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Eplication licensing and the DNA damage checkpoint

Jeanette Gowen Cook

Department of Biochemistry and Biophysics and Department of Pharmacology, Lineberger Comprehensive Cancer Center Campus Box 7260, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

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1. ABSTRACT

Accurate and timely duplication of chromosomal DNA requires that replication be coordinated with processes that ensure genome integrity. Significant advances in determining how the earliest steps in DNA replication are affected by DNA damage have highlighted some of the mechanisms to establish that coordination. Recent insights have expanded the relationship between the ATM and ATR-dependent checkpoint pathways and the proteins that bind and function at replication origins. These findings suggest that checkpoints and replication are more intimately associated than previously appreciated, even in the absence of exogenous DNA damage. This review summarizes some of these developments.

2. INTRODUCTION

Normal cell cycle progression requires that the steps of DNA replication and segregation occur in the proper space and time. Cell cycle checkpoints ensure that individual events, such as replication initiation or mitotic entry, only occur under circumstances where the intracellular and extracellular environments are compatible with successful cell division. Failure to properly signal information about these environments puts cells at risk of lethal damage or of generating daughter cells that carry harmful mutations or chromosomal abnormalities. For this reason it is not surprising that checkpoint defects lead to genome instability, which in turn, increases the likelihood of carcinogenesis.

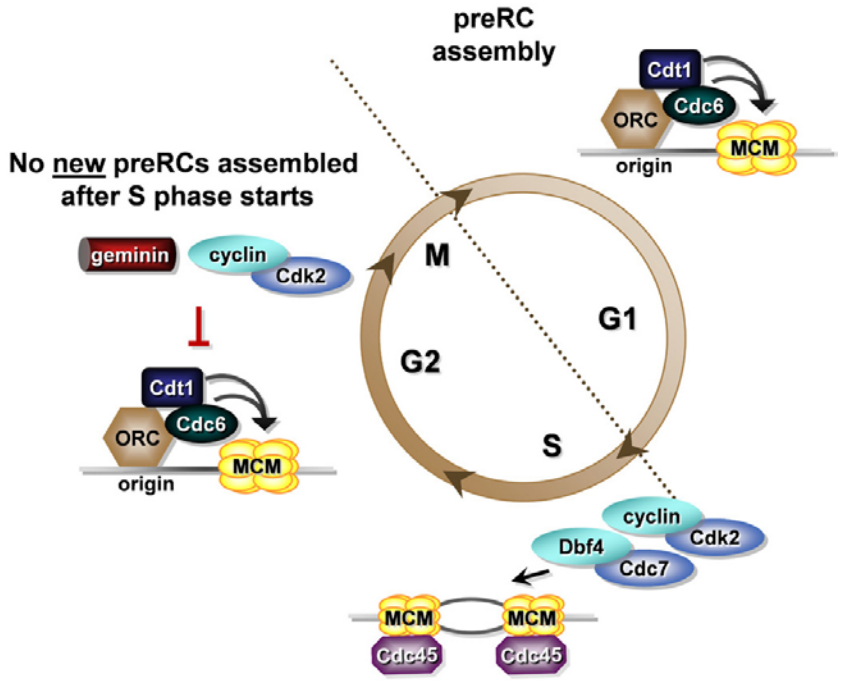

Figure 1. Replication licensing is restricted to G1. Pre-replication complexes (preRC) are assembled at origins during G1 by the loading of MCM complexes by ORC, Cdc6, and Cdt1. Origin firing requires loading of Cdc45 and GINS (not shown) which is induced by the action of Cdk2 and Cdc7 in S phase. After the G1/S transition Cdk2 (and Cdk1) plus geminin inhibition of Cdt1 block any new MCM chromatin loading in order to prevent rereplication.

The checkpoint system to detect and respond to DNA damage has traditionally been studied in cells treated with exogenous genotoxic insults. However, recent molecular genetic investigations of cell cycle checkpoint factors have shown that replication factors and checkpoint signaling molecules are much more intimately associated than previously appreciated. In particular, two signaling kinases, ATR and Chk1, are required for cell viability even in the absence of exogenous DNA damage, and these two kinases influence replication initiation and S phase progression in unperturbed cells. The earliest studies implicated ATR and Chk1 in general cell cycle progression through control of Cdk activation. However, additional findings suggest that ATR and Chk1 influence DNA replication at other steps as well. There are a number of excellent reviews on the individual subjects of DNA replication control (1-6) or the activation of the DNA damage checkpoint pathways (7-11). This review will focus primarily on the interaction of the ATR-Chk1 pathway with the regulation of the events that take place at replication origins.

3. DNA REPLICATION

3.1. Once and only once

Human cells face an enormous challenge as they initiate S phase. They must access and duplicate more than 3 billion base pairs of DNA within a matter of hours. In order to efficiently duplicate so much DNA, replication initiates at thousands of sites throughout the genome. These replication origins do not initiate ("fire") synchronously; instead they fire at various times throughout S phase. Asynchronous origin firing sets up a second challenge: how to prevent any origin from firing a second time within the same cell cycle. This task seems daunting since origins that fire early in S phase exist in an environment containing high levels of all of the necessary enzymes and substrates to support the assembly of a new replication fork. Cells must simultaneously block rereplication from thousands of origins that have already fired while permitting efficient firing of thousands of origins that have not yet fired. Successfully accomplishing that task allows each chromosome to be fully replicated exactly once per cell cycle.

In order to prevent rereplication, eukaryotic cells compartmentalize the steps of replication into different cell cycle phases. First the origin is prepared for replication by the assembly of a chromatin-bound multiprotein complex, the pre-replication complex or preRC. Once this complex has formed that origin is said to be "licensed" for replication, and licensing is only permitted during G1 (Figure 1). Secondly, in S phase DNA synthesis initiates from the licensed origins as a consequence of protein kinase activity from both Cdc7/Dbf4 and cyclin E/Cdk2 which are activated at the G1/S transition. Once S phase has begun, multiple overlapping mechanisms (discussed below) block the assembly of any new preRCs until after chromosomes have segregated.

Experimental manipulations that perturb preRC control can result in substantial rereplication and genome instability. The fact that normal cell have stable genomes

leads to the assumption that rereplication doesn't normally occur. Nevertheless, many thousands of potential sites for rereplication are present in each human cell, and it is not known if rereplication is perfectly blocked or if perhaps a few origins re-fire each cell cycle. If limited rereplication takes place even in normal cells, how do cells deal with the extra DNA? Are some cell types more prone to rereplication than others? Might such rereplication be a source of endogenous mutagenesis? The genome instability that is a hallmark of cancer cells could partially reflect failure to maintain once per cell cycle replication, although a rigorous examination of this aspect of genome stability in cancer cells has not yet been undertaken. A thorough understanding of the ways in which licensing is controlled under a variety of circumstances will shed light on such questions.

