Molecular Cell, Vol. 11, 997–1008, April, 2003, Copyright ©2003 by Cell Press

A p53-Dependent Checkpoint Pathway Prevents Rereplication

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Summary

Eukaryotic cells control the initiation of DNA replication so that origins that have fired once in S phase do not fire a second time within the same cell cycle. Failure to exert this control leads to genetic instability. Here we investigate how rereplication is prevented in normal mammalian cells and how these mechanisms might be overcome during tumor progression. Overexpression of the replication initiation factors Cdt1 and Cdc6 along with cyclin A-cdk2 promotes rereplication in human cancer cells with inactive p53 but not in cells with functional p53. A subset of origins distributed throughout the genome refire within 2–4 hr of the first cycle of replication. Induction of rereplication activates p53 through the ATM/ATR/Chk2 DNA damage checkpoint pathways. p53 inhibits rereplication through the induction of the cdk2 inhibitor p21. Therefore, a p53-dependent checkpoint pathway is activated to suppress rereplication and promote genetic stability.

Introduction

Considerable progress has been made in the last few years in understanding how replication is initiated in eukaryotic cells (Bell and Dutta, 2002; Diffley, 2001; Dutta and Bell, 1997; Kelly and Brown, 2000; Labib and Diffley, 2001; Waga and Stillman, 1998). The current paradigm is that a complex of proteins called the origin recognition complex (ORC) bound to origins of replication facilitates the recruitment of two additional proteins, Cdc6 and Cdt1, to the chromatin (reviewed in Lei and Tye, 2001; Takisawa et al., 2000). The latter two proteins, in turn, help recruit the putative replicative helicase Mcm2-7 complex to the chromatin. The formation of this prereplicative complex (pre-RC) at the origins licenses the chromatin for replication initiation once cyclin-dependent kinases become active at the onset of S phase.

Besides initiating DNA replication at the G1-S transition, it is also important that eukaryotic cells control the initiation machinery so that origins that have fired once in S phase do not fire a second time within the same cell cycle (Diffley, 2001; Dutta and Bell, 1997; Kelly and Brown, 2000). Failure to exert this control could have deleterious consequences. In mammalian cancer cells in particular, failure to prevent rereplication at even a handful of origins might initiate amplification of segments of the genome. Thus, it is of particular interest to determine how rereplication is prevented in normal mammalian cells and how these mechanisms might be overcome during tumor progression. In the yeasts, the assembly of the pre-RC is critically dependent on low cyclin-dependent kinase (cdk) activity, a condition that holds in the cell only from the end of mitosis to the beginning of S phase. The activation of cdk at the G1-S transition results in phosphorylation and inactivation of several components of the pre-RC (Dahmann et al., 1995; Diffley, 2001; Nguyen et al., 2001). Conditions for assembling new pre-RCs are reestablished only at the end of mitosis when the cdks are inactivated through the degradation of cyclins. Through this mechanism, the same protein kinase that triggers replication initiation simultaneously inactivates the pre-RC components to prevent reinitiation of replication on already replicated DNA. Mutations in the *Saccharomyces cerevisiae* genes for ORC, Cdc6, and the Mcm2-7 complex which render these proteins resistant to inactivation by cdks allow new pre-RCs to form in late S and G2 and permit rereplication of the genome (Nguyen et al., 2001). Inactivation of the high cyclin-cdk activity in G2 of *Schizosaccharomyces pombe* and *S. cerevisiae* also results in rereplication of the genome (Correa-Bordes and Nurse, 1995; Jallepalli and Kelly, 1996; Noton and Diffley, 2000). Similar experiments confirm the importance of high cyclin B-cdc2 activity in G2 for preventing rereplication in mammalian cells (Bates et al., 1998; Itzhaki et al., 1997).

Besides the elevated cdk activity seen in the latter half of the cell cycle, higher eukaryotes have evolved a second mechanism that could prevent rereplication. A protein called geminin appears at the G1-S transition and increases in abundance through the latter half of the cell cycle to be ultimately degraded in mitosis (McGarry and Kirschner, 1998). In *Xenopus* egg extracts, addition of geminin prevents the loading of the Mcm2-7 complex on the chromatin by interacting with the Cdt1 protein (McGarry and Kirschner, 1998; Tada et al., 2001; Wohlschlegel et al., 2000). Thus, limitation of Cdt1 activity through the action of geminin could be a second mechanism by which rereplication is prevented in metazoans during late S and G2. Indeed decreasing the amount of geminin in *Drosophila* tissue culture cells by small interfering RNAs (siRNA) led to overreplication of the genome (Mihaylov et al., 2002).

Rereplication of segments of the genome in the same cell cycle could lead to significant genomic instability.

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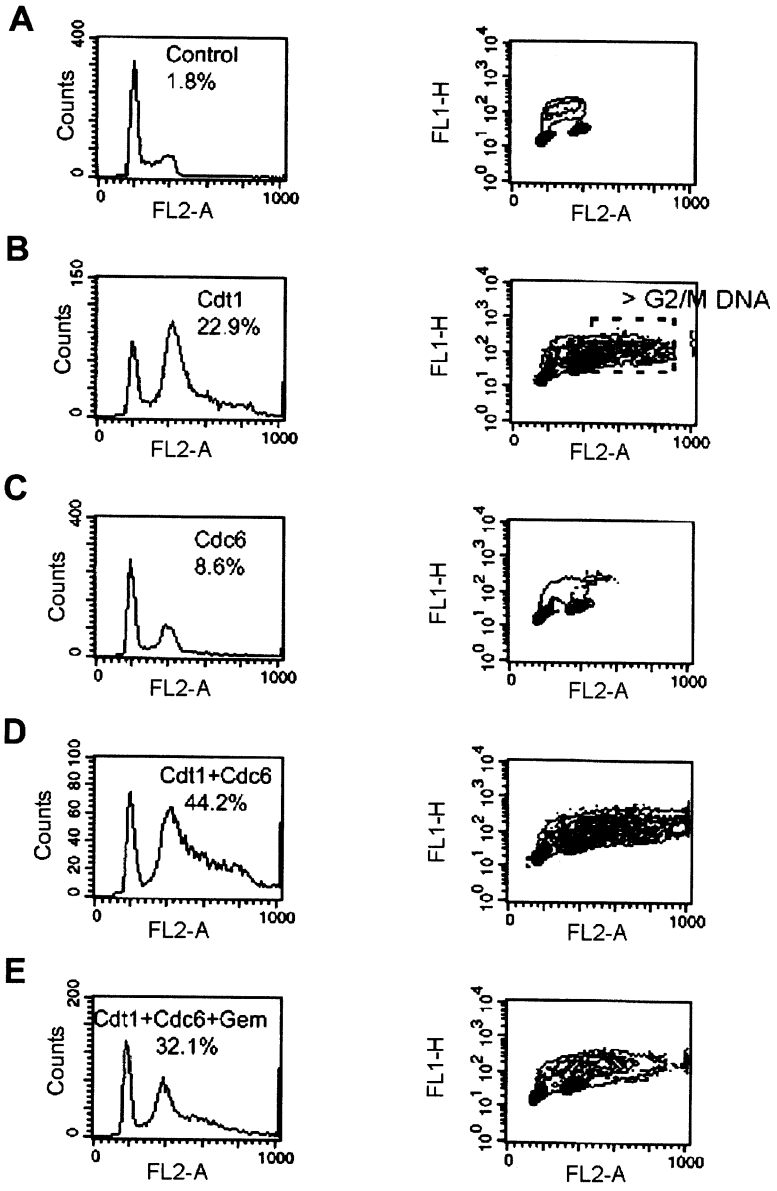


Figure 1. Overexpression of Cdt1 and Cdc6 Shows the Appearance of Cells with Greater than 4n DNA Content

(A–E) FACS analyses of H1299 cells infected with adenoviruses expressing indicated proteins. Left panels: histograms of cells stained with propidium iodide for DNA content. *y* axis, cell count; *x* axis, propidium iodide fluorescence. Right panels: dot plot of same cells labeled with BrdU and stained with anti-BrdU antibody and propidium iodide. *y* axis, intensity of staining with anti-BrdU antibody; *x* axis, propidium iodide fluorescence. The dotted box in (B), right panel, indicates the cells included to calculate the percentage of cells with >G2/M DNA content. This percentage is indicated inside each left panel and is a measure of the number of cells demonstrating rereplication. Control in (A), adenovirus expressing GFP. Geminin is referred to as "Gem" in (E).

Genomic instability of mammalian cancer cells is manifested in chromosomal translocations, microsatellite instability, gene amplifications, and aneuploidy (Lengauer et al., 1998). Defects in mismatch repair and in mitotic checkpoint control have been shown to be responsible for some of this instability. The tumor suppressor protein p53 is a major protector against genomic instability and is frequently inactivated in cancer cells (Prives and Hall, 1999; Vogelstein et al., 2000). It is not known, however, whether p53 plays any role in preventing rereplication.

