60–69 Nucleic Acids Research, 2009, Vol. 37, No. 1 Published online 14 November 2008
doi:10.1093/nar/gkn912

Analysis of re-replication from deregulated origin licensing by DNA fiber spreading

Elizabeth S. Dorn[1], Paul D. Chastain II[2], Jonathan R. Hall[1] and Jeanette Gowen Cook[1,*]

[1]Department of Biochemistry and Biophysics and [2]Department of Pathology and Laboratory Medicine, Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7260, USA

Received August 21, 2008; Revised October 22, 2008; Accepted October 29, 2008

ABSTRACT

A major challenge each human cell-division cycle is to ensure that DNA replication origins do not initiate more than once, a phenomenon known as re-replication. Acute deregulation of replication control ultimately causes extensive DNA damage, cell-cycle checkpoint activation and cell death whereas moderate deregulation promotes genome instability and tumorigenesis. In the absence of detectable increases in cellular DNA content however, it has been difficult to directly demonstrate re-replication or to determine if the ability to re-replicate is restricted to a particular cell-cycle phase. Using an adaptation of DNA fiber spreading we report the direct detection of re-replication on single DNA molecules from human chromosomes. Using this method we demonstrate substantial re-replication within 1 h of S phase entry in cells overproducing the replication factor, Cdt1. Moreover, a comparison of the HeLa cancer cell line to untransformed fibroblasts suggests that HeLa cells produce replication signals consistent with low-level re-replication in otherwise unperturbed cell cycles. Re-replication after depletion of the Cdt1 inhibitor, geminin, in an untransformed fibroblast cell line is undetectable by standard assays but readily quantifiable by DNA fiber spreading analysis. Direct evaluation of re-replicated DNA molecules will promote increased understanding of events that promote or perturb genome stability.

INTRODUCTION

In each cell-division cycle, a human cell must duplicate over three billion DNA base pairs precisely once. In order to efficiently copy a large genome in a single cell cycle, eukaryotic cells initiate replication at thousands of chromosomal locations known as origins of DNA replication. Initiation of DNA synthesis, or origin 'firing', takes place in the S phase of the cell cycle with individual origins firing at different times during that period. Each origin that fires must simultaneously be prevented from firing again until the next cell cycle. Even modest *re-replication* from failure to maintain this 'once and only once' rule results in DNA damage and genome instability which has been linked to oncogenesis (1–5).

Origins are licensed for DNA replication during the G1 cell-cycle phase by the assembly of an origin-bound pre-replication complex (preRC). PreRCs are assembled by the recruitment of the Mcm2–7 complex through the combined action of the Origin Recognition Complex (ORC) and the Cdc6 and Cdt1 proteins. Once S phase begins, licensed origins containing a preRC are stimulated to fire by the S phase-specific protein kinases, Cdk2 and Cdc7, but no new preRCs can be assembled, thus avoiding relicensing and reinitiation of origins that have already fired (6,7). To prevent re-replication a variety of overlapping non-redundant mechanisms restrict origin licensing in all cell-cycle phases except G1 by directly affecting the activity or abundance of individual preRC components. These mechanisms include ubiquitin-mediated degradation, Cdk-mediated phosphorylation and the accumulation of the Cdt1 inhibitor, geminin (1–3,8).

Overexpression of Cdt1 or depletion of the Cdt1 inhibitor geminin can induce substantial re-replication in human cancer cell lines that is detectable as an aberrant increase in the overall amount of DNA per cell (9–12). It is presumed that re-replication at more physiological (sublethal) levels promotes genomic instability. In support of that assertion, modest overproduction of Cdt1 or Cdc6 did not induce detectable re-replication in cultured cells but markedly increased tumorigenesis in xenograft assays (4,5). The increased tumorigenesis may have been

*To whom correspondence should be addressed. Tel: +1 919 843 3867; Fax: +1 919 966 2852; Email: jean_cook@med.unc.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

the result of limited re-replication, but it is unclear if re-replication actually occurred in those studies or if the tumorigenesis was related to potential other functions of Cdt1 and Cdc6. Conventional cell-based techniques to detect re-replication are restricted to the subpopulation of cells that accumulate a DNA content greater than 4C (more than the normal G2 DNA content) and require lethal extents of re-replication to reach detectable levels. For this reason, detection of re-replication has required extensive origin refiring and fork elongation over periods of time longer than the normal S phase to allow hyper-accumulation of chromosomal DNA. It is thus impossible to determine *when* in the cell cycle the re-replication actually occurred. In addition, during these long incubations DNA becomes fragmented triggering a secondary cell-cycle DNA damage checkpoint and/or apoptosis (9,11,13,14). Moreover, most primary and nontransformed cells appear to be resistant to re-replication induction when analyzed for total DNA content, though cell-cycle checkpoints are still activated (9,14). Re-replication in these cells can only be inferred from cell-cycle checkpoint activation, but it has not been demonstrated that these cells re-replicate after geminin depletion or Cdt1 overproduction.

The limits of available re-replication assays prompted us to develop a more sensitive method to directly quantify re-replication. We report here a protocol for detecting re-replication by single molecule DNA fiber analysis, also known as 'fiber spreading'. We have used this technique to demonstrate for the first time that re-replication can occur in very early S phase, in geminin-depleted untransformed cells, and further that HeLa cells may re-replicate at a low level even in unperturbed cell cycles.

MATERIALS AND METHODS

Cell manipulations

Normal human fibroblasts immortalized with human telomerase (NHF1-hTert) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Sigma, St Louis, MO) and 2 mM L-glutamine (Sigma). Purified adenovirus producing HA_2-tagged Cdt1 was previously described (15), and a derivative truncating Cdt1 after amino acid 321 was constructed by standard methods. siRNA oligonucleotides were previously described (16) and introduced into cells using Dharmafect 1 reagent (Dharmacon). Cells to be analyzed for flow cytometry were trypsinized, fixed with ethanol and treated with propidium iodide/RNAse solution by standard methods. DNA content was analyzed using the CyAn (DakoCytomation) and cell-cycle distributions were determined using Summit v4.3 software (DakoCytomation). Total cell lysates were separated by SDS–PAGE and transferred to PVDF membrane (Millipore) and probed with antibodies to detect the following proteins: anti-geminin (FL-209) and anti-HA (y-11) purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-tubulin (DM1A) purchased from Sigma, phosphospecific antibodies to p53 and Chk1 purchased from Cell Signaling Technology (Beverly, MA) and polyclonal anti-Cdt1 described in Cook *et al.* (17).

Fiber spreading

Culture medium was supplemented with CldU to 100 μM for 30 min, the medium containing CldU was removed and fresh medium was added. After 30 min, IdU was added to 50 μM for 10 min. Cells were trypsinized and resuspended in PBS to a density of 250 cells per microliter. DNA spreads of approximately 500 cells per slide and the staining of the fibers were as previously described (18,19) with the following modifications: the amount of antibody that detects IdU was diluted 1:500 instead of 1:250 and the length of the stringency buffer wash increased to 15 min. In addition, IdU-only and CldU-only slides were also stained alongside the slides from the experimental conditions. If more than 5 of 100 tracks stained with both antibodies in the IdU-only or CldU-only slides (i.e. appreciable staining from both antibodies when only one nucleotide was used), then slides from the whole set were not analyzed. IdU and CldU were considered coincident if the IdU and CldU fluorescence were equal to each other (i.e. the red and green signals were similar). To determine whether they were equal in intensities, the red channel and green channels were visually estimated simultaneously using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2006)) alongside the composite image.