3.2. Origin licensing by preRC assembly

The preRC assembles in G1 in a step-wise fashion (reviewed in (5, 12, 13)). The first step is the binding of the Origin Recognition Complex (ORC) to the origin. ORC is a heterohexamer composed of subunits Orc1-Orc6, and the complex has DNA-dependent ATPase activity. ORC association with origins promotes the subsequent association of both the Cdc6 ATPase and the Cdt1 protein. Cdt1 and Cdc6 are then responsible, in cooperation with ORC, for the recruitment and loading of the Mini-chromosome Maintenance complex (MCM). The loading of MCMs at origins is what distinguishes licensed from unlicensed origins. The MCM complex consists of six polypeptides, Mcm2-Mcm7, and the MCM complex is the current best candidate for the DNA helicase at replication forks. Consistent with this model, MCM subunits, but not other preRC proteins, have been shown to travel with the replication fork (14). Multiple MCM complexes are loaded at each origin (15), and the enzymatic activity of both Cdc6 and ORC are required to fully load each origin with its complement of MCM (16). Assays of preRCs assembled *in vitro* indicate that once MCMs are loaded, they no longer require ORC, Cdc6, or Cdt1 to remain functionally associated with origins (17, 18). Ultimately however, the MCM complex is released from chromatin by an as yet undetermined mechanism as the cell completes S phase (19, 20).

All origins that will be licensed in a given cell cycle – regardless of whether they will fire in early or late S phase - receive MCM in G1. The MCM complexes that are loaded at origins are tightly associated with chromatin, but are inactive throughout G1. The purified MCM complex has weak, if any, DNA helicase activity, but DNA helicase activity is stimulated when MCM associates with the Cdc45 protein and the GINS heterotetrameric complex (consisting of Sld5, Psf1, Psf2, and Psf3) (21-25). Consistent with its function as an MCM activator, Cdc45 does not arrive at any individual origin until just before or during the actual initiation; thus Cdc45 is found at early-firing origins in early S phase and at late-firing origins in late S phase (14).

The loading of GINS and Cdc45 to activate MCM and allow the establishment of a productive replication fork requires the activity of the Cdc7/Dbf4 protein kinase and cyclin E/Cdk2 activity. Cdc7 phosphorylates MCM to promote Cdc45 binding, and Cdk2 activity stimulates the loading of the GINS complex, which in turn, is required for stable association between Cdc45 and MCM (22). While it is clear that phosphorylation by both Cdc7/Dbf4 and cyclin E/Cdk2 are required for the association of these replication fork proteins at origins, precisely how replication initiates is incompletely understood. Two recent studies of yeast replication initiation defined phosphorylation of the Sld2 and Sld3 proteins as the minimal Cdk-dependent phosphorylation events for S phase entry. These phosphorylations are required for recruitment of Cdc45 to origins (26, 27). Sld3 has no clear ortholog in metazoan genomes, so the search for an analogous factor to fill that role in human cells is an important goal.

3.3. Restricting origin licensing to G1

Multiple mechanisms act after the G1/S transition to block licensing in order to prevent rereplication. In theory, robust inhibition of just one component of the preRC would be sufficient to block preRC assembly since each component is absolutely essential for replication. However, no single control mechanism is perfect, and even limited rereplication is potentially harmful in terms of maintaining a stable genome. For those reasons, ensuring that little to no rereplication takes place requires the regulation of more than one preRC component. These regulatory strategies include regulated protein degradation, Cdk-dependent inhibitory phosphorylation, transcriptional control, and inhibition by direct binding. This multifaceted approach to preRC inhibition is a characteristic of all eukaryotic systems, but the details of the individual regulatory events vary across species, particularly when comparing the budding and fission yeasts to multicellular eukaryotes. Nevertheless, general strategies such as phosphorylation to induce protein degradation are broadly conserved even when the species-specific protein targets of that phosphorylation vary.

3.3.1. Transcriptional control

Each of the metazoan preRC subunits is the product of a gene under control of the E2F-Rb transcriptional program. The genes encoding preRC proteins are suppressed by Rb-E2F-mediated repression in quiescence and derepressed in late G1 as cells re-enter the cycle (28-31). At least some of these genes *(cdc6, mcm2,-4 and 6)* also display cell-cycle regulated fluctuations in mRNA abundance with peak expression occurring in late G1 followed by down-regulation in S phase and G2 (30). Presumably the phosphorylation of Rb (and the related p107 protein) in S phase and G2 contributes to the down-regulation of those genes. However, because the MCM and Orc2-6 proteins are relatively stable, they show minor if any cell cycle-dependent fluctuations even when their mRNA levels rise and fall. On the other hand, the Cdt1, Cdc6, and Orc1 proteins are unstable and degraded each cell cycle, so transcriptional control of these genes likely contributes to controlling licensing competence during each cell cycle.

Extensive analyses of a wide variety of human cancers have revealed near-universal deregulation of the E2F-Rb pathway. One consequence of loss of Rb-mediated repression is the overproduction of preRC components, often to quite high levels (28, 29, 31, 32). This overproduction has provided potentially useful markers of DNA replication competence in clinical samples (33, 34). Moreover, the deregulated expression of preRC components – sometimes by as much as 30-fold (31, 35) – could promote inappropriate licensing simply by overwhelming the normal regulatory controls. Hyperaccumulation of preRC proteins in transformed cells may induce rereplication and contribute to the genome instability characteristic of cancer cells. In support of that idea, even moderate overproduction of Cdt1 and Cdc6 in cell lines is sufficient to promote carcinogenesis when implanted in mice (36, 37). Moreover, using DNA fiber analysis, we have recently observed that the transformed HeLa cell line continually produces DNA replication tracks consistent with rereplication at a rate 3-times higher than an untransformed fibroblast cell line (38).

3.3.2. Cdk-dependent phosphorylation

A highly conserved feature of replication licensing control is the inhibition of licensing once S phase Cdks become active. Since these kinases trigger the firing of individual origins, coupling their activity to the cessation of origin licensing effectively creates a clear transition from the licensing step to replication initiation while simultaneously preventing origin relicensing. Inhibition of Cdk activity after the G1/S transition can induce inappropriate MCM reloading onto chromatin (39, 40) and subsequent rereplication (41-43), and Cdk suppression can further enhance rereplication associated with other perturbations in licensing control (44). Furthermore, high levels of Cdk2 during the licensing period block MCM loading onto *Xenopus* chromatin (45), and ectopic Cyclin A/Cdk2 expression in human cells delays MCM chromatin loading (46). Despite the clear role of Cdk-dependent phosphorylation in preventing rereplication, the precise mechanism to explain this role remains undetermined in many cases. Every component of the preRC (ORC, Cdc6, Cdt1, and MCM) is a target for Cdk-dependent phosphorylation, but in many cases the phosphorylation sites and the molecular consequences of those modifications are still largely unclear.

