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Regulation of DNA Replication Origin Licensing

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1. Introduction

DNA replication is a fundamental biological process that serves to create two copies of the genetic material during each cell division. Complete and precise replication enables identical sets of genes to be faithfully delivered to daughter cells during each cell division. To achieve rapid duplication of the entire genome, eukaryotic cells initiate DNA replication at multiple locations on each chromosome termed *origins of DNA replication*. Origin DNA is unwound and complementary DNA is then synthesized from bi-directionally moving replication forks. The replication forks eventually merge to form two identical chromosomes.

The cell expends tremendous energy ensuring that a single origin of replication does not initiate replication twice within the same cell cycle. One of the most highly regulated steps in DNA replication is assembly of pre-replication complexes (pre-RCs). Pre-RC assembly begins as cells exit mitosis and continues through G1 phase, culminating in chromosomes poised for replication by the end of G1. At the onset of S phase, origins fire and replication begins. During this time, several overlapping mechanisms prevent pre-RC assembly on origins that have already fired to avoid utilizing any origins twice.

An abnormal situation in which replication is triggered multiple times from the same origin during a single cell cycle is termed *re-replication* (Figure 1). Re-replication is detrimental to genome stability, because it generates multiple replication forks on the same DNA strand. Ultimately such structures result in double strand breaks, genome instability, and in some cases, tumorigenesis (Arentson et al., 2002; Karakaidos et al., 2004; Xouri et al., 2004; Lontos et al., 2007). This chapter focuses on mechanisms to prevent re-replication during normal and perturbed cell cycles.

2. Pre-replication complex (pre-RC) assembly

To faithfully replicate its genomic information in a timely manner, a cell must initiate replication at thousands of sites across the genome. These origins of replication are prepared for replication through assembly of pre-RC complexes, beginning in late mitosis and continuing through G1 phase of the cell cycle. Origins with a fully assembled pre-RC are said to be “licensed” for replication. It is essential that origins assemble pre-RCs *only* in G1 because assembly of pre-RCs in S or G2 can lead to re-replication.

Pre-RC assembly begins when the six-subunit origin recognition complex (ORC) binds to an origin of replication (Figure 2). ORC is composed of the constitutively-expressed subunits Orc2-6, as well as the cell cycle-regulated Orc1 protein, and acts as an ATPase (Dhar et al.,

2001; Vashee et al., 2001; Bowers et al., 2004; Mendez et al., 2002). Once bound to origins, ORC recruits the remaining licensing factors Cdc6 and Cdt1 to origins (Cocker et al., 1996; Nishitani et al., 2000).

Fig. 1. Re-replication leads to double strand breaks. In a normal replicating cell (left), origins are licensed only in G1 phase (indicated by the green diamonds). Replication begins in S phase, and origins are inhibited from firing again (indicated by the red diamonds); replication is completed by G2 phase. In re-replicating cells (right), origins are licensed again in either S or G2 phase, leading to origin re-firing and re-replicated stretches of DNA. The consequences of re-replication include DNA damage, genome instability, and tumorigenesis.

Cdc6 was discovered in *Saccharomyces cerevisiae* and is essential for DNA replication; if Cdc6 is absent, yeast cells not only fail to replicate but also undergo reductional anaphase in which mitosis initiates without genome replication (Hartwell, 1976; Zwierschke et al., 1994; Piatti et al., 1995). Cdc6 is a member of the AAA+ ATPase family, and is closely related to Orc1 (Gaudier et al., 2007; Liu et al., 2000). ATP hydrolysis by Cdc6 and ORC is needed to load the helicase complex onto DNA (Randell et al., 2006; Bowers et al., 2004; Weinreich et al., 1999; Herbig et al., 1999; Donovan et al., 1997; Cook et al., 2002). Due to its tight association with ORC and its partially conserved DNA binding domain, it has been suggested that Cdc6 may also play a role in defining ORC binding sites (Mizushima et al., 2000).

Cdt1 was first discovered in *Schizosaccharomyces pombe* and, while possessing no enzymatic activity, is essential for the licensing reaction (Hofmann & Beach, 1994; Nishitani et al., 2000). Cdt1 binds the core replicative helicase Mini-Chromosome Maintenance (MCM) complex and recruits MCM to origins through direct interactions with ORC and Cdc6 (Tanaka & Diffley, 2002; Cook et al., 2004; Bruschi et al., 1995; Chen & Bell, 2011). While both Cdc6 and Cdt1 are needed to load the MCM complex, they bind in a sequential manner; Cdt1 can only bind to chromatin-bound Cdc6 and ORC (Tsuyama et al., 2005). Both Cdc6 and ORC hydrolyze ATP to load MCM complexes onto DNA (Randell et al., 2006). ATP hydrolysis by Cdc6 also releases Cdt1 to recruit additional MCM complexes (Randell et al., 2006). Once MCM complexes are loaded, the origin is licensed and can initiate replication once the MCM helicase is activated in S phase. After MCM complexes have been loaded, ORC, Cdc6, and Cdt1 are no longer needed, and replication can continue in their absence

(Donovan et al., 1997; Rowles et al., 1999; Maiorano et al., 2000). This property of the loaded MCM complex is key to preventing re-replication because, as discussed below, ORC, Cdc6, and Cdt1 are inactivated beginning in S phase.