The length of the yellow tracks were measured using Image J software, the length converted to micrometers using five arbitrary units per micrometer and the micrometers were converted to kilo base pairs by multiplying the micrometers by 2.5 kb as previously described (19). The tracks were scored as being all green (green-only), all red (red-only), tracks containing red adjoined to green (red-green tracks), or tracks containing more than one micrometer of yellow (tracks that contained red and green signal with substantial overlap). Red-green tracks on occasion contained yellow at the joint, but the length of the yellow track was less than 1 μm (2.5 kb in length) and were due to a single replication fork being active during both pulses (which is rare due to the gap between signals). True re-replication tracks containing yellow were always much larger and not due to a single replication fork that was active during the first pulse and continued to be active during the second pulse. Any closely spaced tracks that appeared to have come from separate DNA strands due to their angles relative to each other or thickness of signal were ignored.

Statistical analysis

In experiments characterizing re-replication levels and re-replication track length, a minimum of 200 individual fibers was analyzed between at least two independent experiments. Because re-replication level studies compared two categorical values, Fisher's two-tailed exact test was utilized to determine statistical significance. Re-replication track length studies compared populations of lengths so

Student's two-tailed *t*-test was used to determine statistical significance.

RESULTS

Cdt1 overproduction induces an S phase delay

Re-replication induced by Cdt1 overproduction can be demonstrated in human cells by the appearance of a subpopulation of cells with DNA contents greater than 4C (9,14,20). We recapitulated this result by infecting an asynchronous population of HeLa cells with adenovirus expressing HA epitope-tagged Cdt1 and quantifying the amount of DNA per cell using flow cytometric analysis of propidium iodide-stained nuclei. Cdt1-overproducing cells displayed the typical re-replication phenotype characterized by a reduction in the G1 population (2C) and an increase in both the G2 population (4C) as well as a subpopulation with DNA content greater than 4C (Figure 1A). Due to the long incubations required to produce the re-replication phenotype (typically 24 h or more) (9,14,20), it is not clear if the re-replication only occurred after S phase was completed (i.e. in G2 phase) or if origins continuously reinitiated throughout S phase. If re-replication does occur early in S phase, it is not possible to detect the re-replicated DNA by flow cytometry because cells still have DNA content less than 4C.

To determine if there might be cell-cycle restrictions on the opportunities for re-replication, we evaluated synchronized cells overproducing Cdt1 in S phase by the protocol outlined in Figure 1B. In early S phase, endogenous Cdt1 levels are kept low by ubiquitin-mediated proteasomal degradation (21–23) (Figure 1C, lane 1). High-level expression of ectopic Cdt1 presumably overwhelmed the capacity of the cells to properly degrade Cdt1 allowing it to persist into S phase (Figure 1C, lane 2). Control cells completed S phase 8 h after release (Figure 1D), but cells overproducing Cdt1 failed to complete S phase on schedule (Figure 1D). Instead, the majority of Cdt1-overproducing cells still harbored near-G1 DNA content, indicating that Cdt1 overproduction interferes with S phase progression. By 24 h after release (and held in nocodazole to block mitosis), these cells showed a heterogeneous DNA content with some cells accumulating DNA to levels greater than 4C (Figure 1D). Previous studies in asynchronous cells or using in vitro replication assays had indicated that high levels of Cdt1 can interfere with S phase progression, presumably due to re-replication (13,24). One interpretation of these results is that Cdt1 overproduction induced re-replication in early S phase shortly after S phase entry and that this re-replication interfered with S phase progression.

An alternate interpretation hinges on the fact that Cdt1 has multiple binding partners including other components of the preRC, geminin and cyclin A/Cdk2. Early studies suggested that cyclin A/Cdk2 activity is critical for S phase entry and/or progression (25,26), so it was possible that the S phase progression defect in Cdt1 overproducing cells was due to interference with cyclin A/Cdk2 rather than an immediate consequence of re-replication. To address this question, we constructed a recombinant adenovirus in which Cdt1 was truncated after amino acid 321 'Cdt1ΔC' (Figure 1E). The corresponding *Xenopus laevis* truncation is defective for origin licensing, but retains geminin binding and the cyclin A binding motif (24,27). We have also recently demonstrated that this truncation is defective for induction of re-replication in asynchronous cells (28). We confirmed the binding properties of Cdt1ΔC using bacterially expressed GST-Cdt1 fusions incubated with HeLa cell lysate. Both full length ('FL') and the truncation mutant ('ΔC') bound cyclin A and geminin as expected (Figure 1F).

A derivative of the Cdt1 adenovirus bearing the 'ΔC' truncation was constructed and tested for the ability to delay S phase. Overproduction of Cdt1ΔC to levels similar to that of full-length Cdt1 (Figure 1G, immunoblot) failed to induce a substantial S phase progression defect, whereas full-length Cdt1-producing cells again progressed very slowly through S phase (Figure 1G). Taken together the results described above are consistent with excess Cdt1 in S phase immediately re-licensing previously fired origins followed by a re-replication-induced S phase progression defect. Nevertheless, these indirect assays do not definitively demonstrate that re-replication took place *within* S phase.

Single fiber analysis quantifies early S phase re-replication in Cdt1-overproducing cells

DNA replication can be directly detected by incubation with halogenated nucleotide analogs followed by spreading DNA fibers on glass slides and staining with fluorescent antibodies to the nucleotide analogs (29,30). This procedure is referred to as 'fiber spreading' because the DNA fibers are uniformly stretched on a glass slide so that individual replication tracks can be identified. Cells can be sequentially incubated with two different halogenated nucleotides detected by two different fluorescent antibodies so that ongoing replication tracks can be distinguished from replication terminations and origin firing events. Ongoing replication forks produce tracks with the first label adjoining the second label, forks that terminated during the first incubation contain only the first label and origins that fired during the second incubation contain only the second label (31). We hypothesized that re-replication would produce a unique signal where both labels coincide on a single replication track. Since the antibodies to detect the nucleotide analogs are labeled with either red or green fluorescent dyes, the coincidence of these signals would produce a yellow track on merged micrographs.