ORC associates with chromatin throughout the cell cycle, though there are indications that the affinity of *Xenopus* ORC for chromatin may weaken during mitosis as consequence of Cyclin A-dependent activity (47). Cdk-dependent phosphorylation of *Drosophila* (48) and human (49) ORC subunits may be responsible for similar observations. Yeast Orc2 and Orc6 are phosphorylated during S phase, and mutational alteration of the Cdk target sites induced substantial rereplication in a yeast strain background where MCM and Cdc6 are also altered to prevent Cdk dependent inhibition (50). The requirement for Orc2 and Orc6 mutational alteration indicates that those phosphorylation events block ORC function in some as yet unknown way. It is not known if human Orc2 and Orc6 are phosphorylated in a similar manner or if phosphorylation of human ORC subunits contributes to rereplication inhibition. The coincident suppression of preRC assembly with the rise in Cdk activity certainly creates the potential for a link between ORC phosphorylation and restricting ORC chromatin association or function.

Human Cdc6 is phosphorylated by Cdk2 in association with Cyclin E (51) or Cyclin A (52, 53). This phosphorylation has at least two consequences: First, Cdc6 is protected from ubiquitin-mediated degradation (see details below). Second, at least a fraction of the Cdc6 molecules are exported from the nucleus to the cytoplasm. In stark contrast, Cdk-mediated phosphorylation of yeast Cdc6 induces its ubiquitination and degradation. The Cdk binding and phosphorylation sites of both yeast and human Cdc6 are found in their respective N-terminal regions, but these domains are otherwise virtually unrelated which is consistent with the divergence in cell cycle regulation of human and yeast Cdc6. In human cells at least a portion of the Cdc6 protein remains nuclear and chromatin bound throughout S phase (54, 55), raising the question of just how important nuclear export is in restricting rereplication. On the other hand in the nematode *C. elegans*, phosphorylation-induced Cdc6 export appears to play a critical role in restricting rereplication (56). It may be that nuclear export of human Cdc6 could be equally important for preventing rereplication in some human cell types. The fact that nearly all human replication studies are conducted in cultured fibroblasts or cancer cell lines (mostly epithelial) means that our view of the relative contributions of different control mechanisms in different cell types is still unfortunately narrow.

Cdk-mediated phosphorylation of human Cdt1 induces association of Cdt1 with the Skp2 ubiquitin ligase complex. As a result, Cdt1 is ubiquitinated and degraded. Cdt1 preferentially associates with cyclin A –containing complexes as opposed to cyclin E or cyclin B complexes (57, 58), suggesting that its phosphorylation is primarily catalyzed by cyclin A/Cdk2 from mid-S phase through G2. The down-regulation of Cdt1 levels by Skp2-mediated poly-ubiquitination may not be the only consequence of Cdk-mediated phosphorylation however. Cdt1 has affinity for DNA (at least *in vitro* (59), and phosphorylation by Cdk2 diminishes this ability (57).

At least three subunits of the human MCM complex, Mcm2, Mcm3, and Mcm4, are phosphorylated during S phase and G2, and these phosphorylations have been attributed to Cdk activity (60, 61). Importantly, the hyperphosphorylated forms of these subunits are not associated with chromatin, implying that phosphorylation inhibits MCM chromatin association. In yeast, phosphorylation of at least one subunit of the MCM complex induces its translocation from the nucleus to the cytoplasm (62, 63), but this mechanism does not apply to cultured human cells (64, 65). It appears that the regulatory mechanism by cytoplasmic accumulation was "transferred" to human (and nematode) Cdc6 instead. It is not clear if phosphorylation of human MCMs blocks their ability to bind to other members of the preRC during assembly, though such a mechanism is attractive. Interestingly,

Mcm2 has intrinsic histone-binding affinity, and it may be that phosphorylation influences that interaction (66). Not all Cdk-mediated MCM phosphorylation is inhibitory however. Recent identification of a Cdk1-specific site on Mcm3, Ser 112, demonstrated a positive role for Cdk phosphorylation in assembly of the MCM heterohexameric complex in G2 and M phases. The fact that Cdk activity can be both a positive and a negative factor in different individual steps in the replication process creates significant challenges for interpreting the precise outcome from changes in Cdk activity with particular regard to DNA replication. Continued effort to map specific phosphorylation sites and determine the molecular consequences of those modifications is clearly needed to distinguish activating and inhibitory phosphorylation events.

3.3.3. Ubiquitin-mediated degradation

Active degradation plays a large role in preventing inappropriate preRC assembly. Human Cdt1 is degraded in S phase by the combined action of two ubiquitin ligases, Skp2 and Cul4 (67). (Yeast Cdt1 is not degraded but rather exported from the nucleus to the cytoplasm along with MCM during S phase (68).) Skp2 interaction with Cdt1 requires prior phosphorylation of Cdt1 by Cdk2 (69-71), and ectopic Cyclin A/Cdk2 activity in G1 is sufficient to prematurely downregulate Cdt1 and delay MCM loading (46). Cul4-mediated ubiquitination of Cdt1 requires that Cdt1 be associated with chromatin-bound Proliferating Cell Nuclear Antigen (PCNA) (72-74). Thus, activation of Cdk2 at the G1/S transition promotes Skp2-mediated ubiquitination of Cdt1 whereas loading of PCNA at active replication forks promotes Cul4-mediated ubiquitination of Cdt1 on chromatin. These two ubiquitination pathways operate independently of each other leading to significant destruction of Cdt1 in S phase cells. It is clear that destruction of Cdt1 at the onset of S phase contributes to preventing rereplication because mutational alterations that stabilize Cdt1 result in origin re-firing and accumulation of cells with greater than 4C DNA content (67, 74). Several studies have shown that the human Orc1 subunit can also be targeted for Skp2-mediated ubiquitination and degradation during S phase, and that this ubiquitination involves prior phosphorylation by cyclin A/Cdk2 (reviewed in (75)).

Like Cdt1, Cdc6 is degraded in each cell cycle, but unlike Cdt1, Cdc6 is degraded in late mitosis rather than during S phase. Cdc6 levels remain high and actually increase during S phase and G2. Cdc6 is then degraded after ubiquitination by the Cdh1-associated form of the Anaphase Promoting Complex/Cyclosome (APC/C), APC^{Cdh1}. Mailand and Diffley discovered that the recruitment of Cdc6 to APC/C by Cdh1 is blocked by Cdk-mediated phosphorylation of Cdc6. In late G1, the rise in cyclin E/Cdk2 activity promotes Cdc6 phosphorylation rendering it resistant to APC^{Cdh1} (51, 76). Cdc6 phosphorylation in late G1 then provides a window of opportunity for Cdc6 accumulation and MCM loading before S phase when much of the Cdt1 is destroyed. APC/C itself is inhibited during S phase and G2 by multiple mechanisms, one of which is phosphorylation by Cdks (77, 78). APC/C inhibition allows Cdc6 to accumulate significantly throughout S phase and G2, but without active Cdt1, MCM loading is blocked. Cdc6 remains chromatin-bound in S phase and G2 (54, 79), and has been implicated in some aspects of the G2/M checkpoint ((79-81) discussed below). The abundance of chromatin-bound Cdc6 in S phase likely explains why aberrant accumulation of Cdt1 so potently induces rereplication.