At each origin, at least two MCM hexamer complexes are loaded at a time, with multiple rounds of loading at each origin (Evrin et al., 2009; Remus et al., 2009; Edwards et al., 2002; Lei et al., 1996). The exact mechanism of MCM loading is not currently understood, but electron microscopy images suggest ORC and Cdc6 form a structure similar to known clamp loaders such as RFC (Chen et al., 2008; Speck et al., 2005). While multiple MCM complexes can be loaded at each origin, perhaps as many ten copies per origin, the majority of the MCM complexes that associate with chromatin do not travel with the replication fork suggesting that they are not normally activated (Edwards et al., 2002; Krude et al., 1996; Dimitrova et al., 1999). These additional MCM complexes may be loaded as a backup mechanism to ensure that a sufficient number of origins fire in S phase (Ge & Blow, 2010).

Fig. 2. Pre-RC assembly in G1 phase. Pre-RC assembly begins when the Origin Recognition Complex (ORC) binds to origin DNA. ORC recruits Cdc6, which in turn recruits Cdt1 bound to the Mini-Chromosome Maintenance (MCM) core helicase complex. Through the ATPase activity of ORC and Cdc6, the MCM complex is loaded onto DNA and the origin is licensed for replication.

MCM loading is highly regulated by multiple overlapping mechanisms (summarized in Table 1). Cdc6 and Cdt1 protein levels peak at different stages of the cell cycle; Cdt1 levels peak in G1 phase whereas Cdc6 peaks in S/G2 phase in mammalian cells (Nishitani et al., 2000; Petersen et al., 2000). Additionally, a member of the ORC complex, Orc1, is degraded or inactivated at the onset of S phase (Mendez et al., 2002; Li & DePamphilis, 2002; Li et al., 2004).

Due in part to these alternating protein levels, there are only two short windows in the cell cycle when pre-RC formation can occur. Pre-RC assembly begins at the end of mitosis, before the Anaphase Promoting Complex/Cyclosome (APC/C) becomes active in G1, and targets Cdc6 for degradation. The second round of pre-RC assembly occurs in late G1 phase when activated Cdk2 stabilizes Cdc6 but before Cdt1 is degraded at the onset of S phase (Diffley, 2004). Furthermore, the MCM subunits undergo post-translational modifications that facilitate MCM complex formation as well as their ability to be loaded onto DNA (Lin et al., 2008; Chuang et al., 2009). These mechanisms will be discussed in depth in the subsequent sections with specific emphasis on the regulation of metazoan pre-RC assembly.

Regulator	Substrate	Function
Cyclin/Cdk		
cyclin A/ Cdk2	Cdt1	ubiquitination by SCF ^{Skp2}
	Cdc6 (Ser106)	nuclear export of Cdc6
	Orc1	nuclear export and/or degradation depending on cell type
cyclin E/ Cdk2	Cdc6 (Ser54)	stabilization by protection from APC/C ^{Cdh1} ubiquitination
	Mcm3	facilitates Mcm (2-7) complex formation
Ubiquitin ligases		
SCF ^{Skp2}	Cdt1	ubiquitination in S phase
APC/ C ^{Cdc20}	cyclin A	ubiquitination in G2 phase
APC/ C ^{Cdh1}	geminin	ubiquitination in M and G1 phases
	Cdc6	
CRL4 ^{Cdt2}	Cdt1	ubiquitination in S phase and after DNA damage
	Set8	
Acetyltransferases		
Gcn5	Cdc6	Promotes phosphorylation of Ser6 by cyclin A/Cdk2
Hbo1	histone H4	promotes pre-RC formation (Cdt1 stimulation?)
	pre-RC proteins?	
Methyltransferases		
Set8	histone H4 (K20)	Monomethylation promotes chromatin condensation and pre-RC assembly

Table 1. Regulation of pre-RC components and related factors.

3. Pre-RC regulation

Pre-RC formation and origin licensing occurs during late mitosis and early G1 phase of the cell cycle (Bell & Dutta, 2002). It is important to limit the assembly of pre-RCs to only the G1 phase since failure to restrict pre-RC formation results in re-replication. Re-replication can

cause increases in double strand breaks and ploidy. Over time, damaged DNA could lead to genome instability, ultimately increasing the propensity for tumorigenesis. In this section we will discuss several mechanisms by which metazoan cells avoid re-replication.

3.1 Regulation of pre-RCs by cyclin-dependent kinases

Cyclin-dependent kinases (Cdks) are a family of serine-threonine protein kinases essential for timely and appropriate progression through different stages of the cell cycle. Cdks are activated by association with cyclins, whose expression and stability are cell cycle-regulated. In budding and fission yeast, a single Cdk controls the G1/S and G2/M transitions, while in metazoans different Cdks are active in different phases of the cell cycle (Figure 3, reviewed in Malumbres & Barbacid, 2009). In metazoans, passage through G1 phase is governed by cyclin D/Cdk4 (or cyclin D/Cdk6) and cyclin E/Cdk2 (Braden et al., 2008; van den Heuvel & Harlow, 1993). S phase, and therefore DNA replication, is regulated by cyclin A/Cdk2 (Wheeler et al., 2008). Finally, mitotic entry is triggered by Cdk1 first binding to cyclin A and then cyclin B.

