Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication

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The six main minichromosome maintenance proteins (Mcm2–7), which presumably constitute the core of the replicative DNA helicase, are present in chromatin in large excess relative to the number of active replication forks. To evaluate the relevance of this apparent surplus of Mcm2–7 complexes in human cells, their levels were down-regulated by using RNA interference. Interestingly, cells continued to proliferate for several days after the acute (>90%) reduction of Mcm2–7 concentration. However, they became hypersensitive to DNA replication stress, accumulated DNA lesions, and eventually activated a checkpoint response that prevented mitotic division. When this checkpoint was abrogated by the addition of caffeine, cells quickly lost viability, and their karyotypes revealed striking chromosomal aberrations. Single-molecule analyses revealed that cells with a reduced concentration of Mcm2–7 complexes display normal fork progression but have lost the potential to activate ''dormant'' origins that serve a backup function during DNA replication. Our data show that the chromatin-bound ''excess'' Mcm2–7 complexes play an important role in maintaining genomic integrity under conditions of replicative stress.

DNA combing | DNA replication | origin licensing

Rapidly proliferating cells start to prepare for DNA replication several hours before the actual S-phase, with the assembly of prereplication complexes (pre-RCs) at origins in telophase and early G_1. Pre-RC assembly, also referred to as ''origin licensing,'' consists in the recruitment of Mcm2–7 protein complexes by initiator proteins ORC, CDC6, and CDT1. ORC and CDC6 likely constitute a structural module with ATPase activity that opens and closes the ring-shaped MCM hexamer, facilitating its topological engagement with the DNA, whereas CDT1 cooperates in the loading reaction as a molecular chaperone (reviewed in refs. 1 and 2). Different lines of evidence indicate that Mcm2–7 constitute the core of the replicative DNA helicase in eukaryotic cells in association with CDC45 and the GINS complex (3, 4).

The maximum number of origins available in the subsequent S-phase is predetermined at the licensing stage, because additional pre-RCs cannot be assembled later in the cell cycle because of the inhibitory activity of the S, G_2 and M-phase cyclin-dependent kinases. This regulation establishes a temporal alternation of origin licensing and firing that is important to prevent DNA overreplication. In yeast, blending the licensing and firing periods by deletion of the CDK inhibitor Sic1 or by overexpression of the G_1 cyclin Cln2, greatly increased genomic instability (5, 6). In human cells, premature expression of Cyclin E during mitosis and G_1 interfered with the association of MCM proteins with chromatin and at the same time promoted the firing of the limited number of licensed origins, effectively accelerating the G_1–S transition (7). Nevertheless, cells continued to proliferate under these challenging conditions and accumulated karyotypic defects (8). These results are highly relevant because cyclin E deregulation is very common in cancer cells (9), and aberrant DNA replication has been observed during early tumorigenesis (10).

Although, in principle, only two DNA helicase activities are required to establish a bidirectional replication fork from each origin, a relatively large excess of MCM complexes are loaded at origins of replication and distributed along the chromatin. Their function is not well understood, and most of them are displaced from the DNA during S-phase, apparently without having played an active role in DNA replication. The ''MCM paradox'' refers to the fact that, at least in yeast, *Xenopus*, and *Drosophila*, it is possible to reduce the concentration of MCM proteins without impairing DNA replication (11–13) and also refers to the observation that the majority of MCM complexes do not localize to the sites of DNA synthesis in mammalian cells (reviewed in refs. 14 and 15). In the cell-free system of DNA replication in *Xenopus* egg extracts, the excess MCM proteins appear to activate dormant origins of replication under conditions of stress (16).

To evaluate the importance of MCM concentration in human cells, we have used RNA interference to modulate the expression of *Mcm2–7* genes and effectively reduce the concentration of Mcm2–7 proteins on chromatin. We have found conditions in which cells are capable of apparently normal DNA replication with a very reduced concentration (<5% of the normal level) of Mcm2–7 complexes. However, under these conditions of ''limited licensing,'' the cells progressively accumulated DNA lesions and displayed chromosomal fragility. An analysis of origin density revealed that the ''excess'' MCM proteins, although not necessarily active during an unperturbed S-phase, might activate a reservoir of backup origins that are required to recover from DNA replication stress. This function is essential to maintain genomic integrity in human cells.