3.3.4. Geminin

Not all of the Cdt1 is degraded during S phase by Skp2 and Cul4-mediated ubiquitination. The remaining Cdt1 is associated with geminin, a protein unique to metazoan species that accumulates during S phase and G2. High levels of geminin prevent licensing during S phase by blocking the association of Cdt1 with Cdc6 and the MCM complex (59, 82). Surprisingly, lower levels of geminin are compatible with MCM loading in *X. laevis* egg extracts, though it is not clear if geminin actively promotes licensing or if it is recruited to facilitate subsequent preRC inhibition later in S phase (83). It is also not clear if geminin plays a similar positive role in human cells. Geminin is regulated through poly-ubiquitination by APC/C at the metaphase to anaphase transition (84). Degradation of geminin in anaphase releases Cdt1 to permit a new round of MCM loading beginning in telophase and throughout the subsequent G1 phase (85). Geminin is sensitive to both the Cdc20 and Cdh1-activated APC complexes, whereas Cdc6 is only sensitive to the Cdh1-activated form. Since the Cdc20 form appears before the Cdh1 form, an additional brief window opens in telophase between the destruction of geminin and the destruction of Cdc6 where MCM loading can occur (1).

Though geminin was originally named because it is expressed from two highly related "twin" genes in *X. laevis* (84), the name turned out to be particularly appropriate. Geminin has at least two distinct functions, one in preRC regulation and the other in transcription. Specifically, geminin antagonizes both the expression and function of Hox transcription factors (86, 87), and associates with Brg1 and Brm-containing SWI/SNF chromatin remodeling complexes to regulate differentiation (88). Presumably, when geminin is degraded each cell cycle, it becomes limiting for both replication inhibition and for transcriptional control. The interplay between these two roles of geminin in replication and transcription is still mysterious, but a common feature in both processes is chromatin and its modifications.

Loss of geminin by RNAi-mediated knockdown can induce significant rereplication by releasing Cdt1 from inhibition and permitting inappropriate licensing during S phase and G2. Similarly, Cdt1 overproduction, and to a lesser extent, Cdc6 overproduction, also stimulate rereplication (89). Recent advances in understanding the molecular details of MCM loading with purified yeast proteins suggest that the role of Cdt1 might best be described as an MCM "escort" from the nucleoplasm to the ORC-Cdc6 complex on chromatin. Once the MCM complex is delivered to ORC-Cdc6 for loading, Cdt1 is released, but ORC, Cdc6 and (of course) MCM remain behind (16). In that regard, a very small amount of free

Cdt1 may be sufficient to facilitate MCM loading at multiple origins. This pseudo-catalytic role of Cdt1 could explain why disruptions in Cdt1 regulation have by far the greatest effects on rereplication. The potency of free Cdt1 in promoting rereplication may also explain why the Cdt1-specific inhibitor, geminin, evolved in the rise of metazoan species.

4. DNA DAMAGE AND REPLICATION STRESS

Clearly replication or segregation of damaged DNA would be deleterious. DNA damage blocks cell cycle progression in order to create time for repair, or if the damage is too extensive, induce apoptosis. A wide variety of genotoxic agents elicit this checkpoint response including ultraviolet light (UV), ionizing radiation (IR), chemical crosslinkers, and reactive compounds that generate bulky adducts at bases. Checkpoint-induced cell cycle arrest is accomplished by inhibiting the activity of both Cdk2 and Cdk1 to simultaneously block new origin firing and prevent premature entry into mitosis. Decades of research have elucidated signaling pathways that are activated in response to DNA damage to both block cell cycle progression and induce DNA repair activities (for reviews see (7-11, 90)), a few key aspects as they relate to preRC regulation are briefly summarized below.

Two parallel signaling cascades are activated by DNA damage. Ionizing radiation and other treatments that induce double-strand breaks induce the ATM kinase to phosphorylate and activate the Chk2 kinase. ATM activation requires that the double-strand break be bound by the MRN complex (Mre11/Rad50/Nbs1) as part of the damage recognition process (91). UV irradiation and a wide variety of other DNA damaging agents activate the ATR kinase (in association with its partner, ATRIP) to phosphorylate and activate the Chk1 kinase. In addition, treatments that cause replication fork stalling, such as drugs that suppress nucleotide pools (e.g. hydroxyurea) or inhibit DNA polymerase (e.g. aphidicolin) also potently activate ATR and Chk1 although such treatments do not directly attack DNA. Emerging evidence indicates that the generation of single-stranded DNA bound by RPA is a common intermediate in these forms of damage and is a major contributor to ATR activation (92, 93). ATR and ATM are primarily activated by different types of DNA damage, but considerable crosstalk between the two pathways often results in both branches ultimately responding to a single type of exogenous damage (94).

ATM and ATR, as well as Chk1 and Chk2, phosphorylate and stabilize the p53 transcription factor by interfering with p53 polyubiquitination. p53 regulates a cohort of genes involved in DNA repair, apoptosis, and cell cycle progression as well as the Cdk inhibitor, p21, which functions to prevent entry into S phase after damage (95). Independently of p53, Chk1 and Chk2 phosphorylate the Cdc25 family of dual-specificity phosphatases to inhibit their activity and/or promote their degradation, depending on which of the Cdc25 isoforms is targeted. Cdc25 is responsible for removing inhibitory threonine and tyrosine phosphorylations from both Cdk2 and Cdk1. The combined results of these interactions are to block Cdk activity after DNA damage (Figure 2). In the absence of sufficient Cdk2 activity, GINS and Cdc45 are not recruited to any origins that remain unfired, thus blocking replication initiation. Furthermore, the inhibition of cyclin B/Cdk1 by DNA damage prevents entry into mitosis. In addition to inhibiting Cdk-dependent Cdc45 loading at origins, Cdk-independent inhibition of Cdc45 loading has also been suggested through inhibition of Cdc7/Dbf4 protein kinase activity (96-98).

High levels of damage not only block replication initiation, but can also cause Chk1-dependent inhibition of DNA chain elongation and premature termination of ongoing forks (99, 100). A potential target for Chk1 in elongation is the tousled-like kinase, Tlk1. Tlk1 phosphorylates and activates Asf1, an essential chromatin assembly factor (101). Tlk1 activity normally peaks in S phase, but is strongly inhibited by DNA damage in a Chk1-dependent manner, and Tlk1 can be phosphorylated by Chk1 *in vitro* (102, 103). DNA synthesis and chromatin assembly are tightly coordinated, though the intricacies of that coordination are not well understood. It may be that Tlk1 inhibition by Chk1 slows replication fork progression by blocking chromatin assembly at replication forks. Activation of ATR and Chk1 also protects stalled replication forks so that DNA synthesis can proceed once conditions return to normal. The mechanism by which ATR and Chk1 prevent replication fork collapse is incompletely understood, but could involve suppression of homologous recombination (9) and direct regulation of replication fork components (90).