DNA replication is both positively and negatively governed by Cdk activity. High Cdk2 and Cdk1 activities, which are found from early S phase through mid-mitosis, block pre-RC assembly; thus pre-RC assembly begins as cells exit from mitosis when these kinases are not active. Cyclin E protein peaks in early S phase, triggering Cdk2 activity and replication initiation from licensed origins; replication begins also with the help of a dedicated replication kinase, Cdc7/Dbf4 (Bochman & Schwacha, 2009; Remus et al., 2009). (Additional chapters related to origin firing and S phase progression appear elsewhere in this book.) Simultaneously, S phase Cdk activity inhibits pre-RC formation at origins that have already fired. Premature expression of cyclins E or A during G1 phase blocks normal pre-RC assembly (Wheeler et al., 2008; Ekholm-Reed et al., 2004). Thus S phase Cdks promote replication initiation but block pre-RC assembly after G1 (described below), resulting in one genome duplication per cell cycle.

Fig. 3. Cyclin levels fluctuate throughout the cell cycle. Cell cycle progression is regulated through the action of cyclin/Cdk complexes. In G1 phase, the cyclin D/Cdk4 and cyclin D/Cdk6 complexes are nuclear and active and regulate the transcription of genes for pre-RC components. Cyclin E/Cdk2 activity stimulates replication in S phase while simultaneously inhibiting re-licensing of origins. Cyclin A/Cdk controls S phase progression and also inhibits relicensing. Entry into mitosis is triggered by cyclin B/Cdk1 complexes. Darker shading indicates peak protein expression or activity.

3.1.1 Cyclin-Cdks as negative regulators of pre-RC assembly

Several experimental findings support a role for Cdks in preventing re-replication. For example, transient over-expression of the Cdk inhibitor, p21, in G2 caused re-replication (Bates et al., 1998). In addition, ORC and MCM complexes were recruited to human chromatin in G2 when Cdk activity was inhibited genetically or pharmacologically (Coverley et al., 1996; Fujita et al., 1998; Ballabeni et al., 2004; Li et al., 2004; Sugimoto et al., 2004). Finally, chemical inactivation of Cdk1, combined with genetic ablation of Cdk2, allowed pre-RCs to assemble inappropriately during mitosis (Ballabeni et al., 2004). These observations highlight a role for Cdks in preventing inappropriate origin licensing.

Re-licensing of origins during S phase is prevented, in part, by Cdk2, in association with cyclin A (Wheeler et al., 2008). Cdt1 interacts with the S phase cyclin A/Cdk2 complex, which results in Cdt1 phosphorylation at threonine 29 (Li et al., 2004; Liu et al., 2004). Phosphorylated Cdt1 binds to the F-box protein, Skp2, the substrate receptor for the ubiquitin ligase SCF^{Skp2}. Cdt1 is polyubiquitinated by SCF^{Skp2} and targeted for degradation by the 26S proteasome, thus reducing the pool of Cdt1 protein available to participate in origin licensing (Takeda et al., 2005; Sugimoto et al., 2004; Kim & Kipreos, 2007).

Coincident with Cdt1 destruction in S phase, Cdc6 is acetylated by Gcn5 on lysines 92, 105, and 109 which promotes cyclin A/Cdk2 phosphorylation on Cdc6 at serine 106 (Paolinelli et al., 2009; Mailand & Diffley, 2005). Serine 106 phosphorylation results in exclusion of Cdc6 protein from the nucleus, preventing re-replication (Paolinelli et al., 2009; Saha et al., 1998; Coverley et al., 2000; Kim et al., 2007; Fujita et al., 1999; Jiang et al., 1999; Petersen et al., 1999). The small amount of Cdc6 that remains nuclear throughout S phase is chromatin-bound and likely participates in the ATR-dependent intra-S phase checkpoint by mechanisms that are not yet understood (Mendez & Stillman, 2000; Lau et al., 2006).

Additionally, the Orc1 subunit of ORC is phosphorylated by cyclin A/Cdk1 during S phase, and this phosphorylation promotes Orc1 degradation in HeLa cells (Mendez et al., 2002). The same phosphorylation on Orc1 in Chinese Hamster Ovary cells (CHO) does not affect Orc1 stability, but lowers the affinity of Orc1 for chromatin (Li et al., 2004). In both HeLa and CHO cells, Orc1 phosphorylation allows the export of Orc1 to the cytoplasm (Saha et al., 2006). Over-expression of cyclin A from Kaposi's Sarcoma-associated herpes virus also facilitates re-localization of Orc1 to the cytoplasm. These results show that Orc1 is subject to phosphorylation by cyclin A/Cdk1, and this event modulates the stability and/or localization of Orc1, thereby contributing to the prevention of re-replication.