To test this hypothesis, we infected synchronized HeLa cells with control adenovirus or adenovirus overproducing Cdt1 as in Figure 1B. These cells were released from the aphidicolin block immediately into medium containing the thymidine analog CldU. Thirty minutes later, the medium was removed and fresh medium lacking nucleotide analogs was added for an additional 30 min. Cells were then labeled with IdU for 10 min and harvested and subjected to fiber spreading as described in Materials and Methods. The chase period between pulses was introduced to ensure that any residual CldU was exhausted from the

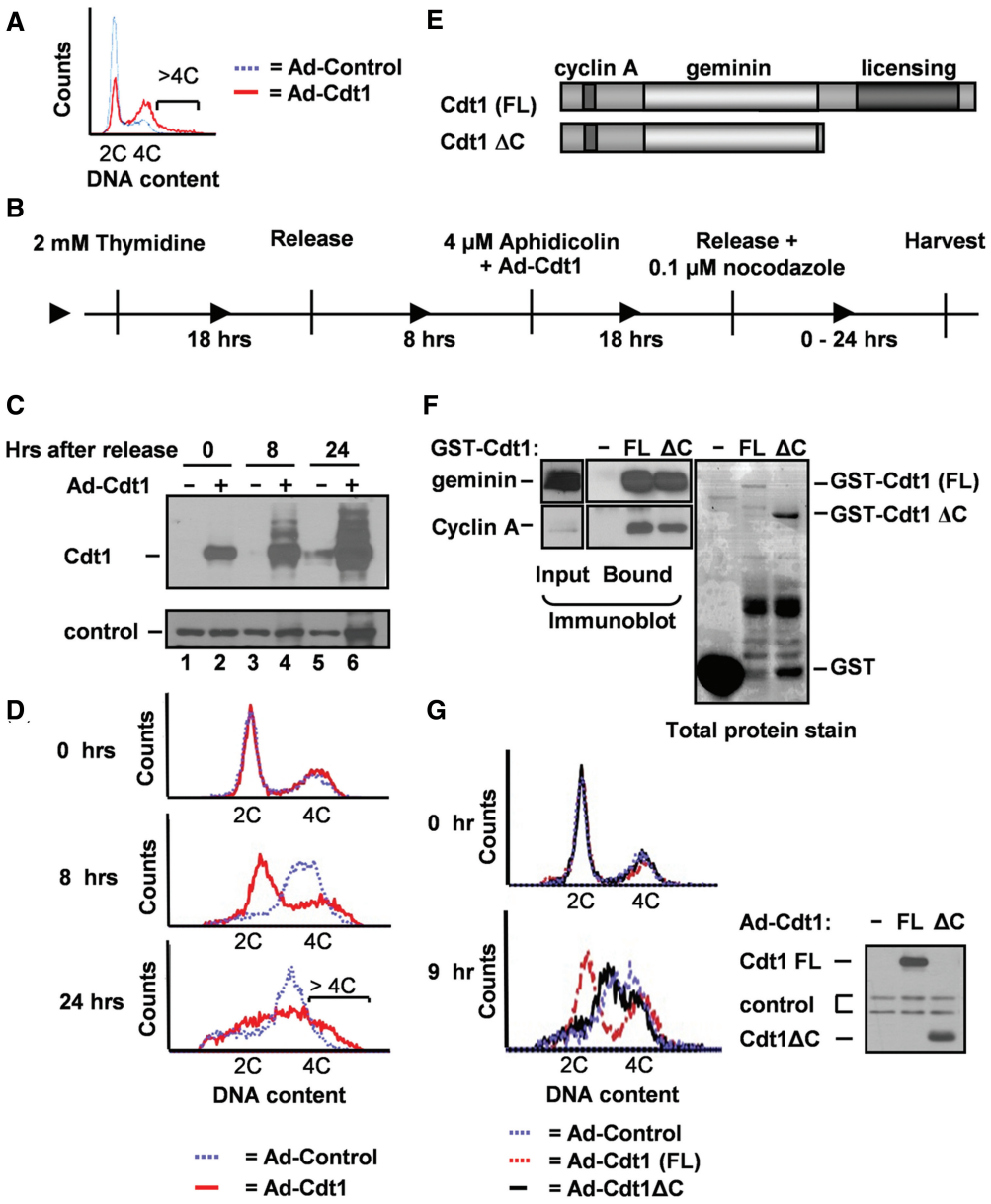


Figure 1. Cdt1 overproduction induces slow S phase progression. (**A**) Asynchronously growing HeLa cells were infected with control adenovirus (Ad-CMV) or adenovirus expressing epitope-tagged Cdt1 (HA_2-Cdt1) at a multiplicity of infection of 500. Cells were harvested 48 h post-infection and evaluated for DNA content by flow cytometric analysis of propidium iodide-stained nuclei. The percentage of cells with greater than 4C DNA content is 1.2% for Ad-Control and 9.4% for Ad-Cdt1. (**B**) Workflow of the cell synchronization. HeLa cells were synchronized in early S phase with a thymidine-aphidicolin double synchronization protocol and infected with recombinant adenovirus expressing either HA_2-tagged Cdt1 or empty virus (CMV promoter only) as a control at a multiplicity of infection of 500. This viral dose leads to Cdt1 overproduction by ~25–30-fold over endogenous Cdt1 in asynchronous cells (data not shown). About 18 h post-infection, aphidicolin was removed and cells were collected at various times after release. (**C**) Immunoblot of Cdt1 in cells collected at 0, 8 and 24 h after release from aphidicolin; 'control' is a non-specific band serving as a loading control. (**D**) DNA content of synchronized HeLa cells from C determined by flow cytometry. The percentage of cells with greater than 4C DNA content was the following: for Ad-Control, 0 h 0.3%, 8 h 3.6%, 24 h 5.4%; for Ad-Cdt1, 0 h 0.6%, 8 h 8.7%, 24 h 14.9%. (**E**) Diagram of human Cdt1 identifying the cyclin A binding motif, geminin binding domain and the replication licensing domain. The truncation to remove the licensing domain 'Cdt1ΔC' is illustrated below. (**F**) Fusions of full-length Cdt1 ('FL') and Cdt1ΔC to glutathione-S-transferase (GST) were produced in *E. coli*, bound to glutathione agarose, then incubated with lysates of asynchronous HeLa cells. Endogenous geminin and cyclin A were detected in the lysate ('input') or bound fractions by immunoblotting and the purified GST fusions were detected by Coomassie staining. (**G**) HeLa cells were infected with control Ad-CMV (control virus), Ad-HA2-Cdt1, or Ad-HA2-Cdt1ΔC and synchronized in early S phase as in (B). Cells were collected at the 0 and 9 h timepoints and analyzed for DNA content. Portions of the cells from the 0 h samples were analyzed for ectopic Cdt1 expression by immunoblotting with anti-HA antibody. Non-specific bands serve as a loading control.