5. THE ATR-CHK1 PATHWAY IN UNPERTURBED CELL CYCLES

While some signaling components, such as ATM and Chk2, are only needed in cells exposed to exogenous DNA damage (104), other components such as ATR and Chk1 are essential even in the absence of DNA damage. Mice nullizygous for either ATR or Chk1 die during very early embryonic development (105-107). Thus far, the role of the ATR/Chk1 pathway in unperturbed cell cycles appears to be highly related to its role in the DNA damage response, namely the regulation of Cdk activity and control of DNA replication. Since ATR-Chk1 pathway contributes to the regulation of Cdk2 and Cdk1 activity in normal cell cycles, and since Cdk activity regulates licensing, the ATR-Chk1 pathway may have a role in preRC control in the absence of damage.

In cultured cells, elimination of Chk1, ATR, or its essential partner ATRIP, leads to premature mitosis before replication has completed (108, 109). This uncoupling of mitotic entry with S phase progression is "mitotic catastrophe," and is a lethal event. The ATR-Chk1 pathway is required to block mitosis when replication forks are stalled by treatment with hydroxyurea or aphidicolin. Niida *et al.* employed a knockout/knock-in approach to directly demonstrate that mitosis is restrained by phosphorylation of Chk1 in unperturbed cells indicating that at least one of the essential functions of Chk1 is to

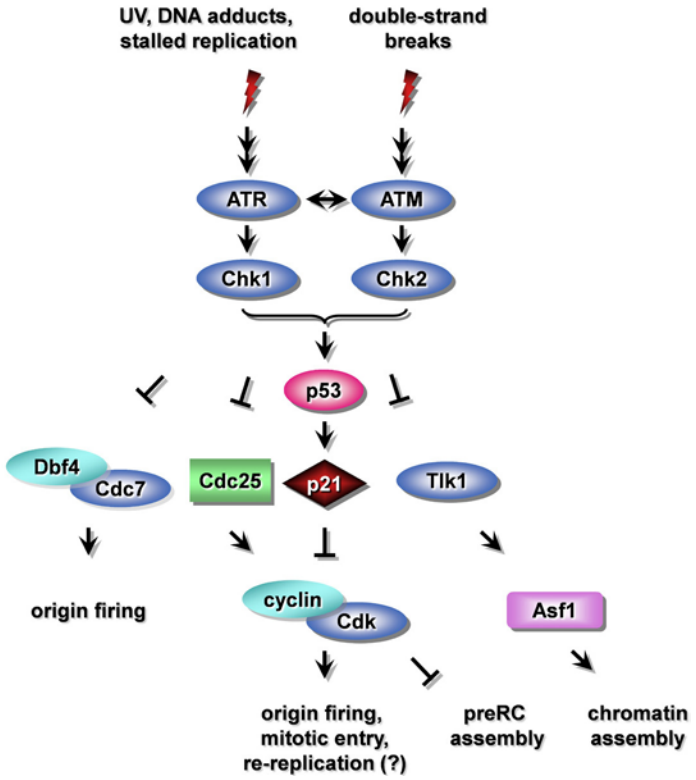

Figure 2. DNA damage induces intracellular signaling cascades that block DNA replication and cell cycle progression. Two parallel branches, the ATR-Chk1 and ATM-Chk2 pathways are activated by different forms of damage. Cdk activity is inhibited by degradation/inhibition of Cdc25 phosphatases and by the p53-dependent induction of the p21 Cdk inhibitor. Cdk-independent events also block origin firing through inhibition of Cdk and Cdc7 kinase activity. The Tlk1 kinase which promotes Asf1-dependent chromatin assembly is also inactivated in response to DNA damage.

control the timing of mitotic entry relative to the completion of replication (110). Chk1 also regulates Cdc25A and Cdc25B stability in unperturbed cell cycles as well as after DNA damage (111, 112) which may prevent premature activation of both Cdk2 and Cdk1. Moreover, the fact that ATR and Chk1 are activated by slow or stalled replication forks, suggests that these kinases could function in unperturbed cell cycles to stabilize forks at natural pause sites. Regions of altered chromatin, DNA secondary structure, transcription machinery, and matrix attachment sites are all likely to influence the ability of a replication fork to proceed. It may be that in the absence of the ATR-Chk1 pathway, forks collapse much more frequently during S phase leading to an inability to complete DNA replication and contributing to cell death.

Recent investigations have indicated that ATR and Chk1 can restrain origin firing in normal cell cycles as well as after DNA damage perhaps by controlling the ability of Cdc45 to load at origins. Inhibition of ATR-Chk1 activity or depletion of Chk1 protein results in a shorter distance between active origins, suggesting that origins that are normally dormant fire in the absence of Chk1 (113-116). The ability of these dormant origins to fire during S phase requires that they were licensed in the preceding G1, and they may be important under conditions where replication is slowed. Precisely how ATR-Chk1 is regulated to permit the firing of these dormant origins under appropriate conditions and how that regulation differs from the strong origin inhibition by ATR-Chk1 during a checkpoint response is still unclear.

Chk1-depleted cells have been reported to exhibit not only more origin firing but also slow fork progression (99, 100). If Chk1 is responsible for the regulation of Asf1 phosphorylation by Tlk1 to control chromatin assembly during a DNA damage response, then it may also play that role in normal S phases. The interpretation of these findings is complicated by the fact that Chk1 depletion also promotes replication stress and even frank DNA damage that may be the result of stalled and collapsed forks (114). It is not clear if the effects of Chk1 depletion on replication dynamics reflects a direct role of Chk1 or if these effects are the indirect consequence of fork collapse initiating a DNA damage response. The fact that the loss of Chk1 induces DNA damage suggests that Chk1 both responds to DNA damage and plays a role in preventing the damage in the first place.

Chk1 can be detected on chromatin, though the requirements for that chromatin association are not yet known. Strikingly Chk1 chromatin binding is inhibited when Chk1 becomes phosphorylated after DNA damage, raising the possibility that Chk1 release from chromatin

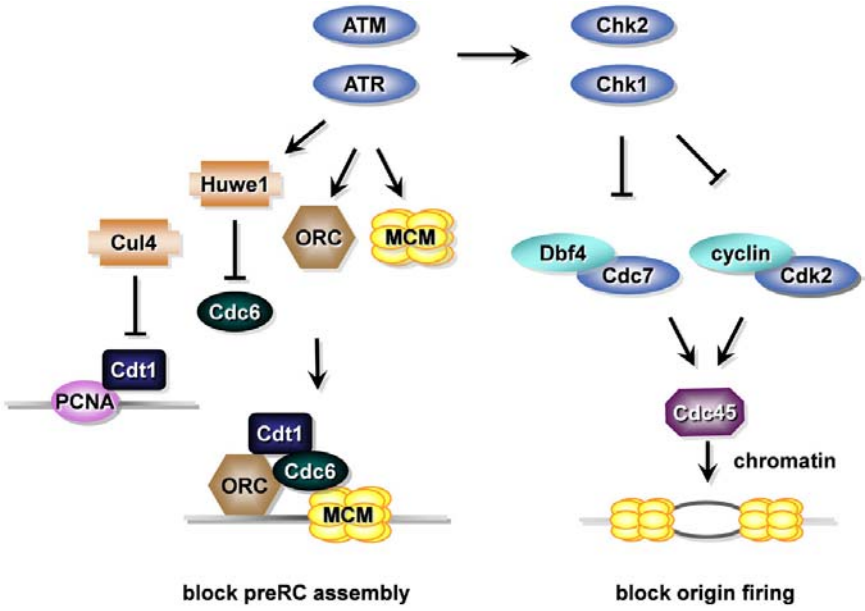

Figure 3. DNA damage regulates both origin licensing and origin firing. DNA damage induces the phosphorylation of MCM and ORC subunits, while stimulating the ubiquitination and degradation of Cdt1 and Cdc6. The DNA damage-activated checkpoint also blocks Cdc45 loading at origins to prevent replication initiation.