Recent evidence from *S. cerevisiae* suggests that Orc2 and Orc6 may also be targets of cyclin/Cdk inhibition. Phosphorylation of these subunits leads to a marked decrease in MCM loading (Green et al., 2006; Tanny et al., 2006; Nguyen et al., 2001). Interaction between Orc6 and the S phase Cdk, Clb5, is needed to prevent MCM loading outside of G1 phase; this interaction occludes the Cdt1 binding site on the ORC complex (Wilmes et al., 2004; Tanny et al., 2006; Chen & Bell, 2011). In addition to steric hindrance, Clb5 phosphorylates Orc6; this modification also partially blocks the Cdt1 binding site and prevents MCM loading (Chen & Bell, 2011). It remains to be determined if similar mechanisms also apply to Cdk regulation of mammalian ORC (DePamphilis, 2005).

3.1.2 Cyclin-Cdks as positive regulators of pre-RC assembly

Cdc6 protein levels are very low in both quiescent cells and in early/mid G1 phase cells due to ubiquitin-mediated proteolysis, but Cdc6 protein accumulates in late G1 just prior to a

burst of MCM loading. In late G1, cyclin E/Cdk2 phosphorylates human Cdc6 on serine 54, which protects Cdc6 from the ubiquitin E3 ligase APC/C^{Cdh1} (Maidland & Diffley, 2005). Many cancer cells have both high cyclin E/Cdk2 activity and high Cdc6 protein levels which may reflect not only the transcriptional up-regulation of *cyclin E* and *cdc6* genes in tumors but also the stabilizing effect of cyclin E/Cdk2 on Cdc6 protein (Nielsen et al., 1996). Cyclin E may also have an as yet poorly understood direct role in MCM loading through association with Cdt1 and MCM subunits independently of its association with Cdk2. When cyclin E was depleted from mammalian cells, MCM loading did not occur as cells re-entered G1 from quiescence (Geng et al., 2007; Geng et al., 2003).

In addition to regulating Cdc6, Cdk complexes also facilitate the formation and loading of the MCM helicase complex. Mcm3 is phosphorylated on serine 112 by Cdk1, which triggers MCM complex assembly (Lin et al., 2008). Cdk activity affects MCM loading in an indirect fashion as well. The activity of cyclin E/Cdk2 is required for the accumulation of Cdc7 mRNA, which in turn functions in origin firing (Chuang et al., 2009; Francis et al., 2009; Jiang et al., 1999; Masai et al., 2000; Sheu & Stillman, 2006). In quiescent cells, phosphorylation of Mcm2 at serine 5 is necessary to promote MCM loading (Chuang et al., 2009; Geng et al., 2007).

3.2 Cdk-independent regulation of pre-RCs

3.2.1 Regulation of Cdt1 by CRL4^{Cdt2}

Cdk-independent mechanisms also prevent re-replication by targeting Cdt1. Non-phosphorylatable (Cdk-resistant) Cdt1 mutants are degraded during S phase despite being unable to bind to Skp2 (Takeda et al., 2005; Nishitani et al., 2006; Senga et al., 2006). An alternate, DNA-dependent, mechanism for Cdt1 degradation was subsequently uncovered (Jin et al., 2006; Arias & Walter, 2006; Senga et al., 2006; Higa et al., 2006; Nishitani et al., 2006; Hu & Xiong, 2006; Ralph et al., 2006). Proliferating Cell Nuclear Antigen (PCNA) is a cofactor required to increase the processivity of DNA pol δ during leading strand synthesis (Maga & Hubscher, 2003). Thus, PCNA travels with active replication forks. Cdt1 interacts with PCNA through a highly conserved region called the PIP box during S phase. Cdt1 binding to DNA-loaded PCNA is essential for Cdt1 recognition by the CRL4^{Cdt2} ubiquitin E3 ligase. CRL4 associates with Cdt1 via direct binding to the Cdt2 substrate adapter which links to the Cul4 scaffold. Since Cdt1 only binds PCNA on chromatin, it is only ubiquitinated by chromatin-associated CRL4^{Cdt2}. In this manner Cdt1 degradation is directly coupled to DNA synthesis. A Cdt1 mutant that cannot bind either PCNA or cyclin/Cdk is stable during S phase and causes re-replication (Nishitani et al., 2006). Likewise, Cdt2 depletion stabilizes Cdt1 in S phase, causing re-licensing of fired origins, and extensive re-replication (Jin et al., 2006; Higa et al., 2006; Ralph et al., 2006).

3.2.2 Regulation of Cdt1 by geminin accumulation

As another layer of regulation, metazoan S phase cells accumulate the protein geminin, which binds to Cdt1 and blocks the Cdt1-MCM interaction (Lee et al., 2004; Wohlschlegel et al., 2000; Tada et al., 2001). Geminin is expressed throughout the S, G2 and M phases of the cell cycle when origin licensing is inhibited (McGarry & Kirschner, 1998; Wohlschlegel et al., 2000). Geminin is targeted for degradation in G1, but begins to accumulate at the end of G1 when the ubiquitin ligase responsible for its degradation (APC/C^{Cdh1}) is inactivated (McGarry & Kirschner, 1998; Rape et al., 2006). In S phase, geminin binds to the residual Cdt1 that escaped degradation and renders it unavailable to re-license origins.