In this paper we demonstrate that overexpression of Cdt1, with or without its partner Cdc6, promoted rereplication, indicating the importance of the geminin-Cdt1 balance for preventing rereplication. In situ hybridization identified segments of the genome particularly susceptible to rereplication, and by extension, to genomic instability through this pathway. Cyclin A stimulated rereplication, suggesting that active S phase cdk2 was a promoter of rereplication rather than a deterrent to the process. Finally our results reveal a third mechanism by which eukaryotic cells avoid replicating their chromosomes more than once in a cell cycle: rereplication results in DNA damage, which causes DNA damage checkpoint pathways to activate the tumor suppressor protein p53, ultimately resulting in suppression of rereplication.

Results

Overexpression of Cdt1 and Cdc6 Promotes Rereplication

Since Cdt1 is the primary target of geminin for preventing reinitiation, we speculated that overexpression of Cdt1 might bypass the rereplication control imposed by geminin. Cdt1 was overexpressed in H1299 lung cancer cells by infection with a recombinant adenovirus.

Forty-eight hours after infection, the cells were labeled with BrdU to selectively label cells in active DNA synthesis. Flow cytometry of propidium iodide stained cells revealed a significant fraction of the cells with DNA content greater than 4n, indicating some rereplication without mitosis (Figure 1B). The cells with greater than 4n DNA incorporated BrdU during the hour-long pulse before harvest, indicating that most of them were alive and in active S phase (Figure 1B). Since Cdt1 cooperates with Cdc6 to promote replication initiation, we overexpressed Cdc6 in these cells. Cdc6 alone induced very subtle rereplication, but it cooperated with Cdt1 to produce robust rereplication (Figures 1C and 1D).

Extracts of H1299 cells infected with adenoviruses expressing green fluorescent protein (GFP; control), Cdt1, or Cdt1+Cdc6 were examined for the levels of expression of Cdt1 and Cdc6. Cdt1 was indeed overexpressed relative to control cells (Figure 2A), and cooverexpression of Cdc6 did not increase rereplication by indirectly increasing the level of Cdt1.

Geminin Inhibits Rereplication

To test whether geminin inhibits rereplication induced by Cdt1, we overexpressed geminin along with Cdt1 and Cdc6 (Figure 1E). Overexpression of geminin partially inhibited the rereplication mediated by Cdt1+Cdc6. Overexpression of Cdt1 (by itself) led to a paradoxical increase in geminin levels in the rereplicating cells (Figure 2A). In order to confirm that there was free Cdt1 (uncomplexed with geminin) in the cell lines, we precleared all the geminin from these cell extracts before immunoblotting for residual Cdt1 in the supernatant (Figure 2B). The results show that despite the induction of geminin, not enough of the protein is produced to associate with and inhibit all the overexpressed Cdt1. The increase in geminin was attributed to a 10-fold induction of geminin mRNA (Figure 2C) seen upon overexpression of Cdt1. The mechanism of this induction is currently unclear but suggests the existence of a feedback loop between Cdt1 and its antagonist geminin.

Rereplication Is Stimulated by Cyclin A

Based on observations in yeast, the progressive increase in cyclin A-cdk2 kinase activity seen during S phase could be expected to protect the cells from rereplication. The S phase cdk2 activity is, however, also necessary for the firing of origins, most likely due to the phosphorylation of initiation factors like Sld2 (Masumoto et al., 2002). The level of the S phase cyclin, cyclin A, and associated kinase activity were increased in H1299 cells undergoing rereplication (Figure 2A), suggesting a positive role of the kinase in rereplication. Cdk1 kinase activity was not increased (data not shown), suggesting that the elevated cyclin A-associated kinase was due to cyclin A-cdk2. To test whether cyclin A-cdk2 kinase was a positive factor for rereplication, the H1299 cells were coinfected with viruses expressing cyclin A alone or in combination with Cdt1 and/or Cdc6 (Figure 2D). Cyclin A alone promoted some rereplication and stimulated rereplication seen with Cdt1 alone, Cdc6 alone, or Cdt1+Cdc6. This result suggests that the S phase cdk2 kinase activity promotes rereplication in much the same way it is required for initiation of replication. Cdt1 levels were not stimulated by the coexpression of cyclin A (data not shown), ruling out an indirect mechanism by which cyclin A could stimulate rereplication. It is also of interest that overexpression of the G1 cyclin, cyclin E, did not promote rereplication (Figure 2D), a result consistent with data in *Xenopus* egg extracts where excess cyclin E-cdk2 inhibited replication initiation (Findeisen et al., 1999; Hua et al., 1997).

Cdc6 is a bona fide substrate of cyclin A-cdk2 (Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998; Takeda et al., 2001). Given the stimulation of rereplication by cyclin A-cdk2, we examined whether phosphorylation of Cdc6 by cyclin A-cdk2 is essential for rereplication. Cdc6A4 carries nonphosphorylatable alanines in place of the phosphoacceptor serines at the cdk2 phosphorylation sites (Delmolino et al., 2001). Cdc6CyΔ has a mutation in a cyclin binding motif that is an essential part of the substrate recognition signal for cdks (Delmolino et al., 2001). Both these forms of Cdc6 cooperated with Cdt1 to promote rereplication, indicating that phosphorylation of Cdc6 by cdk2 is not essential for rereplication (data not shown). The likely targets of cyclin A-cdk2 are Sld2 and related proteins that have to be phosphorylated to stimulate origin firing at the onset of S phase.

Rereplication Occurs without the Cells Passing through an Abortive Mitosis

The positive role of cyclin A-cdk2 in promoting rereplication could be because the kinase drives the cells into an abortive mitosis, followed by degradation of the cyclin before chromosome segregation. The cells entering the next cell cycle without chromosome segregation would then accumulate greater than 4n DNA content in the next S phase. To rule this out, we overexpressed Cdt1 and Cdc6 in cells arrested in S phase with aphidicolin, an inhibitor of DNA polymerase α. Following the removal of aphidicolin, there was a progressive accumulation over 24 hr of cells with greater that 4n DNA content (Figure 2E). During this period, however, there was (1) no entry of cells into the early stages of mitosis as marked by phospho-H3 staining (Figure 2E) and (2) no point when the cyclin A protein dropped in amount (Figure 2F). These experiments argue against the possibility that the rereplication is a manifestation of the cells passing into the next cell cycle through an abortive mitosis.

Rereplication Is Seen within 2–4 hr of the First Cycle of Replication

Further support that the greater than 4n DNA content was the result of relicensing of origins in the same cell cycle came from measurement of the time to rereplicate segments of the genome. To determine how quickly origins refire, H1299 cells overexpressing Cdt1 and Cdc6 were labeled with BrdU for 2, 4, and 8 hr prior to harvest. Density gradient centrifugation of the cellular DNA enabled us to separate heavy-heavy (rereplicated) DNA from heavy-light (singly replicated) DNA (Figure 3A). Heavy-heavy DNA was detected in cells infected with adenoviruses expressing Cdc6 and Cdt1, but not control viruses expressing GFP, confirming the occurrence of rereplication in the former. Although the heavy-heavy peak was not distinct with 2 hr of BrdU labeling,