disseminates the checkpoint signal throughout the cell (110, 117). Could chromatin-binding sequester Chk1 to allow late origin firing and normal S phase progression? Moreover, in normal cell cycles Chk1 shifts its localization from the nucleus to the cytoplasm during late S/G2 (118). It is becoming increasingly clear that the localization of Chk1 is an important aspect of its role in regulating mitosis (119, 120); localization may be similarly important in the control of origin firing. If one of the normal functions of Chk1 is to suppress origin firing, and if that function requires nuclear Chk1, then relocalization of Chk1 to the cytoplasm could permit the firing of any dormant origins that still remain in late S phase to ensure completion of S phase.

6. MAINTAINING LICENSING CONTROL DURING CHECKPOINT ACTIVATION

The inhibition of Cdk activity during a DNA damage response eliminates an important mechanism to control replication licensing and prevent rereplication. Once Cdks are inhibited by the checkpoint, phosphorylation of ORC, MCM, and Cdt1 would all be suppressed promoting the inappropriate reloading of MCM complexes onto replicated chromatin. When the DNA damage is extensive, cells are likely to initiate apoptosis, so rereplication from these MCM complexes would likely be inconsequential in a cell that is destined to die. The real danger may come from sub-lethal DNA damage – after S phase has already begun - that is sufficient to activate the checkpoint and transiently inhibit Cdk activity. During the period of low Cdk activity, MCM complexes may be loaded at origins that have already fired. Once the damage is repaired and the checkpoint is quenched, Cdks are reactivated, and such relicensed origins may fire a second time.

Despite the fact that geminin levels are unchanged after DNA damage (35, 69, 121), early studies of Cdk inhibition suggested that the presence of geminin alone is not sufficient to inhibit rereplication (41, 42). Indeed, pharmacological inhibition of Cdk activity in nocodazole-arrested cells permits robust MCM re-association with chromatin in prometaphase (39). On the other hand, in a recent study by Hochegger *et al.*, Cdk inhibition during periods of peak geminin expression were insufficient to permit MCM chromatin loading, suggesting that (at least in the transformed avian cells tested) some cell cycle periods are less dependent on Cdk regulation for preRC inhibition than others (43). Another potentially sensitive time when Cdk activity may be particularly important is in early S phase when geminin levels are still rising, but perhaps insufficient to fully block MCM loading. Furthermore, the relative levels of geminin and Cdt1 vary quite widely between different tumor cell lines (122, 123), and may also vary between normal cells of different tissue origin, though the relative abundance of geminin in different normal cell types has not yet been explored. It seems likely that in cells that express lower levels of geminin, the contribution of Cdk activity in restricting licensing is greater and vice versa.

Given the potential danger of relicensing previously replicated origins during a checkpoint response, it would seem prudent to have additional means to inhibit rereplication that do not rely on Cdk activity or geminin. Recent investigations have shown that components of the preRC itself are targets for additional regulation in response to DNA damage (Figure 3). Most notable among these are

Cdt1 and Cdc6 which are ubiquitinated and degraded after damage.

6.1. Cdt1 ubiquitination and degradation

Cdt1 is eliminated from cells within minutes of a high dose of a DNA damaging agent such as UV or IR. DNA damage-induced Cdt1 ubiquitination relies on one of the same ubiquitin ligases that controls S phase degradation, Cul4 (121, 124) in association with DDB1 (DNA Damage Binding protein 1) and a substrate adaptor, Cdt2 (125-128). Skp2-dependent ubiquitination likely plays a minor role in DNA damage induced Cdt1 degradation, although its contribution may be more prominent in some cell lines or cell types (67, 69). Strikingly, in order for Cdt1 to be a target of Cul4/DDB1/Cdt2 it must associate with PCNA (72-74), and both PCNA and Cul4/DDB1 are chromatin-associated not only during S phase, but also during DNA repair (reviewed in (129, 130)). Furthermore, core proteasome components have been reported to localize to chromatin after DNA damage in yeast (131) and possibly in human cells as well (132). This convergence of factors prompted a model for the regulation of Cdt1 ubiquitination in which PCNA chromatin localization at DNA repair sites positions Cdt1 near an active DDB1/Cul4/Cdt2 ligase (129, 133, 134). In support of that model, evidence for an interaction between *X. laevis* Cdt1 and PCNA could only be detected on chromatin (74). On the other hand human Cdt1 can interact with soluble PCNA (67, 72). It will be important to sort out exactly where Cdt1 ubiquitination occurs in order to determine the requirements for that event, particularly in human cells.

Given that Cul4 is brought to Cdt1/PCNA by the direct association of Cdt1 with DDB1, which as its name implies can bind directly to damaged DNA, Cdt1 ubiquitination by Cul4 likely occurs independently of the activation of the damage checkpoint pathway. Chromatin localization may not be the only regulatory event that governs Cdt1 after DNA damage however. In at least one study, caffeine treatment which can override the DNA damage checkpoint or treatment with the Chk1 kinase inhibitor UCN01 blocked the UV induced - but not the IR-induced - degradation of Cdt1 (69). Another study confirmed that IR-induced degradation is independent of checkpoint kinases using both caffeine and RNAi-mediated knockdown of checkpoint kinases, but did not test UV-induced degradation (121). It is thus possible that Chk1 contributes to the Cdt1-PCNA interaction on chromatin or to the activation of Cul4/DDB1/Cdt2 after UV irradiation, though such regulation has not yet been reported. As mentioned earlier, geminin levels do not change in response to DNA damage. What is the fate of the Cdt1-geminin complex in this context? Is geminin brought with Cdt1 to chromatin, but not ubiquitinated? Does the interaction of Cdt1 with PCNA or Cdt1 ubiquitination release geminin into the nucleoplasm to inhibit additional Cdt1 molecules? In other words, is the actual target of Cul4/DDB1/Cdt2 free Cdt1 or geminin-bound Cdt1 (or both)? The answers to such questions are relevant to our complete understanding of how Cdt1 regulation is achieved in order to prevent rereplication.