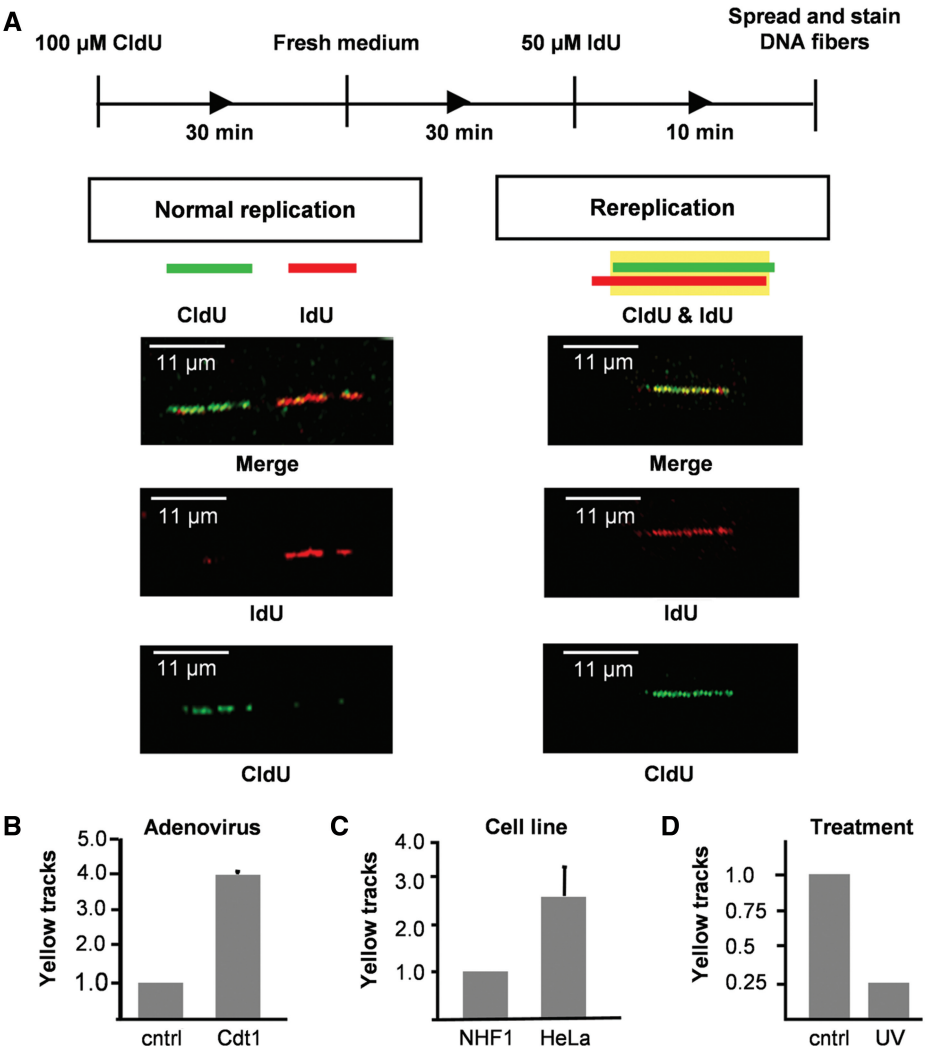
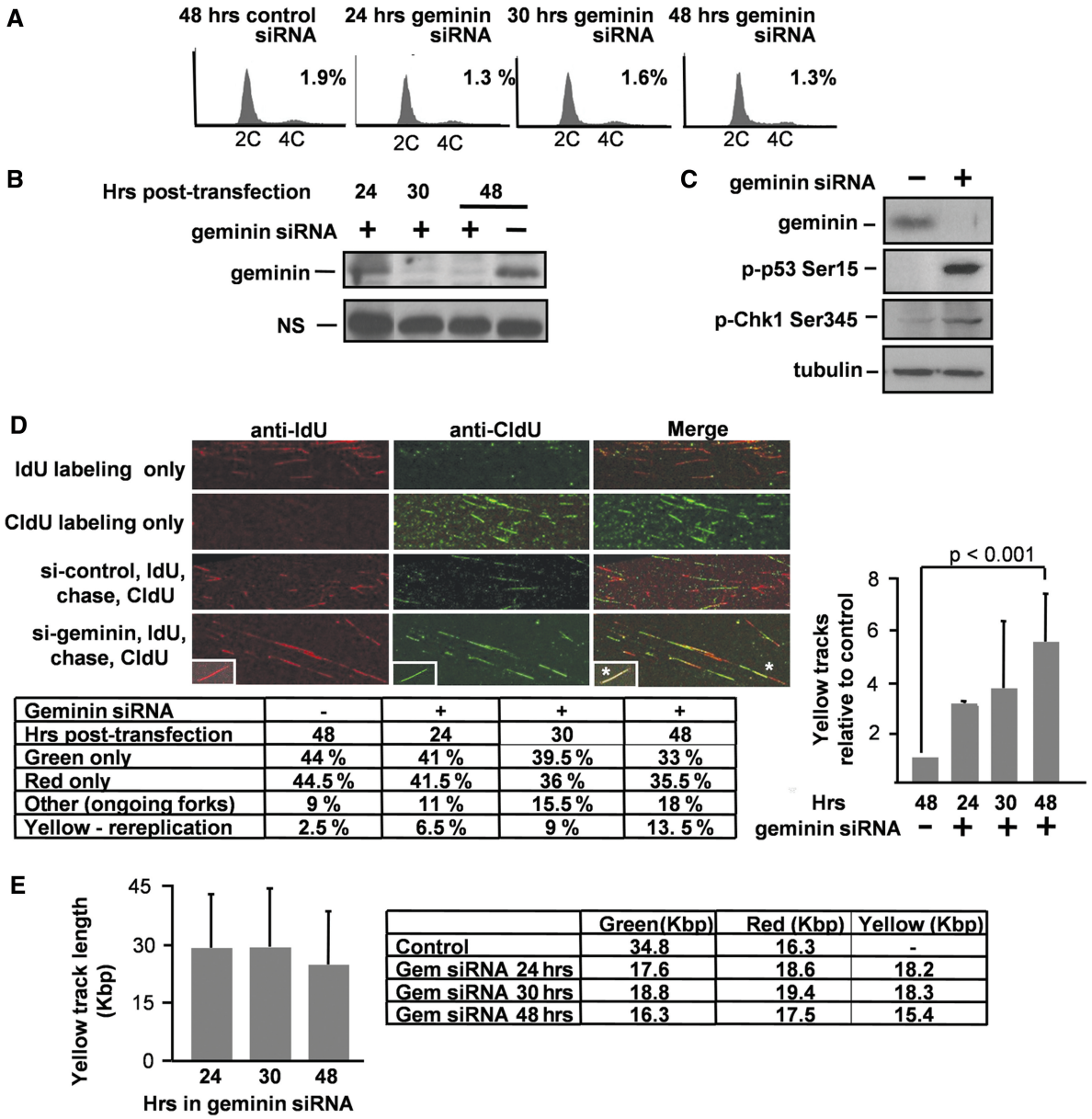


Figure 2. Fiber spreading detects re-replication. (**A**) Workflow of the labeling protocol and representative replication tracks. See Materials and methods section for details. (**B**) DNA fibers from HeLa cells synchronized and transduced with control adenovirus or Cdt1 adenovirus as in Figure 1A. Labeling was initiated immediately after the release from aphidicolin and cells were harvested approximately 70 min after release. Bar graph: a total of at least 200 replication tracks were analyzed from each sample and the relative fraction of yellow tracks (re-replication) in Cdt1-overproducing cells compared to control is plotted; $P < 0.001$. (**C**) Quantification of re-replication in asynchronous unperturbed HeLa cells and NHF1 cells. The number of yellow tracks produced by NHF1 cells was set to 1 and the relative number of yellow tracks in HeLa cells is plotted; $P < 0.05$. (**D**) Asynchronous HeLa cells were treated with 1 J/m^2 UV immediately before labeling with CldU and IdU by standard protocols; the 30 min chase period was omitted. Yellow tracks were scored as in B and reported as the fold-change in the irradiated cells compared to unirradiated control cells.

intracellular nucleotide pools when the second thymidine analog was added. With this protocol, actively progressing replication forks produce two distinct tracks with a clear unlabelled region between them and no overlap (Figure 2A, left panels). We confirmed the staining specificity by labeling a set of control cells with either CldU only or with IdU only but staining with both antibodies (for an example, see Figure 3D). Small numbers of cells were applied to the slides and only tracks that were clearly separate from neighboring tracks were analyzed. Finally, the fluorescence emissions of the two secondary antibodies were scanned sequentially by confocal microscopy rather than at the same time to minimize spectral overlap.