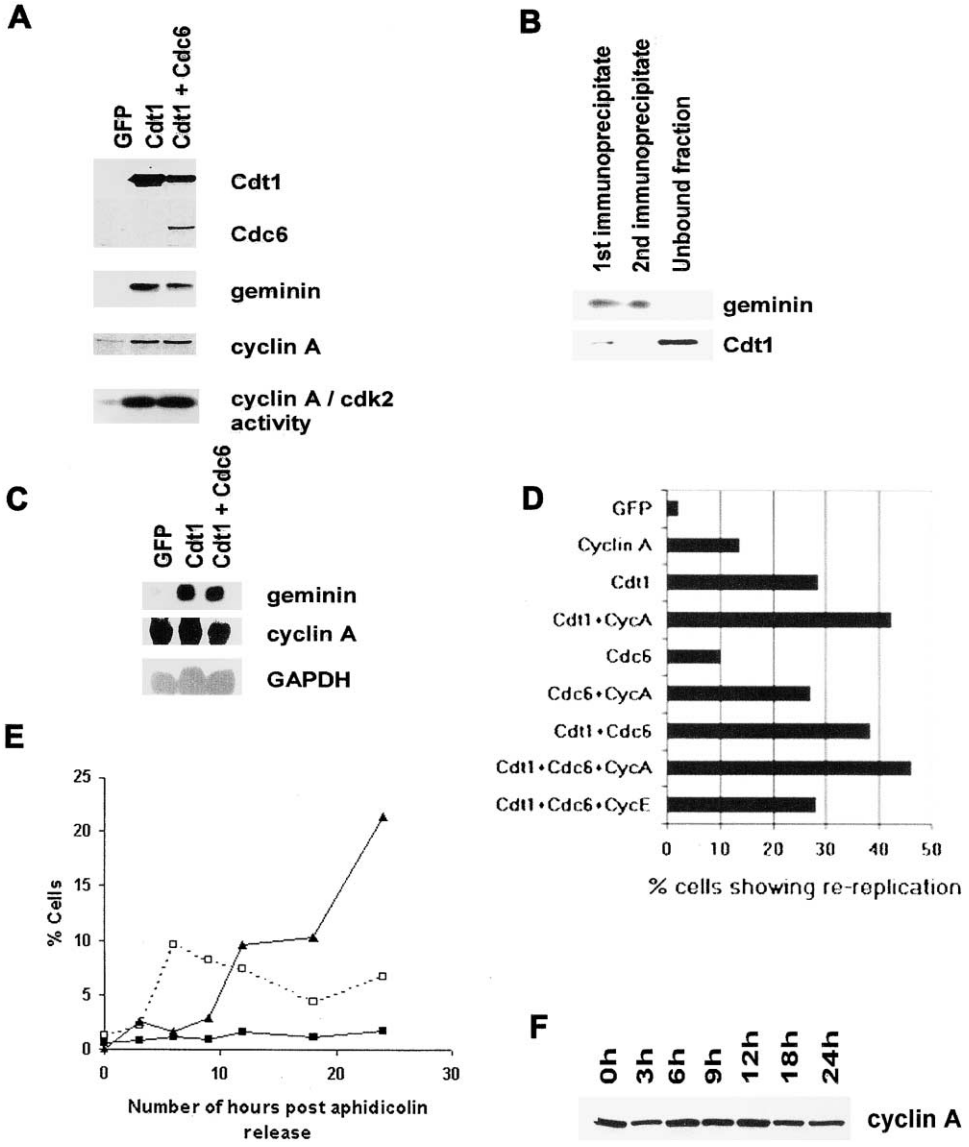


Figure 2. Analysis of H1299 Cells Infected with Various Adenoviruses

(A) Cells infected with viruses overexpressing proteins indicated at the top. Top four panels: immunoblots for indicated proteins. Bottom panel: histone H1 phosphorylation by an anti-cyclin A immunoprecipitate. Identical results are obtained by an anti-cdk2 immunoprecipitate. The level of exposure of the immunoblots is such that endogenous Cdt1, Cdc6, and geminin proteins cannot be detected.

(B) Presence of geminin-free Cdt1 in H1299 cells overexpressing Cdt1 and Cdc6. Cell lysate was immunoprecipitated twice with anti-geminin antibody. The two precipitates and the supernatant from the final precipitation were immunoblotted for geminin and Cdt1. Two rounds of immunoprecipitation clear all the geminin from the extract but still leave geminin-free Cdt1 in the supernatant.

(C) Geminin mRNA is induced upon Cdt1 overexpression. Northern blot of cells overexpressing proteins indicated on top.

(D) Cyclin A stimulates rereplication. Percent of cells showing rereplication upon overexpression of indicated proteins. Rereplication was measured as in Figure 1.

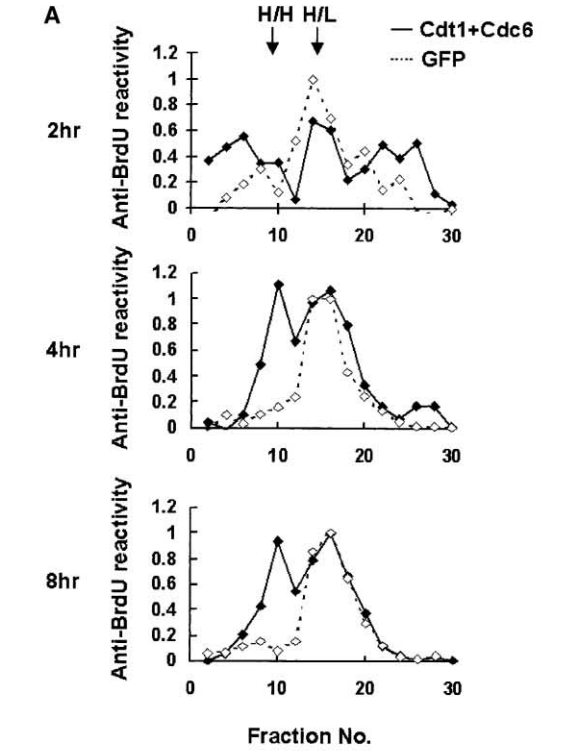
(E) H1299 cells expressing Cdt1 and Cdc6 enter rereplication without entering mitosis. Cells infected with the adenoviruses for 24 hr in the presence of aphidicolin were released from the drug at 0 hr. The cells were analyzed by FACS at indicated time points to determine the percentage of cells with DNA content >4n (triangles, Cdt1+Cdc6-overexpressing cells) and the percentage of cells positive for phospho-H3 (closed squares, Cdt1+Cdc6-overexpressing cells; open squares, GFP-overexpressing cells).

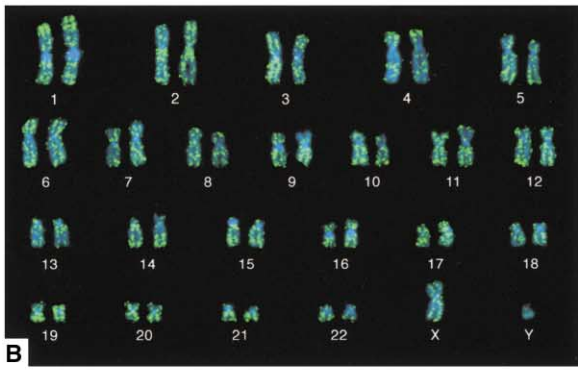
(F) Cyclin A is not abruptly degraded in the Cdt1+Cdc6-overexpressing H1299 cells as they begin rereplication; lysates from the experiment in (E) were immunoblotted for cyclin A.

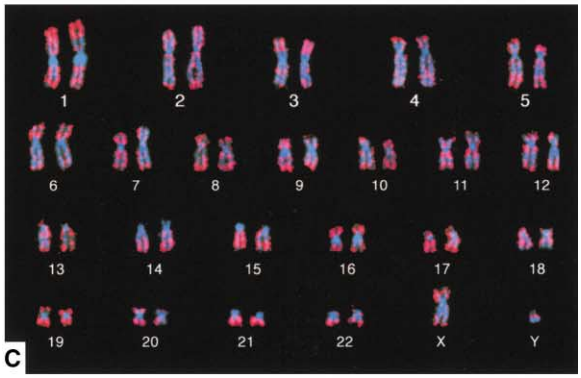
it was very distinct with a 4 hr label, indicating that origins refire in 2–4 hr, a time interval insufficient for these cells to pass through G2, M, and G1. Therefore, the origin refiring is due to replication relicensing in the same cell cycle.

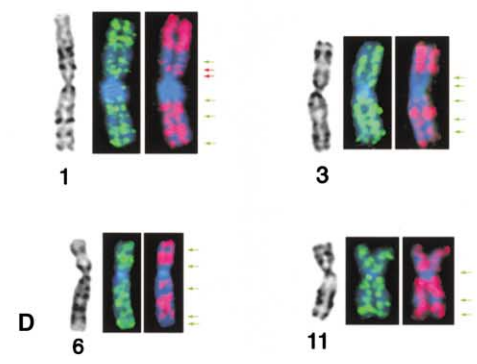
Cytogenetic Localization of Rereplicating DNA Segments

An important question is whether all origins in the genome are equally susceptible to rereplication upon overexpression of Cdt1 and Cdc6. To answer this question,









heavy-heavy DNA obtained from rereplicating cells was used as a probe for fluorescence in situ hybridization (FISH) on metaphase chromosome spreads (Figure 3B). Discrete chromosomal segments are hybridized to the heavy-heavy DNA, in contrast to the "chromosomal paint" seen when total genomic DNA from a specific chromosome is hybridized under similar conditions (data not shown). Therefore, although the rereplicating origins are widespread, all origins are not uniformly susceptible to this type of deregulation.