6.2. Cdc6 ubiquitination and degradation

Like Cdt1, Cdc6 is ubiquitinated and degraded after all forms of DNA damage. Also like Cdt1, a ubiquitin E3 ligase that controls the cell cycle-dependent degradation of Cdc6 plays a role in damage-induced ubiquitination, though not exclusively. As outlined above, Cdc6 is ubiquitinated during anaphase by APC^{Cdh1}. Importantly the interaction of Cdc6 with Cdh1 is inhibited by Cdk-dependent phosphorylation of Cdc6. This phosphorylation-induced stabilization of Cdc6 allows Cdc6 to accumulate during G1 despite the presence of active APC^{Cdh1}. A mechanism to stimulate Cdc6 ubiquitination by APC^{Cdh1} after DNA damage which relies on the dephosphorylation of Cdc6 after ionizing radiation and the subsequent acquisition of APC^{Cdh1} sensitivity was explored by Duursma and Agami (76). In that study Cdc6 degradation after ionizing radiation required the induction of the p21 Cdk inhibitor by p53 in order to promote Cdc6 dephosphorylation. On the other hand, Blanchard *et al.* found that DNA damage induced p53-independent ubiquitination of Cdc6 (135). It thus appears that Cdc6 can be degraded by both APC/C-dependent and APC/C-independent pathways.

A clue to how Cdc6 might be targeted for degradation by a Cdk and APC-independent mechanism came from the discovery of an interaction between Cdc6 and the E3 ligase, Huwe1. Huwe1 is a member of the HECT family of E3 ligases that includes E6-AP. Huwe1 has been implicated in the ubiquitination of a number of other factors involved in both cell growth and cell death control, including p53, c-myc, core histones, and Mcl-1. (These investigations have each given the E3 ligase a different name: ARF-BP1, HectH9, Lasu1, and Mule (136-141).) Huwe1 ubiquitinates Cdc6 *in vitro*, and when Huwe1 expression is suppressed, Cdc6 is significantly stabilized after DNA damage (35). Important questions about the regulation of the Huwe1-Cdc6 interaction remain to be addressed. Is Huwe1 activity regulated by the DNA damage checkpoint? Huwe1 protein levels are constant throughout the cell cycle and do not change upon DNA damage. Moreover, DNA damage prevents Huwe1 from ubiquitinating p53, but promotes ubiquitination of Mcl-1, suggesting that the absolute enzymatic activity of Huwe1 is not the target of regulation. Nevertheless, a recent proteome-scale analysis of protein phosphorylation in response to DNA damage identified both Huwe1 and two subunits of ORC as likely substrates of ATM and/or ATR by virtue of their induced phosphorylation on the ATM and ATR consensus sequence S/TQ (142). Phosphorylation of Huwe1 may affect its protein-protein interactions in ways that alter substrate specificity, localization, or interaction with potential activators or inhibitors, but the functional consequences of that phosphorylation have yet to be explored.

Another possibility is that the Huwe1 substrates themselves are the primary targets of regulation. In support of that model, we found that Cdc6 is released from chromatin after DNA damage (35). Since Huwe1 is a soluble nucleoprotein, movement of Cdc6 from the chromatin to the nucleoplasm could promote ubiquitination

by Huwe1. Precisely how Cdc6 chromatin binding is inhibited by DNA damage is not yet clear. Cdc6 association with chromatin requires interaction with ORC, so an attractive explanation is that phosphorylation of ORC by ATM or ATR inhibits Cdc6 binding and blocks further preRC assembly. Furthermore, the fact that ORC is phosphorylated by a checkpoint kinase raises the possibility that the kinase could be brought to origins by an ORC-ATM/ATR interaction in order to carry out that phosphorylation. The recruitment of a checkpoint kinase to origins may promote the phosphorylation of additional preRC components, though that idea has not yet been addressed.

6.3. MCM phosphorylation

Another potential mechanism to block rereplication after DNA damage involves the phosphorylation of multiple MCM subunits by the ATM and ATR kinases. Cortez *et al.* detected Mcm3 in a biochemical screen for proteins phosphorylated on the ATM/ATR consensus sequence S/TQ (143). These investigators went on to demonstrate that Mcm2 is also an ATR substrate and furthermore, that Mcm7 associates with the ATR binding partner ATRIP. Similarly, *X. laevis* ATM and ATR phosphorylate Mcm2 in egg extracts (144), and Mcm4 is heavily phosphorylated after UV irradiation or replication inhibition (145). Though phosphorylation of both Mcm2 and Mcm3 after UV and IR-induced damage was robust, the physiological consequences of these events regarding preRC assembly remains to be determined. Unlike Cdt1 and Cdc6, DNA damage did not induce the degradation of any of the MCM subunits, nor did it detectably alter their chromatin association shortly after damage. It may be that phosphorylation of MCM proteins inhibits the DNA helicase activity of the complex rather than inhibiting its loading onto chromatin (145). Alternatively, phosphorylation of the MCM complex by ATR may contribute to maintaining MCM at a stalled fork to prevent replication fork collapse. It is not yet known if ATR targets only the MCM complexes that are actively participating at replication forks, or if origin-bound MCM or soluble MCM are also substrates. A recent report by Trenz *et al.* suggested that the binding of a polo-like kinase, Plx1, to *Xenopus* chromatin is required for full replication in the presence of low amounts of replication inhibitors such as aphidicolin. The precise stimulatory effects of Plx1 binding on replication activity are not yet known, but its induced association with chromatin required the ATR-dependent phosphorylation of Mcm2 (146).

7. MUTUAL REGULATION OF ORIGIN LICENSING AND THE DNA DAMAGE RESPONSE.

PreRC components are not only regulated in response to DNA damage, but they can also induce a DNA damage response. In that regard, preRCs are both "downstream" targets of the DNA damage checkpoint and "upstream" inducers of the checkpoint. This mechanism of checkpoint activation is largely indirect, but reports of interactions among replication factors and checkpoint proteins suggest that preRC components could also have direct effects on the activity of checkpoint activities.

7.1. Rereplication causes DNA damage

Rereplicated DNA ultimately causes DNA damage, including, but not necessarily limited to, double-strand breaks. Manipulations that permit MCM loading in S phase or G2 induce phosphorylation of Chk1, Chk2, and p53 (147-150). Chromosome fragmentation as a consequence of rereplication has also been directly detected in *S. cerevisiae* (151) and in *X. laevis* rereplication assays (148). How exactly does re-firing of an origin cause DNA damage when normal replication does not? Not all origins are equally sensitive to rereplication (89, 147, 151, 152), so limited MCM re-loading at only a subset of origins means that the additional replication forks are likely to be widely spaced from one another. Under such circumstances forks will not always meet their neighbors to converge, and the outcome is eventual fork collapse leading to chromosome fragmentation. It is also possible that the presence of multiple forks on the same chromosomal segment places unusual torsional strain on the DNA itself leading to fragmentation. Davidson *et al.* proposed a straightforward model to explain the appearance of short DNA fragments under conditions of massive rereplication. They suggested that the second forks eventually catch up with the first forks, perhaps as a result of natural pause sites that slow the first fork. MCM helicase activity from the second pair of forks could release a double-stranded DNA fragment containing the re-fired origin (148).