Recent biochemical evidence has suggested that geminin-Cdt1 complexes exist in several forms (De Marco et al., 2009). These forms include a licensing-inhibitory heterohexamer that consists of two Cdt1 molecules and four geminin molecules, and a licensing-permissive heterotrimer, comprised of one Cdt1 molecule and two geminin molecules (Lutzmann et al., 2006). Binding of geminin to Cdt1 in a heterohexamer can tether several Cdt1 molecules together, creating chromatin-bound foci that may cooperatively inhibit licensing (Ode et al., 2011). Depending on the amount of geminin in the cell, geminin may switch from being an inhibitor of origin licensing when geminin levels are high to an activator when levels of geminin are low.

3.2.3 APC/C as a regulator of pre-RC assembly

The cell spends a significant amount of energy to ensure that the correct proteins are expressed at the appropriate time. Before one cell cycle phase begins, cells ensure that the previous step has been properly completed and, in many cases, inactivated by controlling protein activity abundance. One mechanism for enforcing the proper order of events is through regulated protein degradation. The Anaphase Promoting Complex/Cyclosome (APC/C) is uniquely tied to cell cycle progression and control of DNA replication as evidenced by the fact its regulation and activity are modulated in every phase of the cell cycle. APC/C is a RING-type E3 ubiquitin ligase originally discovered through its association with its substrates, the mitotic cyclins (Sudakin et al., 1995; King et al., 1995). Two activator subunits, Cdc20 and Cdh1, interact dynamically with the APC/C holoenzyme to influence substrate recognition (Figure 4). APC/C^{Cdh1} targets in G1 include Skp2, a member of the SCF ubiquitin ligase complex, the licensing factor Cdc6, and the inhibitor protein geminin (Wei et al., 2004; Petersen et al., 2000; McGarry & Kirschner, 1998). Degradation of Skp2 results in accumulation of the Cdk2 inhibitors p21 and p27, and prevents premature S phase entry due to low Cdk2 activity (Wei et al., 2004). APC/C^{Cdh1} also acts to limit the amount of Cdc6 that is available in the cell (discussed in 3.1.2).

As cells progress through S phase, Cdh1 is phosphorylated by cyclin A/Cdk2 complexes; since hyper-phosphorylated Cdh1 cannot interact with APC/C, the ubiquitin ligase complex is inactive (Zachariae et al., 1998). This inactivation allows geminin to accumulate and bind any remaining Cdt1 (McGarry & Kirschner, 1998). During S phase, APC/C^{Cdh1} is also bound by its inhibitor protein, Emi1 (Hsu et al., 2002). Interestingly, Emi1 accumulation is not needed to begin S phase but is needed to signal the stop of replication and mitotic entry, even though APC/C^{Cdc20} can still ubiquitinate its targets if Emi1 is present in mitosis (Di Fiore & Pines, 2007). Emi1 remains bound until prophase, when it is phosphorylated by Plk1 (Moshe et al., 2004). While Emi1 accumulation is not needed for S phase entry, it is essential to inhibit re-replication. Depletion of Emi1 leads to re-replication in human cells, due to the untimely activation of APC/C^{Cdh1} (Sivaprasad et al., 2007; Machida & Dutta, 2007). This stabilization allows geminin levels to drop when Cdt1 levels are high; at the same time, increased activity of cyclin A/Cdk2 allows Cdc6 to become stabilized. With both licensing factors present, origins are licensed outside of G1 and re-replication occurs.

4. Re-replication causes DNA damage and cell cycle arrest

Manipulation of key components of the replication machinery can induce re-replication. For instance, over-expression of replication proteins Cdt1 and Cdc6 causes re-replication in human cancer cells (Nishitani & Nurse, 1995; Vaziri et al., 2003; Ekholm-Reed et al., 2004;

Fig. 4. APC/C is cell cycle regulated and controls several important pre-RC proteins. In G1 phase, APC/C is bound to the adaptor protein Cdh1 and APC/C^{Cdh1} ubiquitinates the licensing factor Cdc6 and the Cdt1 inhibitor geminin. During this time, it also targets another APC/C adaptor protein, Cdc20, for degradation. As cells progress into S phase, APC/C^{Cdh1} is bound by its inhibitor protein, Emi1. At the beginning of mitosis, APC/C and Cdh1 become hyper-phosphorylated, leading to dissociation of Cdh1 from APC/C. Phosphorylated APC/C can then bind the adaptor protein, Cdc20. The APC/C^{Cdc20} complex is responsible for degrading cyclin B and securin, thereby promoting sister chromatid separation and mitotic exit.