Different origins of replication are known to fire at discrete times in S phase. Gene-rich segments (which are GC rich and Giemsa light) replicate in the early part of S phase (Fetni et al., 1996). It was of interest whether the rereplicating origins were confined to those that replicate in the early part of S phase or were distributed throughout the genome. HeLa cells synchronized at the G1-S transition were released for 2 hr and labeled simultaneously with BrdU. Heavy-light DNA prepared from these cells represents DNA fragments that replicate in the first quarter of S phase. When this DNA was hybridized to metaphase spreads, the replication was mostly confined to the Giemsa-light bands as published before (Figures 3C and 3D). Comparison of the pattern of hybridization of heavy-heavy DNA with that of heavy-light DNA obtained in the first quarter of S phase (Figure 3D) suggests that the rereplicating origins are enriched in origins that fire early in S phase.

p53+ Cells Are Resistant to Rereplication Induced by Cdt1 and Cdc6

Four primary untransformed cell lines, IMR90, WI38, HDF, and Rat1R12 diploid fibroblasts, were relatively resistant to rereplication induced by Cdt1 and Cdc6 overexpression (Table 1). A major difference between the H1299 cancer cells and the primary cells is that the p53 gene is mutated in the former. Consistent with a protective role of p53 in preventing rereplication, the overexpression of Cdt1 and Cdc6 resulted in significant

Figure 3. Time and Location of Rereplication

(A) Appearance of heavy-heavy rereplicated DNA in H1299 cells overexpressing Cdt1+Cdc6 (solid line) but not in control cells expressing GFP (dashed line). Cells were infected with the indicated adenoviruses for 48 hr and labeled with BrdU for 2, 4, and 8 hr before harvest. The cellular DNA was fractionated as described in Experimental Procedures. ELISA with anti-BrdU antibody was used to measure BrdU-labeled DNA present in individual fractions. *y* axis, anti-BrdU reactivity normalized to the highest BrdU reactivity in the fractions from GFP-expressing control cells; *x* axis, fraction numbers. The positions of the heavy-heavy and heavy-light DNA are indicated. Light-light DNA peaks at fraction 22.

(B–D) Fluorescent in situ hybridization on metaphase spreads to localize sites of rereplicated DNA and DNA replicated early in S phase. The chromosome number is indicated below each chromosome pair. (B) Green: heavy-heavy DNA obtained from an experiment similar to that in (A) was used as probe. (C) Red: HeLa cells synchronized at the G1-S boundary were released from the block for 2 hr in the presence of BrdU. Heavy-light DNA purified by CsCl density gradient centrifugation represents DNA labeled in the first quarter of S phase and used as a probe. (D) Comparison of Giemsa staining (left), FISH with rereplicated DNA (green), and FISH with early replicating DNA (red). The arrows indicate segments that are uniquely labeled with the one or the other probe.

Table 1. Extent of Rereplication Seen in a Panel of Primary and Malignant Cell Lines Infected with Adenoviruses Expressing GFP (Negative Control) or Cdt1+Cdc6

Cell Line	GFP (%)	Cdt1+Cdc6 (%)	p53	Rb
Primary				
IMR90	1.6	5.7	+	+
WI38	2.1	2.4	+	+
HDF	2.0	3.3	+	+
Rat1	1.0	0.8	+	+
Malignant				
H1299 (lung)	2.4	40.7	−	+
H157 (lung)	6.9	29.0	−	+
A549 (lung)	1.1	2.6	+	+

The percentage of cells with greater than G2/M DNA content was determined by two-color FACS for BrdU labeling and DNA content as described in Figure 1. The p53 and Rb statuses of the cells are indicated: wild-type (+) and mutant (−). The source of malignant cell lines is indicated.

rereplication in H1299 lung cancer cells (p53−), H157 lung cancer cells (p53−), but not in A549 lung cancer cells (p53+) (Table 1).

To test whether p53 was truly a deterrent to rereplication in A549 cells, MDM2, an oncogene that is the E3 ubiquitin ligase for p53, was coexpressed in these cells along with Cdt1+Cdc6 (Figure 4A). MDM2 by itself did not induce rereplication, but promoted rereplication in A549 cells overexpressing Cdt1+Cdc6. Therefore, a functional p53 appears to prevent rereplication.

Consistent with a role of p53 in preventing rereplication, the p53 protein level is increased in A549 cells overexpressing Cdt1+Cdc6 (Figure 4B). This increase in protein is not accompanied by an increase in p53 mRNA level (data not shown). MDM2 prevented the induction of p53.

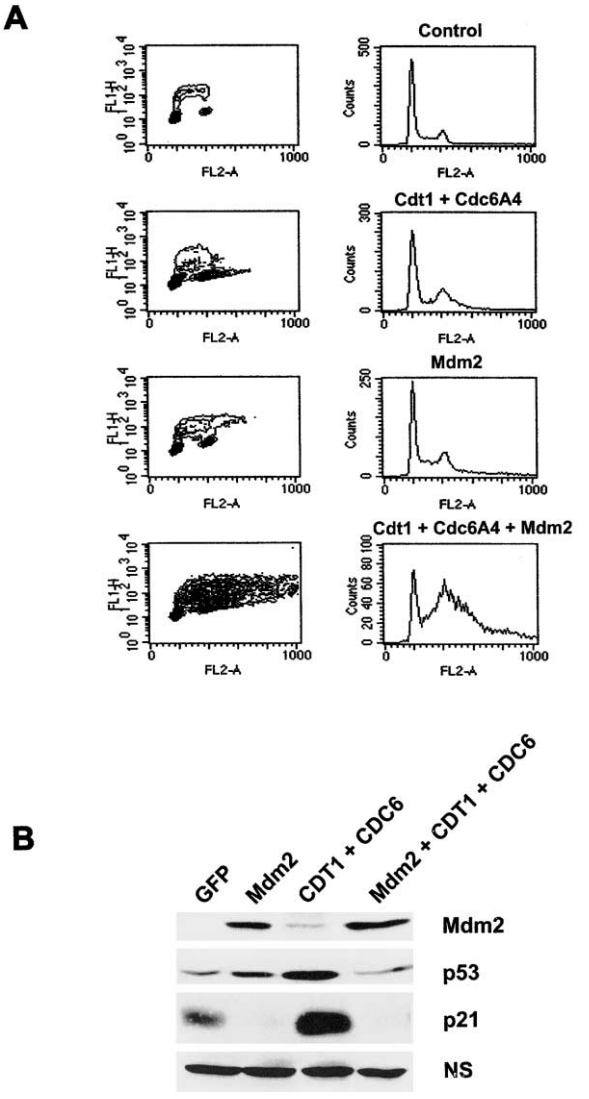


Figure 4. Mdm2 Inactivates p53 in A549 Cells and Promotes Rereplication

(A) Mdm2 overexpression in A549 cells makes the p53+ cell rereplicate its DNA upon Cdt1+Cdc6 overexpression. Dot plots of cells labeled with BrdU and stained with anti-BrdU antibody and propidium iodide (left column) and histograms of cells stained with propidium iodide for DNA content (right column) as described for Figure 1 are shown.

(B) Mdm2 neutralizes the p53 stabilization and p21 induction in A549 cells. Immunoblot of A549 cells overexpressing the proteins indicated at the top, with antibodies to proteins indicated at the side. NS, a nonspecific band in the p21 immunoblot to demonstrate equal loading. Cdc6A4 used in this figure is a mutant form of Cdc6 that is not phosphorylated by cdk2 and gives the same result as wild-type Cdc6 (see text).

DNA Damage Checkpoint Kinases Are Involved in Activation of p53 in Rereplicating Cells

We next examined the upstream pathways by which p53 could be activated by rereplication. Stabilization of p53 protein is often seen following activation of DNA damage-induced checkpoint pathways. We therefore tested whether the aberrant forks involved in rereplication utilize the DNA damage-induced checkpoint pathways to stabilize p53.

The ATM/ATR kinases stabilize p53 by phosphorylating it on serine 15 while the Chk2 kinase stabilizes p53 by phosphorylation on serine 20 (Canman et al., 1998; Hirao et al., 2000). Antibodies to the relevant phosphoserines revealed phosphorylation of p53 on both serine 15 and 20 upon overexpression of Cdt1 and Cdc6 (Figure 5A). Since the amount of p53 protein is also increased concurrently, our results suggest, but do not prove, that overexpression of Cdt1 and Cdc6 induces the phosphorylation of p53. Caffeine, an inhibitor of the ATM/ATR kinases, prevents the phosphorylation of p53 on S15 following γ radiation-induced DNA breaks (Figure 5B). Consistent with a role of the ATM/ATR kinases in sensing rereplication, treatment of the Cdt1+Cdc6-overexpressing cells with caffeine prevented the stabilization of p53 (Figure 5B). Active ATM kinase phosphorylates the Chk2 kinase on threonine 68 and activates its kinase activity. Consistent with both the activation of ATM/ATR kinase and phosphorylation of p53 on serine 20, Chk2 kinase was phosphorylated on threonine 68 in cells overexpressing Cdt1+Cdc6 (Figure 5C).