The analysis of several hundred fibers from each sample clearly revealed a significant number of yellow tracks when Cdt1 was overproduced in very early S phase (Figure 2B). Some tracks containing extensive yellow regions had nearly complete overlap of the red and green signals (an example is shown in Figure 2A, right panels) and some had more complex combinations with flanking single-labeled regions (data not shown). Presumably these differences related to the relative timing of the first and second replication events. Quantification of hundreds of distinct tracks from each sample revealed an approximate four-fold increase in the number of tracks with substantial regions of yellow signal from Cdt1-overproducing cells compared to control cells (Figure 2B). The presence of coincident replication tracks from two distinct labeling pulses is consistent with the immediate refiring of early origins and we suggest



Geminin siRNA	-	+	+	+
Hrs post-transfection	48	24	30	48
Green only	44 %	41 %	39.5 %	33 %
Red only	44.5 %	41.5 %	36 %	35.5 %
Other (ongoing forks)	9 %	11 %	15.5 %	18 %
Yellow - rereplication	2.5 %	6.5 %	9 %	13. 5 %

	Green(Kbp)	Red (Kbp)	Yellow (Kbp)
Control	34.8	16.3	-
Gem siRNA 24 hrs	17.6	18.6	18.2
Gem siRNA 30 hrs	18.8	19.4	18.3
Gem siRNA 48 hrs	16.3	17.5	15.4

Figure 3. Quantification of re-replication in unperturbed and geminin-depleted cells. (**A**) NHF1 cells were transfected with siRNA targeting geminin or GFP as a control and harvested at 24, 30 and 48 h after transfection. DNA content was determined by flow cytometric analysis of a portion of the harvested cells. The percentage of cells with greater than 4C DNA content is indicated on each histogram. (**B**) Immunoblot analysis of a portion of cells from (A) to detect endogenous geminin and a non-specific band as a loading control. (**C**) Immunoblot analysis of phospho-53 (Ser 15) and phospho-Chk1 (Ser 345) from cells in A transfected with siRNA for 48 h. (**D**) Representative fields of stained DNA fibers. Single labeling with CldU or IdU only (stained with antibodies to both) demonstrates minimal cross-reactivity. Examples of representative yellow tracks are marked with asterisks. Table: Quantification of all re-replication tracks in siRNA transfected NHF1 cells from (A). The category 'Other' includes tracks with green into red, green flanked with red and red or green tracks with yellow ends. Bar graph: Comparison of geminin-depleted cells to control cells: 24 h, $P = 0.02$; 30 h, $P < 0.001$; 48 h, $P < 0.001$. (**E**) Measurement of re-replication track lengths from the samples in D. Comparison of yellow track lengths to those in geminin-depleted cells: 24 h; 30 h, $P = 0.98$; 48 h, $P = 0.2$.

that these yellow tracks are the direct visualization of single molecules of re-replicated DNA in early S phase.

HeLa cells produce coincidently labeled tracks in unperturbed cell cycles

In the course of these experiments we were surprised at the high number of yellow tracks that were detectable in the control HeLa cells not overproducing Cdt1. Individual yellow tracks in the control cells were indistinguishable in length or staining pattern from the yellow tracks in Cdt1-overproducing cells (data not shown). The control cells had not been intentionally manipulated to perturb re-replication control and yet they consistently produced yellow tracks (Figure 2B). Because we have shown that the number of yellow tracks increase when HeLa cells are

manipulated to induce re-replication, we were interested to determine if the yellow tracks in these unperturbed cells represented a basal level of re-replication in HeLa cells. First, we considered the possibility that the synchronization procedure that arrested cells in early S phase might have been responsible for this observation, so we repeated the experiment in asynchronously growing HeLa cells. In the absence of cell-synchronizing drugs, the percentage of yellow tracks dropped ~2-fold (~6%), suggesting that holding cells for a period of time in early S phase could promote origin relicensing and re-firing. Importantly however, these findings may suggest that re-replication is not a rare event even in unperturbed HeLa cells.

HeLa cells are transformed cells and as a result suffer multiple disruptions to normal cell cycle and replication controls. Because of these genetic alterations, the HeLa cell line, like most tumor cell lines, shows relative genomic instability (32,33). A higher rate of re-replication in each cell cycle is one potential contribution to genomic instability in cancer cell lines. If so, then HeLa cells would be predicted to re-replicate at a higher rate than non-transformed (more normal) cells. To test that idea, we assayed NHF1-hTert fibroblasts (hereafter NHF1) for re-replication tracks. This cell line was established by telomerase expression in primary human diploid fibroblasts and thus has not been subjected to selection for endogenous mutations to escape senescence or promote aberrant growth (34). Using the same labeling and staining protocol in Figure 2A, samples of asynchronously growing NHF1 and HeLa cells were processed simultaneously and assayed for yellow replication tracks. Strikingly, compared to NHF1 cells, unperturbed HeLa cells are 2.5 times *more* likely to produce yellow tracks (Figure 2C).

While these observations point to re-replication as the most likely source of the increased yellow tracks in HeLa cells, it was also possible that they were produced by some unrelated form of DNA synthesis, such as DNA damage. To determine if DNA damage can induce similar yellow tracks we irradiated asynchronous HeLa cells with 1 J/m^2 of UV and analyzed the replication tracks both before and after UV. This dose of UV is sufficient to induce significant replication stress, but is sublethal (P.D. Chastain, unpublished observations). Rather than stimulating the generation of yellow tracks however, UV irradiation actually *reduced* the number of yellow tracks produced by HeLa cells (Figure 2D). These observations still cannot exclude the possibility that some form of unusual replication could lead to yellow tracks. However, the reduced number of yellow tracks in UV-treated cells argues against the interpretation that damage-induced stress contributes to the generation of yellow tracks and further supports the conclusion that these events include re-replicated DNA.