Tanaka & Diffley, 2002; Liontos et al., 2007). Depletion of the Cdt1 inhibitor geminin also induces re-replication in certain cancer cell types (Melixetian et al., 2004; Zhu et al., 2004). Depletion or inhibition of the CRL4^{Cdt2} ubiquitin E3 ligase, the machinery responsible for the ubiquitination of Cdt1, stabilizes Cdt1 and stimulates re-replication (Jin et al., 2006; Zhong et al., 2003; Lin et al., 2010). Thus, misregulation of pre-RC components or proteins involved in regulating the pre-RC complex can promote re-replication.

Re-replication activates the DNA damage response (Blow & Dutta, 2005). Mammalian cells depleted of geminin, Cdt2, or Ddb1 (another subunit of CRL4^{Cdt2}), fail to properly down-regulate Cdt1 during S phase. These cells, or cells that overproduce Cdt1, also acquire phosphorylated γ H2AX, a histone variant incorporated into chromatin at sites of DSBs (Vaziri et al., 2003; Melixetian et al., 2004; Zhu et al., 2004; Jin et al., 2006; Lovejoy et al., 2006; Zhu & Dutta, 2006). The appearance of double stranded break markers (DSBs) in re-replicating cells suggests that multiple forks on the same DNA result in replication fork collision and/or collapse.

Checkpoint sensors recognize impaired fork activity, generated by either fork collapse or stalled replication forks, and shut down cell cycle progression. This DNA damage response involves activation of the serine-threonine kinases, ATM (Ataxia Telangiectasia Mutated)

and ATR (ATM-related) (Shiloh, 2003). Single-stranded regions are recognized by ATR, which preferentially activates and phosphorylates the Chk1 protein kinase (Guo et al., 2000; Zhao & Piwnica-Worms, 2001; Kramer et al., 2004; Niida et al., 2007; Zou & Elledge, 2003). Chk1 in turn inactivates the Cdc25 phosphatase causing an intra-S or G2/S phase arrest (Donzelli & Draetta, 2003; Jin et al., 2003; Ferguson et al., 2005). Inactivation of Cdc25 is also accomplished by induction of MAPKAP-K2 (MK2), a downstream effector kinase of the p38 MAP kinase pathway, which is stimulated by genotoxic stress (Manke et al., 2005; Reinhardt et al., 2007; Lemaire et al., 2006; Huard et al., 2008). Without active Cdc25, the mitotic cyclin B/Cdk1 complex remains phosphorylated and inactive. Thus, the DNA damage response pathway leads to a G2/M cell cycle arrest (Tang et al., 2006).

Damage caused by DSBs is recognized by ATM (Shechter et al., 2004; Costanzo et al., 2001). ATM activates and phosphorylates the checkpoint protein kinase Chk2 (Melchionna et al., 2000; Zhou & Elledge, 2000). Activated Chk2 then phosphorylates and activates the transcriptional regulator, p53 (Meek, 1994; Milczarek et al., 1997). Active p53 induces expression of many genes needed for DNA repair and cell cycle arrest, including the Cdk inhibitor, p21 (Smith et al., 1995). The accumulation of p21 inhibits Cdk2 function and blocks cells from entering S phase (Taylor & Stark, 2001; Levine, 1997; Vogelstein et al., 2000). DNA damage in G2 phase also leads to p53-dependent p21 accumulation, inhibition of Cdk1, and arrest in G2 (Agarwal et al., 1995; Deng et al., 1995; Brugarolas et al., 1995).

Cells can recover from arrest by activating repair pathways and continuing cell division, but if the damage is too extensive, they die by apoptosis (Hartwell & Weinert, 1989; Weinert & Hartwell, 1990; Nasmyth, 1996; Abraham, 2001). Re-replication induced by Cdt1 stabilization or geminin depletion results in many of the checkpoint events outlined above, including phosphorylation of Chk2, Chk1, and p53, failure to enter mitosis or initiation of apoptosis depending on the degree of re-replication-associated damage (Zhu & Dutta, 2006; Zhu et al., 2004; McGarry, 2002). G2/M arrest caused by loss of geminin is primarily mediated by ATR recognition of single-stranded regions generated early in re-replication. As cells eventually accumulate more damage from more extensive re-replication and generate DSBs, the ATM pathway is induced (Lin & Dutta, 2007). Such sequential activation may permit low-level damage to be repaired during an ATR-mediated G2/M arrest prior to ATM-mediated p53 activation and permanent arrest or cell death.

Interestingly, ATR-deficient cells show a higher propensity to re-replicate than their ATR-proficient counterparts do (Lin & Dutta, 2007). These observations suggest that the ATR pathway can restrict re-replication while re-replication simultaneously activates ATR. Another potential interplay between replication licensing and the ATR pathway is suggested by the binding of Cdc6 to ATR, which promotes ATR chromatin binding (Yoshida et al., 2010). Cdc6 can also activate p21-bound Cdk2 and override the DNA damage checkpoint (Kan et al., 2008).