To directly test whether the DNA was damaged in the rereplicating cells, we measured the levels of phospho-

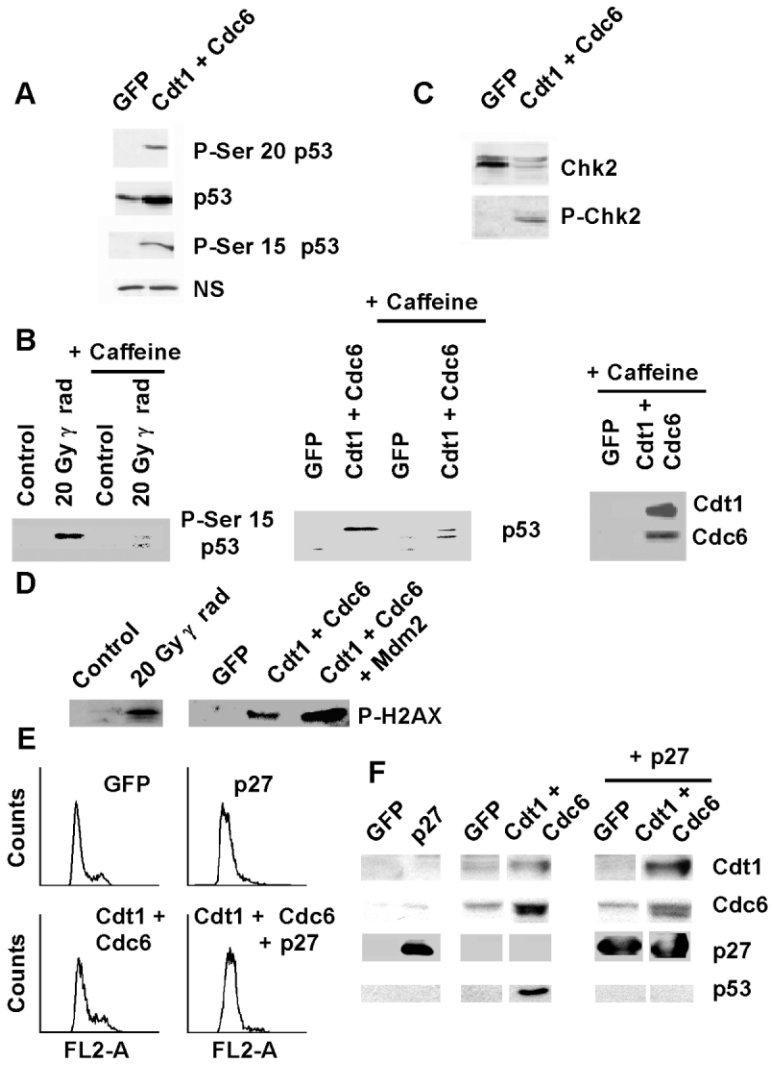


Figure 5. The DNA Damage Checkpoint Pathway Is Activated in Cells Undergoing Rereplication

(A) Immunoblot for total p53, phosphoserine 20-p53, and phosphoserine 15-p53 of extracts from A549 cells overexpressing GFP or Cdt1+Cdc6. NS, a nonspecific band to demonstrate equal loading.

(B) Effect of caffeine. Immunoblots for antigens are indicated at the side. Left panel: caffeine blocks the accumulation of phosphoserine 15-p53 in cells treated with γ radiation. Middle panel: p53 is stabilized by Cdt1+Cdc6 in untreated A549 cells but not those treated with caffeine. Right panel: Cdt1 and Cdc6 proteins are still expressed in the presence of caffeine.

(C) Activation of Chk2 by overexpression of Cdt1 and Cdc6. A549 cell extracts blotted for indicated antigens. P-Chk2, antibody to phosphothreonine 68 of Chk2.

(D) Immunoblot for phosphorylated histone H2AX in cells radiated with 20 grays of γ rays (left panel) or overexpressing indicated proteins (right panel).

(E) Overexpression of p27 blocks A549 cells in G1.

(F) Immunoblots for indicated antigens in cells overexpressing proteins indicated at the top. The p53 stabilization seen in cells overexpressing Cdc6+Cdt1 is suppressed by coexpression of p27.

H2AX (Rogakou et al., 1998). As reported, γ radiation of A549 cells led to an increase in the phospho-H2AX (Figure 5D). A similar increase was detected in cells overexpressing Cdt1+Cdc6, suggesting the presence of damaged DNA in these cells. Phospho-H2AX levels were increased when rereplication was permitted in these cells through inactivation of p53 by MDM2. Therefore, the ATM/ATR-Chk2 checkpoint pathway was most likely activated by DNA damage resulting from the rereplication.

We next tested whether the forks involved in rereplication are essential for DNA damage and activation of the checkpoint pathway. p27, an inhibitor of cdk2, was overexpressed in A549 cells for 48 hr so that the cells were blocked in G1 (Figure 5E). Cdt1 and Cdc6 were then overexpressed in these cells. Because of continued p27 expression, the cells remained arrested in G1 phase, and p53 was not stabilized despite the overexpression of Cdt1+Cdc6 (Figure 5F). This result suggests that DNA damage and activation of the checkpoint response requires actual rereplication and is not simply due to overexpression of replication initiation factors.

Proteins Downstream from Active p53 Protect Cells from Rereplication

Consistent with the activation of p53 in p53+ cells, the p53 responsive gene product, p21, was induced by Cdt1+Cdc6 in the p53+ IMR90 and A549 cells but not in the p53− cells (Figure 6A). The induction of p21 was at the mRNA level and was suppressed by coexpression of MDM2 (Figure 6B), consistent with a role of p53 in the induction. The p53 stabilization was accompanied by the induction of another p53 target, the proapoptotic gene PIG3 (Figure 6B).

To test the protective effect of wild-type p53 in suppressing rereplication, an adenovirus expressing wild-type p53 was added to those expressing Cdt1 and Cdc6 in H1299 cells (Figure 6C). Since cells expressing p53 undergo apoptosis by 48 hr, the cells were harvested 24 hr after infection. Expression of wild-type p53 suppressed rereplication in the H1299 cells in support of the hypothesis that activation of p53 prevents origin refiring in response to Cdt1 and Cdc6 overexpression. Overexpression of p21, one of the targets of p53, also suppressed rereplication induced by Cdt1+Cdc6 (Fig-

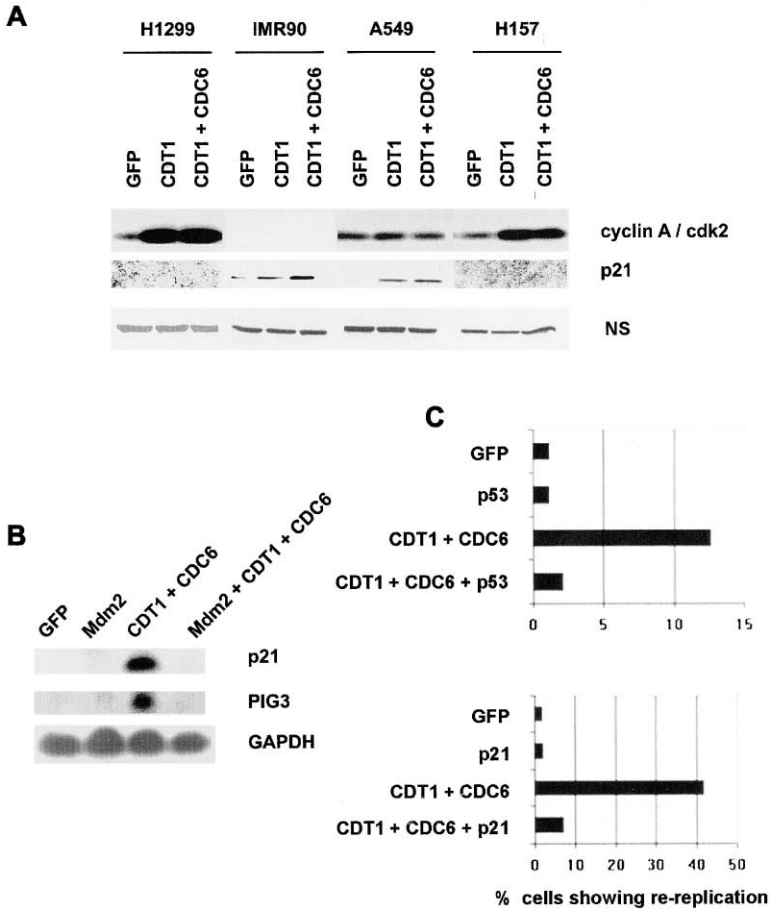


Figure 6. The Pathway Downstream from p53

(A) Cyclin A-cdk2 activity and p21 protein levels in a panel of cell lines overexpressing GFP (control), Cdt1, and Cdt1+Cdc6. Cyclin A-cdk2 activity was measured by the phosphorylation of histone H1 with [γ-^{32}P]ATP and a cyclin A immunoprecipitate from cell lysates. NS, a nonspecific band to demonstrate equal loading.