Is rereplication the only way that preRC components interact with the DNA damage checkpoint pathway? Perhaps not. In a surprising study, Tatsumi *et al.* observed molecular markers of ATR and ATM pathway activation when Cdt1 was overproduced in quiescent cells. These contact-inhibited and serum-deprived cells stained positive for phosphorylated ATM without detectable DNA synthesis. The mechanism to account for this observation has not yet been determined, but the authors suggested that the DNA binding ability of Cdt1 may directly perturb chromatin when Cdt1 is overproduced. Another possibility could be through physical association between Cdt1 and checkpoint factors, either directly or via MCM complexes which are known to interact with both Cdt1(59, 82) and with the ATM and ATR kinases (143).

Exciting possibilities also exist for other direct interactions between preRC components and checkpoint signaling proteins. An early report that overproduction of Cdc6 in G2 cells caused a Chk1-dependent block to mitosis, could indicate a direct role for Cdc6 in Chk1 activation, but might also be explained if Cdc6 induced rereplication which in turn, activated Chk1 (80). More direct evidence for a functional interaction between Cdc6 and Chk1 comes from the work of Oehlmann *et al.* in which *X. laevis* Cdc6 was shown to be required for Chk1 activation by DNA damage independently of its role in MCM loading (79). After S phase entry, Cdc6-depleted extracts showed a defect in Chk1 activation even though MCM loading had already been accomplished in the preceding G1. Moreover, ORC-depleted (Cdc6-containing) extracts were competent for Chk1 activation despite the fact that ORC is required for Cdc6 chromatin binding (79). In human cells, depletion of Cdc6 after the G1/S transition

using siRNA induced a similar defect in Chk1 activation as well as an S phase progression defect (81). These studies fall just short of demonstrating a direct association between Cdc6 and Chk1, but they do suggest that Cdc6 has checkpoint functions that cannot be easily explained solely through its role in MCM loading in G1. Perhaps this novel function is the reason why Cdc6 is so abundant in G2 cells instead of being degraded in S phase like Cdt1.

7.2. The DNA damage response restricts rereplication

Once rereplication has induced sufficient DNA damage to trigger activation of the ATM and ATR pathways, further rereplication should be suppressed by the checkpoint-dependent inhibition of new origin firing. In the same way that exogenous DNA damage blocks origin firing, rereplication-induced damage activates p53 and suppresses Cdk activity (147, 149). In support of this feedback inhibition of rereplication, Cdt1 overproduction induces rereplication much more readily in cells deficient for ATR or p53 than in cells with normal ATR and p53 (89, 153, 154). These assays for rereplication all rely on relatively insensitive methods to detect rereplicated DNA – either by flow cytometry or density gradient centrifugation of total cellular DNA. A significant portion of the genome must be rereplicated in order to be detected. For this reason it is not yet clear, if the role of the ATR pathway in restricting rereplication primarily impacts origin licensing, origin firing, fork elongation, fork stabilization, or delaying mitosis since all of those events are required to produce enough rereplicated genomic DNA to be detected.

As outlined above (sections 6.1 and 6.2), DNA damage induces the ubiquitination and degradation of both Cdt1 and Cdc6. If rereplication induces DNA damage sufficient to activate the ATR and ATM checkpoint pathways, then might that DNA damage also induce Cdt1 and Cdc6 degradation? The first hint that this mechanism may be operating in rereplicating cells came from observations that geminin-depleted human or *Drosophila* cells also have very low levels of Cdt1 (39, 150). We have extended these observations to demonstrate that Cdc6 is also degraded when rereplication is induced (155). Interestingly, the rereplicated genomic DNA that is observed after geminin depletion could only accumulate in the short time period between geminin depletion and the subsequent degradation of Cdt1 and Cdc6. Stabilization of either Cdt1 or Cdc6 by disruption of the ubiquitin ligases that target them during a DNA damage response, Cul4 and Huwe1 respectively, allowed for even more rereplication than could be observed from geminin depletion alone. We argue that rereplication once it begins is then limited by the combination of origin licensing inhibition (destruction of Cdc6 and Cdt1) and checkpoint activation (Cdk inhibition, MCM and ORC phosphorylation, etc.) that blocks origin firing and limits fork progression. Interestingly, Chk1 depletion or inhibition can be sufficient to induce DNA damage from replication fork collapse (114). It is not yet known however if this DNA damage is also sufficient to induce Cdc6 and Cdt1 degradation.

The rereplication induced by geminin depletion or by Cdt1 or Cdc6 overproduction is typically irregular and uneven. Individual cells have widely varying amounts of over-replicated DNA as though only a subset of origins have re-fired. Indeed when the over-replicated DNA was purified and hybridized to human metaphase chromosomes, large regions of the genome were not detectably rereplicated at all. Similarly, induction of rereplication in budding yeast results in uneven origin firing with some origins apparently firing more than one extra time and others not at all (50, 151, 152). What determines the difference between origins that re-replicate and origins that don't? Differential chromatin modification is one attractive explanation, but it may not fully account for the observed differences. A satisfactory model would need to accommodate mechanisms that still permit MCM loading at rereplication-resistant origins in a normal G1. The overall role of chromatin in preRC assembly and replication initiation is another important question that remains to be answered. The relative crudeness of the available rereplication assays can be attributed in part to our lack of knowledge about the location and characteristics of human origins themselves. Progress in mapping and analyzing human origins will allow better rereplication assays based on detection of specific sequences either by PCR or by single fiber probing.

8. CONCLUDING REMARKS

For many years, the pathways that halt cell cycle progression in response to exogenous DNA damage were studied as distinct branches of cell cycle control. Signaling molecules in these pathways have often been described as waiting in an inactive state under normal growth conditions, "watching" for attack on the DNA or for replication forks to stall. Upon signal activation, a cascade of events would then be put in place to prevent new replication initiation events and to block mitosis while simultaneously activating appropriate repair measures. Once such repairs were completed, the signaling molecules presumably returned to their inert states until the next emergency response. In this model, checkpoint factors do not influence or interact with the replication process in the absence of DNA damage.

The view that is currently emerging suggests that the checkpoint proteins are constantly regulating mitotic entry and replication activity in order to prevent cell death and DNA damage. Furthermore, replication itself regulates checkpoint activity at multiple levels. This more integrated relationship between the checkpoint and the processes it monitors provides opportunities for mutual regulation and information sharing between the systems. Some of those connections may be indirectly carried by DNA itself in the form of damage, and some may involve direct protein-protein interactions such as among ORC, MCM and ATM /ATR. Future developments will undoubtedly shed light on how these interactions coordinately ensure genome integrity.

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Abbreviations: ORC: Origin Recognition Complex; MCM: minichromosome maintenance; Cdk: cyclin-dependent kinase; preRC: pre-replication complex; APC/C: anaphase promoting complex

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Send correspondence to: Jeanette G. Cook, Department of Biochemistry & Biophysics, Campus Box 7260, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, Tel: 919-843-3867, Fax: 919-966-2852, E-mail: jean_cook@med.unc.edu