5. Pre-RC regulation during a DNA damage response

As described above, cyclin/Cdk activity prevents inappropriate formation of pre-RC complexes in S and G2 phases of the cell cycle. Exposure to DNA-damaging agents or generation of DSBs during replication elicits the DNA-damage response, which can reduce Cdk activity, particularly in G2 phase. Therefore, DNA damage can perturb the normal Cdk-dependent regulation of pre-RC components such that there is potential danger of

initiating re-replication events. To counteract this risk, the DNA damage induces Cdk-independent mechanisms to inhibit pre-RC assembly.

5.1 Degradation of Cdc6

When cells are exposed to various genotoxins, such as ultraviolet (UV) irradiation, ionizing radiation (IR), base alkylating agents, etc., Cdc6 is actively degraded by two mechanisms. As outlined above, the DNA damage response results in p53-dependent induction of the Cdk inhibitor p21^{Cip1}. Reduced Cdk activity prevents phosphorylation of Cdc6 at serine 54 (Duursma & Agami, 2005). Without this protective phosphorylation, Cdc6 can be ubiquitinated by APC/C^{Cdh1} and degraded, particularly in G1 phase due to the high activity of APC/C^{Cdh1} (Mailand & Diffley, 2005). Cdc6 ubiquitination by APC/C^{Cdh1} is further facilitated by the activation of APC/C^{Cdh1} by the DNA damage checkpoint and cyclin destruction (which also reduces Cdc6 serine 54 phosphorylation) (Sudo et al., 2001).

Cdc6 is also targeted for degradation by a different E3 ubiquitin ligase, Huwe1 (Figure 5). Recognition of Cdc6 by Huwe1 is independent of both p53 induction of p21 and changes in Cdk-mediated phosphorylation (Hall et al., 2007). Huwe1 is a monomeric HECT family E3 ligase that has been linked to the regulation of many proteins central to cell proliferation control, including maintaining low p53 levels in the absence of DNA damage (Chen et al., 2005). Huwe1-mediated Cdc6 degradation is also activated in response to endogenous re-replication-associated DNA damage ultimately leading to loss of Cdc6 from re-replicating cells (Hall et al., 2008).

5.2 Degradation of Cdt1

When cells are exposed to exogenous DNA damaging agents, Cdt1 is rapidly degraded (Higa et al., 2003; Hu et al., 2004). DNA damage-induced Cdt1 degradation is unaffected by Cdk activity, and is therefore unrelated to ubiquitination by the SCF^{Skp2} ubiquitin ligase. Instead, Cdt1 is degraded through association with the CRL4^{Cdt2} ubiquitin ligase (Higa et al., 2003; Hu & Xiong, 2006; Jin et al., 2006; Nishitani et al., 2006; Ralph et al., 2006; Sansam et al., 2006; Senga et al., 2006). PCNA is loaded onto damaged DNA not only during normal replication, but also in response to DNA damage both for DNA repair synthesis and in a checkpoint role (Lee & Myung, 2008). As a result, Cdt1 interacts with loaded PCNA after DNA damage in the same way that it interacts with PCNA during S phase. The interaction of Cdt1 with PCNA at sites of DNA damage recruits CRL4^{Cdt2} via direct binding of Cdt2 to the Cdt1-PCNA complex, leading to Cdt1 ubiquitination and proteasomal degradation.

Like Cdc6, Cdt1 is targeted for destruction in response to both exogenous and endogenous DNA damage. Human or *Drosophila* cells depleted of geminin also rapidly lose Cdt1 and Cdc6 (Mihaylov et al., 2002; Ballabeni et al., 2004). Since the lack of geminin induces re-replication-related DNA damage, Cdt1 and Cdc6 are simultaneously targeted for degradation by CRL4^{Cdt2} and Huwe1, respectively (Hall et al., 2008). In cells depleted of either CRL4^{Cdt2} components or Huwe1, geminin depletion allowed more extensive re-replication, suggesting that the destruction of pre-RC proteins in re-replicating cells protects them from even further re-replication.

5.3 Phosphorylation of pre-RC components by DNA damage-stimulated kinases

Cdt1 and Cdc6 destruction after DNA damage is largely independent of the DNA damage checkpoint, but other pre-RC members are specifically targeted by the ATM and ATR pathways. Mcm2 is phosphorylated in both *Xenopus* and human cancer cells (on serine 92

Fig. 5. DNA damage causes degradation of Cdc6 and Cdt1. Exogenous genotoxic damage and endogenous re-replication-associated damage induce the degradation of the licensing factors Cdc6 and Cdt1. Cdc6 is targeted by the ubiquitin ligase Huwe1. Cdt1 binds to PCNA, which is loaded at sites of DNA damage. This association allows the CRL4^{Cdt2} ubiquitin ligase to bind Cdt1 and target it for degradation.

and serine 108, respectively) in response to many DNA damaging agents (Yoo et al., 2004; Cortez et al., 2004). In *Xenopus* egg extracts, both ATM and ATR phosphorylate the Mcm2 subunit of the MCM complex, whereas in human cells ATR is the primary kinase involved (Yoo et al., 2004; Cortez et al., 2004). Mcm3 and Mcm4 are also phosphorylated in response to DNA damage. Mcm4 becomes phosphorylated after DNA damage in a caffeine-sensitive manner. Caffeine is a known inhibitor of the ATM and ATR kinases, so these kinases or their downstream targets are presumed to be responsible. Mcm3 is phosphorylated on serine 535 by ATM in response to ionizing radiation (Cortez et al., 2004). Additionally, Orc3 is phosphorylated in response to ionizing radiation (Matsuoka et al., 2007). The functional consequences of these phosphorylations are not yet known, but it is reasonable to predict that these modifications prevent pre-RC assembly.