Results

Cell Proliferation After the Acute Reduction of MCM Levels. The six *Mcm2–7* genes are essential in yeast, and the knockout of any of them in mammalian cells is expected to be lethal as well. In fact, one previous attempt at targeted silencing of *hMcm2* caused cell death (17). Our goal was to partially reduce the amount of chromatin-bound Mcm2–7 protein complexes without abolishing cell proliferation. With this purpose, different siRNA oligonucleotides targeting each one of the Mcm2–7 mRNAs were tested in HeLa cells [*Materials and Methods* and supporting information (SI) Fig. S1*A*]. Whereas transfection of the Mcm4–7 siRNAs rapidly prevented

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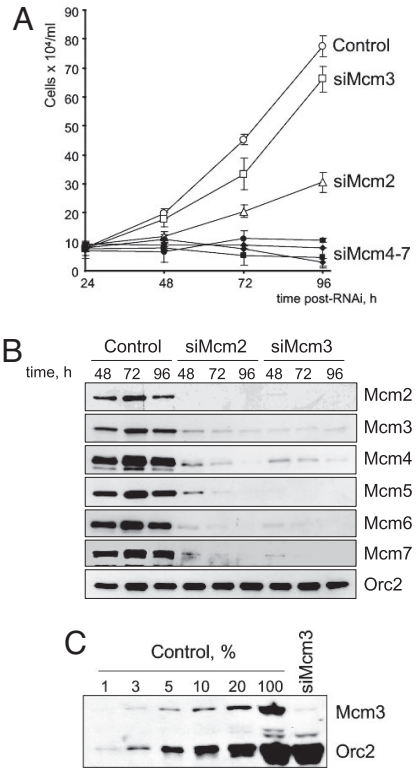

Fig. 1. Cell proliferation after MCM downregulation. (*A*) Proliferation curves after transfection of siRNA oligonucleotides targeting each one of the Mcm2–7 subunits (*Materials and Methods*). (*B*) Levels of individual MCM subunits on chromatin were determined by immunoblotting at 48, 72, and 96h after transfection of the indicated siRNAs. Orc2 is shown as loading control. (*C*) Quantification of the efficiency of Mcm3 knockdown. The amount of Mcm3 remaining in a total cell extract prepared 72 h after Mcm3 siRNA was determined by immunoblotting using different amounts of extract from untreated cells as reference. Orc2 levels are shown as loading control.