(B) p21 and PIG3 mRNA are induced in A549 cells overexpressing Cdt1+Cdc6, and this induction is abrogated by MDM2. Northern blot of A549 cells overexpressing indicated proteins. GAPDH provides the loading control.

(C) p53 and p21 repress rereplication. Percentage of cells showing rereplication measured as described in Figure 1. Because of apoptosis induced by p53 at 48 hr, the experiment in the upper panel was stopped at 24 hr, accounting for the lower percentage of rereplication seen with Cdt1+Cdc6.

ure 6C). Since p21 is a known inhibitor of cdk2, one likely pathway by which p21 could suppress rereplication is through the inhibition of the S phase cdk2 that we have already shown to be a promoter of this process.

Discussion

This study demonstrates that rereplication can be induced in mammalian cells through the direct deregulation of replication initiation factors. The activation of the DNA damage checkpoint pathway and p53 by rereplication was unexpected and suggests a role of checkpoint pathways and this tumor suppressor protein in preventing rereplication in mammalian cells. This study also measures the rate of origin refiring in a rereplicating system and assesses the distribution of rereplicated segments in the genome.

These results illuminate important issues about the barriers to rereplication that have to be overcome by mammalian cancer cells to permit the initiation of gene amplification events. Of the replication initiation factors, excesses of Cdc6 and Cdt1 appear to be sufficient to induce rereplication. In the yeasts, ORC, Cdc6, and probably the Mcm2-7 proteins are phosphorylated in late S and G2 by S phase cyclins, and this modification is important for preventing rereplication (Nguyen et al., 2001). The mammalian Mcm2 and Mcm4 proteins are phosphorylated by cyclin A-cdk2 in vitro, and this phosphorylation is postulated to release the Mcm2-7 complex from chromatin (Ishimi and Komamura-Kohno, 2001; Ishimi et al., 2000). Given that rereplication most likely requires the presence of Mcm2-7 proteins on the chromatin, overexpression of Cdc6 and Cdt1 appears sufficient to promote the binding of Mcm2-7 despite the presence of active cyclin A-cdk2 in these cells. Mammalian ORC subunits are also phosphorylated by cdks, although the functional significance of this phosphorylation is not yet clear (Mendez et al., 2002). Mammalian Orc1 is ubiquitinylated and released from the chromatin as S phase progresses (Li and DePamphilis, 2002). While we cannot rule out that mammalian ORC is inactivated as S phase progresses, our results suggest that overexpression of Cdt1 and Cdc6 bypasses the inactivation of ORC. It should be noted that overexpression of Cdt1 and Cdc18 (the *S. pombe* Cdc6) also permits cells to rereplicate their DNA (Gopalakrishnan et al., 2001; Yanow et al., 2001) despite the inactivation of *S. pombe* ORC by cdk (Vas et al., 2001).

It is interesting that cyclin A-cdk2 is a positive factor for rereplication in human cancer cells. In *S. pombe* and *S. cerevisiae*, the increasing activity of cyclin-dependent kinases in the latter half of the cell cycle is inhibitory for rereplication. Inhibition of cdc2 (the only cdk) in *S. pombe* by overexpression of an inhibitor or by conditional inactivation of a mitotic cyclin promotes rereplication (Correa-Bordes and Nurse, 1995; Jallepalli and

Kelly, 1996). In *S. cerevisiae* the appearance of cdk activity at the G1-S transition is the point of "no return" beyond which new prereplicative complexes cannot be established (Dahmann et al., 1995). In *Xenopus* egg extracts, excess cyclin-cdk activity inhibits the licensing of chromatin (Findeisen et al., 1999; Hua et al., 1997). Finally, inhibition of *Drosophila* cyclin A by RNAi strongly promoted rereplication (Mihaylov et al., 2002). These results are in contrast to the requirement of cyclin A-cdk2 for rereplication in mammalian cancer cells and might suggest that the mitotic cdk, cdk1, has taken over the role of inhibition of rereplication in mammals. Cyclin A-cdk2 most likely promotes rereplication by phosphorylating some as yet unknown mammalian protein whose modification is essential for replication initiation. A recently discovered candidate for such a substrate is the yeast Sld2 protein that appears to require phosphorylation by the yeast cdk to facilitate replication initiation (Masumoto et al., 2002). It is likely that similar substrates of cyclin A-cdk2 must be phosphorylated in mammalian cells to permit rereplication in the presence of excess Cdt1 and Cdc6.

Labeling the rereplicating cells with BrdU and isolation of heavy-heavy DNA allowed us to confirm that segments of the genome were indeed being rereplicated. This assay provided conclusive evidence of origin refiring in mammalian cells in as short an interval as 2–4 hr. Given the normal duration of the mammalian cell cycle of 24 hr and normal duration of S phase of 8 hr, this is an impressively short interval between the cycles of replication initiation. The asynchronous increase in DNA content beyond 4n DNA (instead of a discrete peak of 8n DNA), the lack of appearance of phospho-H3 before rereplication, and the evidence of origin refiring in 2–4 hr confirms that the rereplication is due to bona fide replication relicensing in the same cell cycle.

Isolation of the rereplicated DNA also allowed us to identify which portions of the genome are being rereplicated. The distributed pattern of the rereplicated DNA seen by FISH suggests that Cdt1 and Cdc6 overexpression does not uniquely affect a few isolated origins. On the other hand, despite the fact that the BrdU labeling was carried out over an 8 hr interval, the rereplicated DNA did not completely cover the chromosomes (as in a chromosome paint), consistent with the asynchronous nature of rereplication and suggesting that certain origins or chromatin structure may be resistant to the effects of Cdt1 and Cdc6. The rereplicated segments are enriched in portions of chromosomes that are replicated early in S phase, suggesting a selective effect of Cdt1 and Cdc6 on early firing origins. The reason for the relative susceptibility of certain segments to rereplication will be an interesting question to explore in the future.

Based on the wide distribution of rereplicated DNA, we believe that rereplication events might provide seeds for genetic instability almost anywhere in the genome by an initial increase in gene copy number. Recombination between homologous segments would be favored by the presence of rereplicated DNA in close proximity to each other, and such recombination events are expected to lead to breakage-fusion-bridge cycles shown to be important for the extensive gene amplification in cancer cells (Singer et al., 2000).

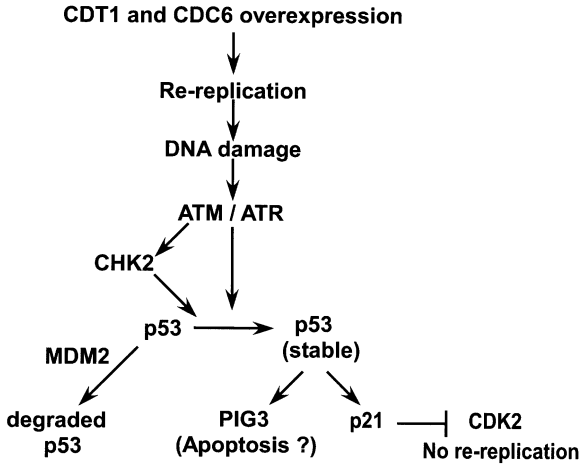


Figure 7. A Checkpoint Pathway Activated by Rereplication

The activation of the DNA damage checkpoint pathway and the tumor suppressor protein p53 provides a pathway by which mammalian cells prevent rereplication (Figure 7). Rereplication appears to lead to DNA damage. Our data suggest that activation of ATM/ATR kinases caused by overexpression of Cdt1 and Cdc6 leads to direct phosphorylation of p53 and indirect phosphorylation of p53 through Chk2 kinase. Phosphorylation of p53 stabilizes the protein and leads to increased transcription and expression of p21. The latter is a potent inhibitor of cyclin A-cdk2 kinase and could therefore prevent any rereplication. Consistent with this hypothesis, overexpression of wild-type p53 or of p21 effectively inhibited rereplication in the p53-negative H1299 cells, while inactivation of p53 in A549 cells by overexpressing Mdm2 prevented p21 induction and permitted rereplication. Because of the concurrent induction of proapoptotic genes like PIG3, p53 could also promote apoptosis of cells that have already undergone significant rereplication. Since mutations in p53 have been widely documented to promote genomic instability and gene amplification (Shao et al., 2000; Vogelstein et al., 2000), our results provide a partial explanation of this observation by proposing a mechanism by which p53 stabilizes the genome. Genes besides p53, however, also prevent gene amplification (Hall et al., 1997), so it is unlikely that p53 is the only barrier to rereplication upon overexpression of Cdt1 and Cdc6 in all cell lines.