6. Novel interfaces between pre-RC regulation and cell cycle control

6.1 Additional control of replication licensing

In addition to the core pre-RC members described thus far, other proteins have also been implicated in regulating DNA replication licensing. A protein related to MCM subunits, Mcm9, is not itself incorporated into the core helicase complex, but has been suggested to be essential for licensing in eukaryotes. Mcm9 contains an ATPase and a helicase domain, and may directly bind and influence Cdt1 function (Lutzmann & Mechali, 2008). Geminin and Mcm9 compete for binding to Cdt1; as such, Mcm9 could protect Cdt1 from geminin during origin licensing (Lutzmann & Mechali, 2009).

Another protein that plays a role in influencing DNA replication is the acetyltransferase, Hbo1. Hbo1 binds both Orc1 and Mcm2, and is thereby recruited to origins (Miotto & Struhl, 2008; Iizuka et al., 2006; Burke et al., 2001). Hbo1 depletion causes licensing defects in human cells and in *Xenopus* egg extracts by preventing the loading of MCM complexes but not the chromatin association of ORC, Cdc6, or Cdt1 (Iizuka et al., 2006; Miotto & Struhl, 2008). These defects can be reversed by over-expressing Cdt1, suggesting that Hbo1 is an activator of MCM loading at the Cdt1 step in pre-RC assembly. Hbo1 acetylates histone H4 which may reflect a role for Hbo1 in modulating chromatin at origins (Miotto & Struhl, 2010). Hbo1 also associates with the tumor suppressor, p53, and this association negatively regulates Hbo1 enzymatic activity. Cellular stress agents other than DNA damage, such as hyperosmotic stress, activate p53 (Vogelstein et al., 2000), and this activation was suggested to inhibit the HAT activity of Hbo1 and consequently, origin licensing (Iizuka et al., 2008). Hbo1 can also acetylate multiple pre-RC components *in vitro* however, so its role in pre-RC assembly may also be through direct modification of licensing proteins (Iizuka et al., 2006).

In addition to histone acetylation, monomethylation of histone H4 facilitates pre-RC formation. Tethering the H4 Lys20 methyltransferase, Set8 (PR-Set7), to an ectopic sequence induced pre-RC formation at that site, suggesting that H4K20 methylation may also promote pre-RC assembly at normal chromosomal origins (Tardat et al., 2010). Set8 is ubiquitinated by the CRL4^{Cdt2} ubiquitin ligase in PCNA-dependent manner during S phase and in response to DNA damage (Jorgensen et al., 2011; Wu & Rice, 2010; Oda et al., 2010). Failure to degrade Set8 in S phase leads to re-replication and lack of chromatin condensation during mitosis (Wu & Rice, 2010; Jorgensen et al., 2011). These data further suggest an involvement of chromatin structure in the regulation of origin licensing.

Each of the genes encoding pre-RC components is transcriptionally regulated by the Rb-E2F pathway. Given that tumor cells frequently exhibit high-level expression of E2F target genes, (Rb or p16 loss, cyclin overproduction, etc.) it is not surprising that Cdt1 and Cdc6 are overproduced in many cancers (Ohta et al., 2001; Karakaidos et al., 2004; Pinyol et al., 2006; Di Micco et al., 2006). Overproduction of Cdt1 or Cdc6 in cultured human cells induces re-replication, raising the possibility that tumor cells also re-replicate *in vivo*. Recently it has been suggested that cancer cells “hyper-replicate” and that this form of replication stress is a driving force in oncogenesis. It has also been suggested that excessive pre-RC assembly may even downregulate expression of the INK4/ARF tumor suppressor locus due to interference between a nearby origin and the INK4 promoter (Gonzalez et al., 2006). Recently, mutations in genes for several components of the pre-RC, including Orc1, Orc4, Orc6, Cdt1, and Cdc6 have been linked to the autosomal recessive primordial dwarfism syndrome, Meier Gorlin syndrome (Guernsey et al., 2011; Bicknell et al., 2011). This is the first report implicating impaired licensing in a developmental disorder. Taken together there are now clear links between pre-RC formation, normal human development, and tumorigenesis.

7. References

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DNA replication, the process of copying one double stranded DNA molecule to produce two identical copies, is at the heart of cell proliferation. This book highlights new insights into the replication process in eukaryotes, from the assembly of pre-replication complex and features of DNA replication origins, through polymerization mechanisms, to propagation of epigenetic states. It also covers cell cycle control of replication initiation and includes the latest on mechanisms of replication in prokaryotes. The association between genome replication and transcription is also addressed. We hope that readers will find this book interesting, helpful and inspiring.

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