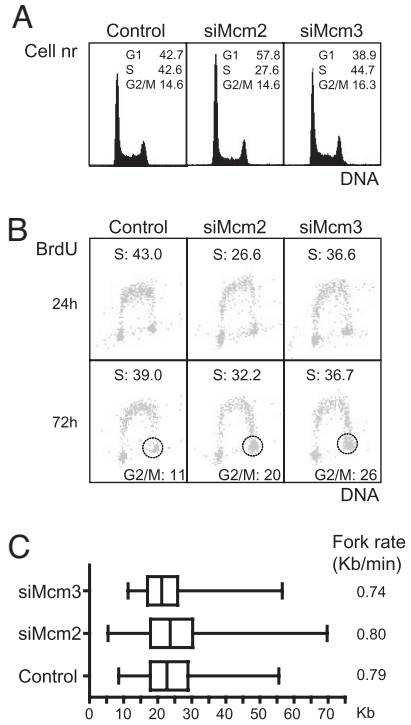

Fig. 2. S-phase dynamics after MCM downregulation. (*A*) DNA content, analyzed by flow cytometry 48 h after transfection with the indicated siRNA. The percentage of cells in each phase of the cell cycle was calculated by using ModFit software. (*B*) Cellular BrdU incorporation assay, carried out 24 or 72 h after transfection with the indicated siRNA, after a 30-min pulse with BrdU (*Materials and Methods*). (*C*) Fork progression rates. Control cells or cells treated with the indicated siRNA were pulsed with BrdU for 30 min and processed for DNA combing analysis 48 h after RNAi (*Materials and Methods*). The length of >100 BrdU tracks were measured in each case. In the box plot, the horizontal line represents the full range of experimental data, the box spans the interquartile range, leaving out the lower and upper quartiles, and the vertical line within the box indicates the median value.

cell proliferation (Fig. 1*A*) and activated an apoptotic response (Fig. S1*B*), cells transfected with siRNA against Mcm3 continued to proliferate normally, and cells transfected with siRNA against Mcm2 proliferated at a slower pace (Fig. 1*A*). The efficiency of both Mcm2 and Mcm3 siRNA treatments was very high for the duration of the experiment, reducing the levels of chromatin-associated Mcm2–7 (Fig. 1*B*) as well as the soluble nucleoplasmic pool (Fig. S1*C*). This point was confirmed by immunofluorescence detection of total and chromatin-bound MCM proteins (Fig. S1*D*).

A quantitative immunoblot at 72 h after transfection revealed that the cellular concentration of Mcm3 was reduced to ≈3% of its normal levels (Fig. 1*C*). Furthermore, down-regulation of Mcm2 or Mcm3 also reduced the levels of other MCM subunits (Fig. 1*B* and Fig. S1), probably because of the destabilization of the Mcm2–7 complex when one of its components is missing.

S-Phase Dynamics in Cells with Reduced MCM Concentration. We concentrated on the effects caused by Mcm2 and Mcm3 siRNAs, which probably create a situation of limited origin licensing compatible with cell proliferation. Analysis of the DNA content of cells treated with Mcm3 siRNA revealed an essentially normal cell cycle profile 48 h after siRNA transfection, whereas cells treated with Mcm2 siRNA had a slightly increased G_1 population (Fig. 2*A*) that could explain in part the slower proliferation rate observed after Mcm2 knockdown. Cellular BrdU incorporation assays confirmed that fewer cells were in S-phase after Mcm2 siRNA (Fig. 2*B*). Remarkably, by 72 h after Mcm2 or Mcm3 knockdown, cells started to accumulate in G_2, probably in response to DNA damage generated during replication (Fig. 2*B*; and see below).

The possibility that the reduction in MCM concentration could slow down replication forks was tested by single-molecule DNA analysis after a pulse of BrdU incorporation. The average length of BrdU stretches within a DNA fiber, divided by the duration of the BrdU pulse, gives an estimation of fork progression rate (Fig. 2*C*). No significant variations were observed (mean values 0.79 Kb/min for control, 0.80 Kb/min after Mcm2 siRNA, 0.74 Kb/min after Mcm3 siRNA), indicating that the exceeding MCM complexes are not required to maintain fork speed during elongation.

Hypersensitivity to Replication Stress and Activation of a Checkpoint Response. The incipient accumulation of cells in G_2 after 3 days of proliferation under conditions of limited licensing hinted at the activation of a cellular checkpoint and prompted us to extend the analysis for several additional days. The proliferative potential of cells treated with Mcm3 siRNA was further reduced 5–7 days after transfection (Fig. 3*A*, black lines). After 7 days, the inhibitory effect of the siRNA started to wear off (data not shown). Analysis of DNA content revealed a significant accumulation of cells in G_2 by day 5 (21.8–41.1%; Fig. 3*B i* and *ii*). The block occurred in G_2 rather than mitosis as indicated by the levels of phosphorylated H3, and it was caused by a checkpoint response because it could be relieved by caffeine, the inhibitor