It is also worth noting that cyclin A is overexpressed in a subset of human cancers (Michalides et al., 2002). In several cancers, e.g., breast cancer, non-Hodgkin's lymphomas, and certain melanomas, overexpression of cyclin A is a predictor of poor prognosis (Bukholm et al., 2001a, 2001b; Florenes et al., 2001; Michalides et al., 2002). In one study, elevated expression of cyclin A correlated with the progression of breast cancers to a more advanced stage with gene amplification (Blegen et al., 2001). Therefore, the observed role of cyclin A in promoting rereplication may be important for promoting genomic instability in cancers.

In summary, these results highlight the importance of the geminin-Cdt1 balance and of the DNA damage-induced checkpoint pathway that activates p53 as addi-

tional layers of protection in mammalian cells preventing rereplication and genomic instability.

Experimental Procedures

Adenovirus Construction and Infections

cDNAs for GFP, Cdt1, Cdc6 (wild-type and mutants), p53, p21, p27, geminin, and cyclins were subcloned into the pACCMV-pLpA plasmid (Becker et al., 1994). The resulting shuttle vectors were cotransfected into 293T cells with the pJM17 plasmid containing the adenovirus genome. Recombinant adenovirus was cloned, and large-scale purification of virus from 293T cell lysates was achieved by PEG precipitation, CsCl density gradient centrifugation, and gel filtration (Becker et al., 1994). The concentration of purified virus was determined by OD_{260} measurements using the equation $1 OD_{260} \approx 10^{12}$ pfu.

Mammalian cell lines plated in 60 mm culture dishes (in DMEM containing 10% fetal calf serum) were infected the day after plating, when the cultures had attained a confluence of ~40%. Purified virus was added directly to the culture medium to give a final concentration of 1–2 $\times$ 10^{10} pfu/ml. Susceptibility of different cell lines to adenovirus-mediated gene transduction was determined by visualization of GFP in cells infected with adenovirus expressing GFP (AdGFP). For most cell lines, 5 $\times$ 10^9 pfu/ml of CsCl-banded AdGFP was sufficient to infect >95% of the population. AdGFP-encoded GFP expression was usually detected 12–16 hr post infection.

FACS Analysis

Cells pulsed with 10 mM BrdU (Roche) for 1 hr were harvested with trypsin-EDTA, fixed in 65% DMEM/35% ethanol for 1 hr at 4°C, and resuspended in 1 ml 2 M HCl for 20 min at room temperature. After centrifugation, the cell pellets were resuspended in 1 ml 0.5 M borax (pH 8.5) to neutralize any residual acid. Following brief centrifugation, the pellets were washed in 1 ml PBS and then resuspended in 50 μl of antibody labeling solution (30 μl PBS containing 0.5% Tween-20/0.5% BSA plus 20 μl FITC-conjugated anti-BrdU antibody) (Pharmingen #33284X). After 30 min at room temperature in the dark, cells were washed in PBS and resuspended in 1 ml PBS containing 10 μg/ml propidium iodide (PI) and 8 μg/ml RNase A. The labeled cells were analyzed for PI and BrdU staining on a Becton-Dickinson flow cytometer using Cellquest software. Anti-phospho-H3 antibody (Upstate) was used similarly.

Protein Analysis

Methods of cell extraction, immunoprecipitation, and immunoblotting have been described before (Dhar and Dutta, 2000; Thome et al., 2000; Wohlschlegel et al., 2000). Antibodies to Cdt1 and geminin have been described earlier (Wohlschlegel et al., 2000). Other antibodies were obtained from Santa Cruz Biotechnologies: anti-p21 (sc 397), anti-cyclin A (sc 751), and anti-Cdc6 (H 304). Antibodies to phospho-serine p53 (#9284S and #9287S) and phospho-Chk2 (#2661S) were obtained from Cell Signaling Technology. For cyclin A-cdk2 assays, 200 μg cell lysate was immunoprecipitated with anti-cyclin A antibody, and H1 kinase assay was carried out as described (Chen et al., 1995, 1996).

Synchronization of Cells and BrdU Labeling for Early Replicated DNA

Exponentially growing cells were treated with 2 mM thymidine for 12 hr at 37°C. Thereafter, the cultures were diluted twice and grown in fresh media without thymidine for 12 hr. The cells are arrested again for 12 hr with 1 μg/ml aphidicolin. The cells were then released from the aphidicolin block and simultaneously labeled with 100 mM BrdU for 2 hr.

CsCl Density Gradient Centrifugation and ELISA with Anti-BrdU Antibody

Four to six micrograms of chromosomal DNA isolated from BrdU-labeled H1299 cells was digested with DNase I to obtain fragments ranging from 500 bp to 4 kb. The digested DNA was loaded on Beckman quick seal tube containing 1 g/ml CsCl and centrifuged at 65,000 rpm for 18 hr in a VTi80 rotor (Beckman) at 25°C. 180 μl fractions were collected from the bottom. Two microliters of each fraction was denatured and bound on a poly-L-lysine-coated 96-well plate (Becton Dickinson Labware), and BrdU-labeled DNA was detected by peroxidase-conjugated anti-BrdU antibody (Roche) and TMB Substrate Solution (Pierce).

Fluorescence In Situ Hybridization

Two hundred nanograms of BrdU-labeled DNA fraction was amplified and labeled by degenerate oligonucleotide-primed (DOP)-PCR (Telenius et al., 1992). Heavy-heavy DNA was labeled with biotin-16-dUTP (Roche), and heavy-light DNA was labeled with digoxygenin-11-dUTP (Roche). One hundred nanograms of labeled DNA was denatured in 20 μl of hybridization buffer containing 50% formamide and 2× SSC for 8 min at 70°C and allowed to preanneal in the presence of unlabeled Cot1 DNA for 3 hr at 37°C before application to denatured chromosomal spreads (70% formamide, 2× SSC at 70°C for 3 min). Hybridization at 37°C for 20 hr in a humidified chamber was followed by washes with 50% formamide, 2× SSC at 45°C. The biotin-labeled probe was detected with FITC-avidin, and the digoxygenin-labeled probe fraction was detected with Rhodamine anti-digoxygenin. Chromosomes were counterstained with 125 ng/ml DAPI and visualized with an Olympus AX-70 fluorescent microscope equipped with a DAPI filter, a FITC cube set, and a Rhodamine cube set. Images were digitally obtained with a Photosensys CCD camera and Cytovision Genus software.

Acknowledgments

This work was supported by grants RO1 CA60499 to A.D. and RO1 ES009558 to C.V. Y.J. was supported by a BK21 Research Fellowship from the Korean Ministry of Education, and N.W. was supported by a Fellowship from the Howard Hughes Medical Institute. K. Munger and J. Chen provided critical antibodies. We thank J. Wohlschlegel and J. Walter for reading the manuscript.

Received: April 24, 2002
Revised: February 6, 2003
Accepted: February 10, 2003
Published: April 24, 2003

References

Bates, S., Ryan, K.M., Phillips, A.C., and Vousden, K.H. (1998). Cell cycle arrest and DNA endoreduplication following p21Waf1/Cip1 expression. Oncogene *17*, 1691–1703.

Becker, T.C., Noel, R.J., Coats, W.S., Gomez-Foix, A.M., Alam, T., Gerard, R.D., and Newgard, C.B. (1994). Use of recombinant adenovirus for metabolic engineering of mammalian cells. Methods Cell Biol. *43*, 161–189.

Bell, S.P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu. Rev. Biochem. *71*, 333–374.

Blegen, H., Ghadimi, B.M., Jauho, A., Zetterberg, A., Eriksson, E., Auer, G., and Ried, T. (2001). Genetic instability promotes the acquisition of chromosomal imbalances in T1b and T1c breast adenocarcinomas. Anal. Cell. Pathol. *22*, 123–131.

Bukholm, I.R., Bukholm, G., and Nesland, J.M. (2001a). Coexpression of cyclin A and beta-catenin and survival in breast cancer patients. Int. J. Cancer *94*, 148–149.

Bukholm, I.R., Bukholm, G., and Nesland, J.M. (2001b). Over-expression of cyclin A is highly associated with early relapse and reduced survival in patients with primary breast carcinomas. Int. J. Cancer *93*, 283–287.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677–1679.

Chen, J., Jackson, P.K., Kirschner, M.W., and Dutta, A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature *374*, 386–388.

Chen, J., Saha, P., Kornbluth, S., Dynlacht, B.D., and Dutta, A. (1996). Cyclin-binding motifs are essential for the function of p21CIP1. Mol. Cell. Biol. *16*, 4673–4682.