Re-replication in geminin-depleted non-transformed cells

Cdt1 overproduction or geminin depletion induces many tumor cell lines to accumulate DNA content greater than 4C, but some tumor cell lines and virtually all non-transformed cells do not (9,14). For this reason, it has been difficult to directly quantify re-replication in non-transformed cells. Re-replication can be induced by depleting cells of the Cdt1 inhibitor geminin and in tumor cell lines this treatment results in a robust re-replication phenotype measurable by flow cytometric analysis of total DNA content (10,11). We attempted to induce re-replication in NHF1 cells by transfecting them with geminin siRNA or a control siRNA targeting GFP and then labeling as in Figure 2A prior to harvesting at 24, 30 or 48 h post-transfection. Samples were analyzed by flow cytometry (Figure 3A), by immunoblotting to confirm geminin depletion (Figures 3B and 3C) and DNA fibers were analyzed for re-replication (Figure 3D). We observed no change in overall DNA content in these cells even 48 h after geminin siRNA transfection (Figure 3A). Nevertheless we inferred that some re-replication took place because two checkpoint markers known to be induced during re-replication, phosphorylated Chk1 and p53 (9–11), were induced in the geminin-depleted NHF1 cells (Figure 3C). Strikingly, the proportion of yellow replication tracks steadily increased from 24 to 48 h after geminin depletion (Figure 3D). By 48 h, more than 13% of all replication tracks contained both nucleotide analogs, a difference of more than 5-fold compared to the control transfected cells ($P < 0.001$). The accumulation of yellow tracks appeared at the expense of 'green only' and 'red only' tracks. These singly-labeled tracks include newly fired origins and replication termination events respectively but due to the 30 min chase period, many of these could also represent ongoing replication forks. Geminin depletion also induced an ~2-fold increase in tracks that contained both labels (Figure 3D, table), but by our stringent standards these did not score as re-replication that was clearly distinguishable from ongoing replication. We thus conclude that robust origin refiring occurred in geminin-depleted NHF1 cells despite the fact that the overall cellular DNA content was not detectably increased.

Since the labeling with IdU followed the labeling with CldU by 40–70 min, we assume that some origins fired at least twice in relatively close succession. Immediate refiring of an origin would generate a second set of bi-directional forks that travel on the same stretch of DNA. Some have speculated that replication forks from re-fired origins could travel faster than normal since the chromatin structure behind the first fork may be temporarily more permissive to fork movement (13). Others have speculated that two replication forks on the same strand would slow fork movement because re-replication triggers DNA damage checkpoints that slow replication (35). Our ability to directly visualize re-replication on single DNA fibers permitted the estimation of replication fork speed from re-fired origins. We measured the length of yellow tracks in NHF1 cells depleted of geminin for 24, 30 or 48 h. The average yellow track at 24 and 30 h was approximately 18 kb, (Figure 3E) and since the pulse of the second label was 10 min, this corresponds to a fork speed of 1.8 kb/min. We note that these tracks are much more than 10 times longer than the 200-bp fragments recently described by Gomez *et al.* (36), although we cannot rule out a relationship between the released origin fragments observed by that group and re-replication detected here. At 48 h post-transfection, geminin-depleted NHF1 cells show robust checkpoint activation as determined by

phosphorylation of Chk1 and p53 (Figure 3C). At this time point, the average yellow track length dropped slightly to 15 kb, but this difference was not statistically significant ($P = 0.2$). Normal replication fork speeds in unperturbed S phase have been measured by us in NHF1 cells and by other investigators and all are in close agreement of ~1.5 kb/min. By this analysis, re-replicating forks travel at close to the same speed as normal forks. We noted that in control cells, the average length of the IdU tracks (second label) was less than half that of the CldU tracks (first label), which is expected given their respective labeling times. Interestingly however, geminin-depleted cells produced shorter CldU tracks (Figure 3E, table) suggesting that these tracks were produced by forks that have slowed in response to the effects of geminin depletion, perhaps due to the effects of Chk1 activation on elongation (37). More importantly for the purposes of this study however, the fact that the yellow tracks are of a similar length to the singly labeled tracks further supports the conclusion that the yellow tracks are the result of re-replication.

DISCUSSION

In this study we have developed a highly sensitive method for single-molecule detection of re-replication and have applied it to the analysis of re-replication in early S phase in HeLa cells and in a non-transformed cell line. Our approach relies on the detection of two nucleotide analogs incorporated at different times in S phase into the same chromosomal DNA. Staining with red and green fluorescent antibodies specific to the different nucleotides produces yellow replication tracks on merged micrographs that can be readily quantified. It is possible that yellow tracks have been noted by other investigators during fiber spreading experiments, but these signals would likely have been attributed to cross-reactivity from antibodies or chance deposition of tracks from different DNA fibers on the slide, and thus might not have received much attention. Several features of this study rule out such trivial explanations for the yellow tracks in our experiments however. First, we increased the staining stringency for our antibodies to reduce cross-reactivity to less than 5% and we confirmed the specificity of the staining with single-label controls (e.g. Figure 3D). Second, we introduced a chase period between the two labeling periods equivalent to the first pulse to ensure that all of the first label was depleted before the second label was added (e.g. the gap in Figure 2A). Third, we were very conservative in scoring yellow signals as true re-replication tracks. A few small foci of yellow signal can sometimes be seen in standard fiber spreading protocols where red signal meets green signal on the same track, but these small signals were not scored as positives in our analysis. Fourth, small numbers of cells were applied to the slides for the combing experiments to ensure that tracks were separated enough to be clearly identified. We have noted extremely rare instances of tracks that lie together on slides, and these pairs are easily identified (and therefore excluded) by the double thickness of the fluorescent signal and the fact that their ends are offset from one another. Fifth, we showed that neither DNA damage nor its associated replication stress can account for the increased yellow tracks produced by Cdt1-overproducing cells. This observation argues against an interpretation that the yellow tracks are the result of DNA repair synthesis. Moreover, the length of the yellow tracks is consistently much longer than any characterized DNA repair synthetic events in eukaryotic cells and this length is quite close to the normal length of tracks produced from fired origins (Figure 3E). Finally and most importantly, any artifacts that could have produced yellow tracks cannot account for the marked increase in yellow tracks that was reproducibly observed when Cdt1 was overproduced or geminin was depleted. We induced re-replication by two entirely different techniques, recombinant adenoviral transduction and siRNA transfection, targeting two different genes, Cdt1 and geminin, so any off-target effects to explain this increase can be ruled out.

Using this fiber-spreading procedure we were able to detect re-replication in early S phase long before cells had accumulated supraphysiological DNA levels. The ability to detect re-replication shortly after it begins (within 1 h) permits the evaluation of immediate effects that are not influenced by the long-term cellular responses to re-replication-induced DNA damage. For example, we observe slow S phase progression from Cdt1 overproduction, but little to no Chk1 or Chk2 phosphorylation within the first 8–9 h of re-replication (E.S.D., unpublished observations), whereas long-term overproduction of Cdt1 induces robust Chk1 and Chk2 phosphorylation (9,14,28). The mechanism by which Cdt1 overproduction slows S phase is likely a direct consequence of re-replication in early S phase. Continual re-licensing and re-firing of early origins may act to titrate replication factors away from the rest of the genome or may induce other forms of a replication stress response not detectable as activation of Chk1 and Chk2.

