et al. 2007). Interestingly, Mcm10 stabilizes interactions between CMG and RecQL4 (Xu et al. 2009), and CDK phosphorylation of Mcm10 may be required to release RecQL4 during origin firing. These data raise the possibility that Mcm10 may be partly responsible for determining the temporal order of replication timing in metazoan cells, and the mechanisms underlying regulation of these interactions will be an important focus of future studies.

CDK-Dependent Control of Replication Licensing

CDK phosphorylation of Cdc6 plays a positive role in promoting licensing during $G_0 \rightarrow G_1$ transition in mammalian cells (Mailand and Diffley 2005). Unlike in yeasts, it is unclear if CDK phosphorylation of the pre-RC proteins prevents rereplication during S phase in metazoans. Mammalian ORC, Cdc6, and Cdt1 all bind cyclin–CDKs directly and are substrates of CDKs in vitro (Saha et al. 1998; Petersen et al. 1999; Mendez et al. 2002; Sugimoto et al. 2004; Hemerly et al. 2009). However, studies using phosphorylation mutants of these proteins in vivo have not conclusively shown a role for such modifications in preventing rereplication during the cell cycle. Deletion of the amino-terminal region of Orc1 abolishes CDK phosphorylation in vitro but has no effect on ubiquitination in vivo (Mendez et al. 2002). Overexpression of Cdt1 mutants that are unable to bind cyclin or are not phosphorylatable shows more rereplication than wild-type protein (Takeda et al. 2005a), but because such mutants are degraded normally during S phase, it is likely that they promote relicensing in other cell-cycle stages (Nishitani et al. 2006). The essential function of CDKs in preventing relicensing, therefore, appears to be during the G_2/M phase of the cell cycle. Chemical inhibition of CDK activity during this period results in relicensing of chromatin even in the presence of geminin (Ballabeni et al. 2004; Vassilev et al. 2006). The essential targets of CDK for this G_2/M-specific inhibition are unknown; however, both Cdt1 and geminin are phosphorylated by CDKs in nocodazole-treated extracts and are potential candidates for this CDK-mediated regulation.

CDK-Independent Control of Replication Licensing

Metazoans have evolved CDK-independent pathways to prevent rereplication, and these are largely devoted to Cdt1 regulation. In contrast to yeast, deregulation of metazoan Cdt1 alone is sufficient to induce significant rereplication during S phase (Melixetian et al. 2004; Zhu et al. 2004; Jin et al. 2006; Lovejoy et al. 2006; Sansam et al. 2006). Consequently, Cdt1 protein levels and activity are tightly regulated. This is achieved by a combination of Cdt2-Ddb1-dependent degradation of chromatin-bound Cdt1 during S phase and interaction of Cdt1 with geminin during S and G_2/M phases. Degrading geminin during S phase by premature activation of the APC/C leads to significant rereplication, and this can be suppressed by expression of a nondegradable mutant of geminin (Di Fiore and Pines 2007; Machida and Dutta 2007), highlighting the important role of this protein in maintaining genome ploidy.

Geminin inhibits licensing by blocking the Cdt1–MCM interaction (Wohlschlegel et al. 2000; Yanagi et al. 2002). Crystal structures have suggested a cell-cycle-dependent transition between two possible conformations of Cdt1-geminin (Lee et al. 2004; De Marco et al. 2009).

A heterotrimeric (Cdt1-geminin-geminin) state exposes the surface on Cdt1 that interacts with the MCMs and thus permits licensing. This alternates with a heterohexameric [Cdt1-(geminin)$_2$]$_2$ state, in which the Cdt1–MCM interaction surface is hidden and is thus inhibitory to origin licensing. These observations are explained by a feedback model, whereby geminin functions cooperatively to inhibit licensing at multiple origins, therefore creating a critical threshold resulting in all-or-none inhibition of replication licensing (Ode et al. 2011). It is proposed that geminin and Cdt1 interact at individual origins and the ability of these two proteins to oligomerize promotes interactions between proteins at adjacent origins, thereby propagating the inhibition of licensing and clustering origins into subnuclear foci. This clustering does not affect MCM complexes that are already loaded onto origins during G_1. Based on the expression profiles of these proteins, geminin serves its essential function in preventing rereplication primarily during G_2/M, when it not only inhibits Cdt1 degradation but also releases it for licensing in the subsequent G_1 (Ballabeni et al. 2004), much like Clb2-Cdc6 in budding yeasts.

CONCLUDING REMARKS

Although CDK-dependent pathways are primarily responsible for maintaining genome stability in budding yeasts, it is clear that CDK-independent mechanisms play a critical role in maintaining genome stability in multicellular organisms. Several cyclin subunits and CDK2 can be knocked out in mice (Sherr and Roberts 2004; Malumbres and Barbacid 2009), suggesting significant redundancy among the functions of cyclin–CDK complexes. Redundant mechanisms targeting ORC, Cdc6, and Cdt1-MCM inhibit rereplication in yeasts. In contrast, it appears that some mammalian cancer cell lines are particularly sensitive to Cdt1 deregulation alone, because RNAi-mediated silencing of geminin or Cdt2 is sufficient to induce significant rereplication in some cell lines but not others (Melixetian et al. 2004; Zhu et al. 2004; Jin et al. 2006; Lovejoy et al. 2006; Sansam et al. 2006). Although deregulating either ORC or Cdc6 alone does not have a similar outcome, overexpression of these proteins can enhance the rereplication seen on Cdt1 overexpression (Vaziri et al. 2003; Sugimoto et al. 2009).

Deregulated licensing has emerged as a sensitive and early indicator of tumor development in human cancers (Freeman et al. 1999; Davies et al. 2002). Recent work has proposed that cancer cells may respond differently to licensing inhibition than primary cells (Shreeram et al. 2002; Zhu and DePamphilis 2009), and this could be exploited in designing therapies that selectively target cancer cells. The next few years will witness the identification of new replication factors (e.g., vertebrate Sld7) and lead to a better understanding as to how the divergence of proteins such as Orc6, Treslin, and RecQL4 confers unique properties to replication control in metazoan cells.

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