Correa-Bordes, J., and Nurse, P. (1995). p25rum1 orders S phase

and mitosis by acting as an inhibitor of the p34cdc2 mitotic kinase. Cell *83*, 1001–1009.

Dahmann, C., Diffley, J.F., and Nasmyth, K.A. (1995). S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. Curr. Biol. *5*, 1257–1269.

Delmolino, L.M., Saha, P., and Dutta, A. (2001). Multiple mechanisms regulate subcellular localization of human CDC6. J. Biol. Chem. *276*, 26947–26954.

Dhar, S.K., and Dutta, A. (2000). Identification and characterization of the human ORC6 homolog. J. Biol. Chem. *275*, 34983–34988.

Diffley, J.F. (2001). DNA replication: building the perfect switch. Curr. Biol. *11*, R367–R370.

Dutta, A., and Bell, S.P. (1997). Initiation of DNA replication in eukaryotic cells. Annu. Rev. Cell Dev. Biol. *13*, 293–332.

Fetni, R., Drouin, R., Richer, C.L., and Lemieux, N. (1996). Complementary replication R- and G-band patterns induced by cell blocking at the R-band/G-band transition, a possible regulatory checkpoint within the S phase of the cell cycle. Cytogenet. Cell Genet. *75*, 172–179.

Findeisen, M., El-Denary, M., Kapitza, T., Graf, R., and Strausfeld, U. (1999). Cyclin A-dependent kinase activity affects chromatin binding of ORC, Cdc6, and MCM in egg extracts of Xenopus laevis. Eur. J. Biochem. *264*, 415–426.

Florenes, V.A., Maelandsmo, G.M., Faye, R., Nesland, J.M., and Holm, R. (2001). Cyclin A expression in superficial spreading malignant melanomas correlates with clinical outcome. J. Pathol. *195*, 530–536.

Gopalakrishnan, V., Simancek, P., Houchens, C., Snaith, H.A., Frattini, M.G., Sazer, S., and Kelly, T.J. (2001). Redundant control of rereplication in fission yeast. Proc. Natl. Acad. Sci. USA *98*, 13114–13119.

Hall, I.J., Gioeli, D., Weissman, B.E., and Tlsty, T.D. (1997). Identification of additional complementation groups that regulate genomic instability. Genes Chromosomes Cancer *20*, 103–112.

Herbig, U., Griffith, J.W., and Fanning, E. (2000). Mutation of cyclin/cdk phosphorylation sites in HsCdc6 disrupts a late step in initiation of DNA replication in human cells. Mol. Biol. Cell *11*, 4117–4130.

Hirao, A., Kong, Y.Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S.J., and Mak, T.W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science *287*, 1824–1827.

Hua, X.H., Yan, H., and Newport, J. (1997). A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. J. Cell Biol. *137*, 183–192.

Ishimi, Y., and Komamura-Kohno, Y. (2001). Phosphorylation of Mcm4 at specific sites by cyclin-dependent kinase leads to loss of Mcm4,6,7 helicase activity. J. Biol. Chem. *276*, 34428–34433.

Ishimi, Y., Komamura-Kohno, Y., You, Z., Omori, A., and Kitagawa, M. (2000). Inhibition of Mcm4,6,7 helicase activity by phosphorylation with cyclin A/Cdk2. J. Biol. Chem. *275*, 16235–16241.

Itzhaki, J.E., Gilbert, C.S., and Porter, A.C. (1997). Construction by gene targeting in human cells of a "conditional" CDC2 mutant that rereplicates its DNA. Nat. Genet. *15*, 258–265.

Jallepalli, P.V., and Kelly, T.J. (1996). Rum1 and Cdc18 link inhibition of cyclin-dependent kinase to the initiation of DNA replication in Schizosaccharomyces pombe. Genes Dev. *10*, 541–552.

Jiang, W., Wells, N.J., and Hunter, T. (1999). Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc. Natl. Acad. Sci. USA *96*, 6193–6198.

Kelly, T.J., and Brown, G.W. (2000). Regulation of chromosome replication. Annu. Rev. Biochem. *69*, 829–880.

Labib, K., and Diffley, J.F. (2001). Is the MCM2–7 complex the eukaryotic DNA replication fork helicase? Curr. Opin. Genet. Dev. *11*, 64–70.

Lei, M., and Tye, B.K. (2001). Initiating DNA synthesis: from recruiting to activating the MCM complex. J. Cell Sci. *114*, 1447–1454.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. Nature *396*, 643–649.

Li, C.J., and DePamphilis, M.L. (2002). Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. Mol. Cell. Biol. *22*, 105–116.

Masumoto, H., Muramatsu, S., Kamimura, Y., and Araki, H. (2002). S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature *415*, 651–655.

McGarry, T.J., and Kirschner, M.W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell *93*, 1043–1053.

Mendez, J., Zou-Yang, X.H., Kim, S.Y., Hidaka, M., Tansey, W.P., and Stillman, B. (2002). Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol. Cell *9*, 481–491.

Michalides, R., van Tinteren, H., Balkenende, A., Vermorken, J.B., Benraadt, J., Huldij, J., and van Diest, P. (2002). Cyclin A is a prognostic indicator in early stage breast cancer with and without tamoxifen treatment. Br. J. Cancer *86*, 402–408.

Mihaylov, I.S., Kondo, T., Jones, L., Ryzhikov, S., Tanaka, J., Zheng, J., Higa, L.A., Minamino, N., Cooley, L., and Zhang, H. (2002). Control of DNA replication and chromosome ploidy by geminin and cyclin A. Mol. Cell. Biol. *22*, 1868–1880.

Nguyen, V.Q., Co, C., and Li, J.J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature *411*, 1068–1073.

Noton, E., and Diffley, J.F. (2000). CDK inactivation is the only essential function of the APC/C and the mitotic exit network proteins for origin resetting during mitosis. Mol. Cell *5*, 85–95.

Petersen, B.O., Lukas, J., Sorensen, C.S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J. *18*, 396–410.

Prives, C., and Hall, P.A. (1999). The p53 pathway. J. Pathol. *187*, 112–126.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S., and Bonner, W.M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem. *273*, 5858–5868.

Saha, P., Chen, J., Thome, K.C., Lawlis, S.J., Hou, Z.H., Hendricks, M., Parvin, J.D., and Dutta, A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. Mol. Cell. Biol. *18*, 2758–2767.

Shao, C., Deng, L., Henegariu, O., Liang, L., Stambrook, P.J., and Tischfield, J.A. (2000). Chromosome instability contributes to loss of heterozygosity in mice lacking p53. Proc. Natl. Acad. Sci. USA *97*, 7405–7410.

Singer, M.J., Mesner, L.D., Friedman, C.L., Trask, B.J., and Hamlin, J.L. (2000). Amplification of the human dihydrofolate reductase gene via double minutes is initiated by chromosome breaks. Proc. Natl. Acad. Sci. USA *97*, 7921–7926.

Tada, S., Li, A., Maiorano, D., Mechali, M., and Blow, J.J. (2001). Repression of origin assembly in metaphase depends on inhibition of RLF- B/Cdt1 by geminin. Nat. Cell Biol. *3*, 107–113.

Takeda, D.Y., Wohlschlegel, J.A., and Dutta, A. (2001). A bipartite substrate recognition motif for cyclin-dependent kinases. J. Biol. Chem. *276*, 1993–1997.

Takisawa, H., Mimura, S., and Kubota, Y. (2000). Eukaryotic DNA replication: from pre-replication complex to initiation complex. Curr. Opin. Cell Biol. *12*, 690–696.

Telenius, H., Pelmear, A.H., Tunnacliffe, A., Carter, N.P., Behmel, A., Ferguson-Smith, M.A., Nordenskjold, M., Pfragner, R., and Ponder, B.A. (1992). Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. Genes Chromosomes Cancer *4*, 257–263.

Thome, K.C., Dhar, S.K., Quintana, D.G., Delmolino, L., Shahsafaei, A., and Dutta, A. (2000). Subsets of human origin recognition complex (ORC) subunits are expressed in non-proliferating cells and associate with non-ORC proteins. J. Biol. Chem. *275*, 35233–35241.

Vas, A., Mok, W., and Leatherwood, J. (2001). Control of DNA rerepli-

cation via Cdc2 phosphorylation sites in the origin recognition complex. Mol. Cell. Biol. *21*, 5767–5777.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.

Waga, S., and Stillman, B. (1998). The DNA replication fork in eukaryotic cells. Annu. Rev. Biochem. *67*, 721–751.

Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C., and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science *290*, 2309–2312.

Yanow, S.K., Lygerou, Z., and Nurse, P. (2001). Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication. EMBO J. *20*, 4648–4656.