A somewhat unexpected result that stems directly from the sensitivity of this assay is the high number of re-replication tracks produced by HeLa cells compared to the more normal NHF1 cells. NHF1 cells produce a small but quantifiable number of yellow tracks in unperturbed cell cycles, but it is difficult to determine if those tracks are from re-replication or if they are produced by some other process related to the experiments themselves, telomerase activity, background staining, etc. Importantly however, these cell lines were labeled and processed simultaneously, so the difference between their re-replication rates almost certainly reflects a real biological difference rather than an effect of the experimental technique. This observation implies that HeLa cells already have perturbations in origin licensing control and routinely re-replicate in culture even when there has been no acute experimental manipulation. If so, then HeLa cells must have some means of accommodating the aberrant additional replication forks without triggering either a permanent checkpoint arrest or acquiring lethal amounts of DNA damage. Moderate deregulation of re-replication control in budding yeast causes no overt growth defect but renders those cells highly dependent on DNA repair

activities (38). This observation suggests that cells with moderate re-replication can appear to grow normally, but these cells are constantly subjected to a level of re-replication-associated DNA damage. It may be that HeLa cells exist in a similar state with a constant amount of low-level re-replication. Repeated rounds of re-replication, DNA damage and repair would likely contribute to genome instability. It is possible that absolute 'once and only once' DNA replication is not actually achieved by HeLa cells and by extension, other tumor cell lines as well.

A number of genetic lesions in the HeLa cell line are likely to account not only for the possible low-level re-replication, but also the ability to re-replicate significant portions of the genome when Cdt1 is overproduced (Figures 1A and 1D). Deregulation of the Rb-E2F transcriptional program by the HPV E7 protein results in high-level expression of the majority of the replication proteins including those that are directly involved in licensing control such as Cdt1 and Cdc6 (39–42). Excessive endogenous amounts of replication factors may not be regulated as tightly as they are in normal cells leading to more opportunities for origin re-licensing. Low expression of p53 as a result of the HPV E6 protein has multiple effects on cell-cycle progression and a variety of DNA metabolic events including replication and repair. Insufficient p53 could promote S phase Cdk activity and increase the likelihood that a relicensed origin actually fires, though we note that the absence of p53 is not a strict requirement for re-replication and therefore is not the sole explanation for differences in the propensity to re-replicate (10,11). In addition, recent studies have demonstrated correlations between the activity of the ATR-Chk1 pathway and the ability of cells to re-replicate when Cdt1 is overproduced (14,43). An exciting implication from our findings is that different cancers may be characterized by different propensities to re-replicate based on individual constellations of genetic abnormalities. If so, then the ability to sensitively quantify endogenous re-replication rates may contribute to predicting differences in overall genome stability.

In assays for increases in DNA content above 4C NHF1 cells did not re-replicate when Cdt1 was overproduced (J.R.H., unpublished observations) or when geminin was depleted (Figure 3A). Similar failure to detect greater than 4C DNA content has also been observed in other untransformed cells (9–11,14). In such cases re-replication can usually only be indirectly inferred from the activation of DNA damage checkpoint markers. NHF1 cells have constitutive telomerase expression, but apparently normal Rb, p53 and ATR pathways (28,34,44). Failure to observe overt re-replication by flow cytometry could have been a consequence of profound resistance to origin relicensing and refiring, or it could have been the result of strong checkpoint effects on replication elongation or other events required to produce cells with greater than 4C DNA content or both. Our finding that NHF1 cells can produce robust re-replication tracks when geminin is depleted suggests that these cells are not extraordinarily resistant to origin relicensing and refiring. The undetectable increase in genomic DNA content from these refired origins could be explained if only a subset of origins is sensitive to origin licensing perturbation. In support of that idea, even when large increases in DNA content were induced by Cdt1 overproduction in tumor cell lines, the re-replication was unevenly distributed across the chromosomes (9). It may be possible in the future to combine this technique with sequence specific probes (once more human origins have been mapped) to determine if some origins are more likely than others to re-replicate. The ability to directly examine re-replication tracks in a wide variety of cell lines is likely to be a useful addition to the available tools to study genome stability.

ACKNOWLEDGEMENTS

NHF1-tert cells were a gift from W. Kaufmann. We gratefully acknowledge helpful discussions from A. Sancar, D. Kaufman and members of the Cook lab as well as technical support from K. S. Luce, E. Heenan, K. McKernan and A. Williamson.

FUNDING

National Institutes of Health (K01-CA094907 to J.G.C.); American Cancer Society (GMC-111880 to J.G.C.); Cancer Cell Biology Training Grant (T32-CA71341 to E.S.D.). Funding for open access charge: American Cancer Society (GMC-111880).

Conflict of interest statement. None declared.

REFERENCES

1. Arias,E.E. and Walter,J.C. (2007) Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. *Genes Dev.*, **21**, 497–518.
2. Blow,J.J. and Dutta,A. (2005) Preventing re-replication of chromosomal DNA. *Nat. Rev. Mol. Cell Biol.*, **6**, 476–486.
3. Machida,Y.J., Hamlin,J.L. and Dutta,A. (2005) Right Place, Right Time, and Only Once: Replication Initiation in Metazoans. *Cell*, **123**, 13–24.
4. Arentson,E., Faloon,P., Seo,J., Moon,E., Studts,J.M., Fremont,D.H. and Choi,K. (2002) Oncogenic potential of the DNA replication licensing protein CDT1. *Oncogene*, **21**, 1150–1158.
5. Liontos,M., Koutsami,M., Sideridou,M., Evangelou,K., Kletsas,D., Levy,B., Kotsinas,A., Nahum,O., Zoumpourlis,V., Kouloukoussa,M. *et al.* (2007) Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. *Cancer Res.*, **67**, 10899–10909.
6. Sclafani,R.A. and Holzen,T.M. (2007) Cell cycle regulation of DNA replication. *Annu. Rev. Genet.*, **41**, 237–280.
7. Bell,S.P. and Dutta,A. (2002) DNA replication in eukaryotic cells. *Annu. Rev. Biochem.*, **71**, 333–374.
8. Diffley,J.F. (2004) Regulation of early events in chromosome replication. *Curr. Biol.*, **14**, R778–R786.
9. Vaziri,C., Saxena,S., Jeon,Y., Lee,C., Murata,K., Machida,Y., Wagle,N., Hwang,D.S. and Dutta,A. (2003) A p53-dependent checkpoint pathway prevents rereplication. *Mol. Cell.*, **11**, 997–1008.
10. Melixetian,M., Ballabeni,A., Masiero,L., Gasparini,P., Zamponi,R., Bartek,J., Lukas,J. and Helin,K. (2004) Loss of Geminin induces rereplication in the presence of functional p53. *J. Cell. Biol.*, **165**, 473–482.

11. Zhu,W., Chen,Y. and Dutta,A. (2004) Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. *Mol. Cell. Biol.*, **24**, 7140–7150.
12. 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.
13. Davidson,I.F., Li,A. and Blow,J.J. (2006) Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision. *Mol. Cell.*, **24**, 433–443.
14. Liu,E., Lee,A.Y., Chiba,T., Olson,E., Sun,P. and Wu,X. (2007) The ATR-mediated S phase checkpoint prevents rereplication in mammalian cells when licensing control is disrupted. *J. Cell. Biol.*, **179**, 643–657.
15. Braden,W.A., Lenihan,J.M., Lan,Z., Luce,K.S., Zagorski,W., Bosco,E., Reed,M.F., Cook,J.G. and Knudsen,E.S. (2006) Distinct action of the retinoblastoma pathway on the DNA replication machinery defines specific roles for cyclin-dependent kinase complexes in prereplication complex assembly and s-phase progression. *Mol. Cell. Biol.*, **26**, 7667–7681.
16. Hall,J.R., Kow,E., Nevis,K.R., Lu,C.K., Luce,K.S., Zhong,Q. and Cook,J.G. (2007) Cdc6 stability is regulated by the Huwe1 ubiquitin ligase after DNA damage. *Mol. Biol. Cell*, **18**, 3340–3350.
17. Cook,J.G., Chasse,D.A. and Nevins,J.R. (2004) The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells. *J. Biol. Chem.*, **279**, 9625–9633.
18. Chastain,P.D., 2nd, Heffernan,T.P., Nevis,K.R., Lin,L., Kaufmann,W.K., Kaufman,D.G. and Cordeiro-Stone,M. (2006) Checkpoint regulation of replication dynamics in UV-irradiated human cells. *Cell Cycle*, **5**, 2160–2167.
19. Unsal-Kacmaz,K., Chastain,P.D., Qu,P.P., Minoo,P., Cordeiro-Stone,M., Sancar,A. and Kaufmann,W.K. (2007) The human Tim/Tipin complex coordinates an Intra-S checkpoint response to UV that slows replication fork displacement. *Mol. Cell. Biol.*, **27**, 3131–3142.
20. Lee,A.Y., Liu,E. and Wu,X. (2007) The Mre11/Rad50/Nbs1 complex plays an important role in the prevention of DNA rereplication in mammalian cells. *J. Biol. Chem.*, **282**, 32243–32255.
21. Nishitani,H., Sugimoto,N., Roukos,V., Nakanishi,Y., Saijo,M., Obuse,C., Tsurimoto,T., Nakayama,K.I., Nakayama,K., Fujita,M. *et al.* (2006) Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. *EMBO. J.*, **25**, 1126–1136.
22. Jin,J., Arias,E.E., Chen,J., Harper,J.W. and Walter,J.C. (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol. Cell.*, **23**, 709–721.
23. Li,X., Zhao,Q., Liao,R., Sun,P. and Wu,X. (2003) The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *J. Biol. Chem.*, **278**, 30854–30858.
24. Takeda,D.Y., Parvin,J.D. and Dutta,A. (2005) Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase. *J. Biol. Chem.*, **280**, 23416–23423.
25. Girard,F., Strausfeld,U., Fernandez,A. and Lamb,N.J.C. (1991) Cyclin a is required for the onset of DNA replication in mammalian fibroblasts. *Cell*, **67**, 1169–1179.
26. Walker,D.H. and Maller,J.L. (1991) Role for cyclin A in the dependence of mitosis on completion of DNA replication. *Nature*, **354**, 314–317.
27. Ferenbach,A., Li,A., Brito-Martins,M. and Blow,J.J. (2005) Functional domains of the Xenopus replication licensing factor Cdt1. *Nucl. Acids Res.*, **33**, 316–324.
28. Hall,J.R., Lee,H.O., Bunker,B.D., Dorn,E.S., Rogers,G.C., Duronio,R.J. and Cook,J.G. (2008) CDT1 and CDC6 are destablized by rereplication-induced DNA damage. *J. Biol. Chem.*, **283**, 25356–25363.
29. Jackson,D.A. and Pombo,A. (1998) Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J. Cell. Biol.*, **140**, 1285–1295.
30. Aten,J.A., Bakker,P.J., Stap,J., Boschman,G.A. and Veenhof,C.H. (1992) DNA double labelling with IdUrd and CldUrd for spatial and temporal analysis of cell proliferation and DNA replication. *Histochem. J.*, **24**, 251–259.
31. Herrick,J., Stanislawski,P., Hyrien,O. and Bensimon,A. (2000) Replication fork density increases during DNA synthesis in X. laevis egg extracts. *J. Mol. Biol.*, **300**, 1133–1142.
32. Tlsty,T.D. (1990) Normal diploid human and rodent cells lack a detectable frequency of gene amplification. *Proc. Natl Acad. Sci. USA*, **87**, 3132–3136.
33. 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.
34. Heffernan,T.P., Simpson,D.A., Frank,A.R., Heinloth,A.N., Paules,R.S., Cordeiro-Stone,M. and Kaufmann,W.K. (2002) An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol. Cell. Biol.*, **22**, 8552–8561.
35. Conti,C., Herrick,J. and Bensimon,A. (2007) Unscheduled DNA replication origin activation at inserted HPV 18 sequences in a HPV-18/MYC amplicon. *Genes Chromosomes Cancer*, **46**, 724–734.
36. Gomez,M. and Antequera,F. (2008) Overreplication of short DNA regions during S phase in human cells. *Genes Dev.*, **22**, 375–385.
37. Seiler,J.A., Conti,C., Syed,A., Aladjem,M.I. and Pommier,Y. (2007) The Intra-S-Phase Checkpoint Affects both DNA Replication Initiation and Elongation: Single-Cell and -DNA Fiber Analyses. *Mol. Cell. Biol.*, **27**, 5806–5818.
38. Archambault,V., Ikui,A.E., Drapkin,B.J. and Cross,F.R. (2005) Disruption of mechanisms that prevent rereplication triggers a DNA damage response. *Mol. Cell. Biol.*, **25**, 6707–6721.
39. Leone,G., DeGregori,J., Yan,Z., Jakoi,L., Ishida,S., Williams,R.S. and Nevins,J.R. (1998) E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev.*, **12**, 2120–2130.
40. Ishida,S., Huang,E., Zuzan,H., Spang,R., Leone,G., West,M. and Nevins,J.R. (2001) Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. *Mol. Cell. Biol.*, **21**, 4684–4699.
41. Polager,S., Kalma,Y., Berkovich,E. and Ginsberg,D. (2002) E2Fs up-regulate expression of genes involved in DNA replication, DNA repair and mitosis. *Oncogene*, **21**, 437–446.
42. Ren,B., Cam,H., Takahashi,Y., Volkert,T., Terragni,J., Young,R.A. and Dynlacht,B.D. (2002) E2F integrates cell cycle progression with DNA repair, replication and G2/M checkpoints. *Genes Dev.*, **16**, 245–256.
43. Lin,J.J. and Dutta,A. (2007) ATR Pathway Is the Primary Pathway for Activating G2/M Checkpoint Induction After Re-replication. *J. Biol. Chem.*, **282**, 30357–30362.
44. Heffernan,T.P., Unsal-Kacmaz,K., Heinloth,A.N., Simpson,D.A., Paules,R.S., Sancar,A., Cordeiro-Stone,M. and Kaufmann,W.K. (2007) Cdc7-Dbf4 and the human S checkpoint response to UVC. *J. Biol. Chem.*, **282**, 9458–9468.