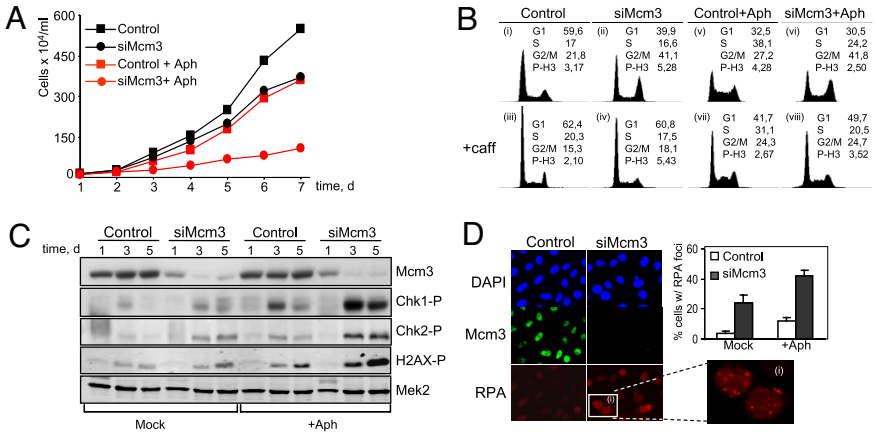

Fig. 3. Hypersensitivity to replicative stress and activation of the DDR. (*A*) Proliferation curves of control or Mcm3 siRNA-treated cells growing in regular medium (black lines) or medium supplemented with 0.1 μM aphidicolin (red lines). (*B*) DNA content of control cells (*i*) or cells treated with Mcm3 siRNA (*ii*), analyzed 120 h after RNAi. *iii* and *iv* show the same analyses in cells treated with 5 mM caffeine for 5 h before cell collection. *v–viii* are similar to *i–iv*, but cells were grown in the presence of 0.1 μM aphidicolin. (*C*) Detection of the activated forms of Chk1 and Chk2 kinases and the phosphorylated form of H2AX at days 1, 3, and 5 after Mcm3 siRNA treatment, in the absence or presence of 0.1 μM aphidicolin. The uppermost row of blots shows the reduction of Mcm3 levels. Mek2, a cytosolic kinase, is shown as loading control. (*D*) Immunostaining of Mcm3 (green) and RPA (red) at 120 h after RNAi. DNA was stained with DAPI (blue). The bar graph indicates the percentage of cells that score positive for two or more RPA foci ($n > 200$ in each case) either in the absence or presence of 0.1 μM aphidicolin.

of ATM–ATR kinases (Fig. 3*B iv*). Consistent with this idea, activation of checkpoint kinases Chk1 and Chk2 was observed (Fig. 3*C*).

Considering that cells were forced to proliferate under limited licensing conditions, the checkpoint response could be triggered by abnormal structures generated during DNA replication, such as collapsed replication forks, long stretches of ssDNA, or unresolved structures with complex topology. This point was confirmed by measuring the appearance of RPA foci, one of the first signaling events of the DNA damage response (DDR) during S-phase (18). RPA foci were detected in 25% of the cells after MCM knockdown, a 6-fold increase over control cells (Fig. 3*D*). The frequency of dsDNA breaks was also higher, as indicated by the levels of phosphorylation of histone H2AX (Fig. 3*C*) and the detection of 53BP1 foci, many of which overlapped with RPA foci (Fig. S2*A*).

We anticipated that if cells with reduced MCM concentration were sensitized to replicative stress, they would become hypersensitive to DNA replication inhibitors. Indeed, a low concentration (0.1 μM) of aphidicolin allowed the proliferation of control cells but virtually impaired proliferation after MCM knockdown (Fig. 3*A*, red lines). The treatment with aphidicolin caused a 2.2-fold increase in the S-phase in control cells (Fig. 3*B*, compare *i* and *v*), whereas cells with low MCM concentration continued to accumulate preferentially in G_2 (Fig. 3*B vi*), suggesting a strong checkpoint response induced by replicative stress. In this sense, the frequency of cells with RPA foci was higher in the presence of aphidicolin (Fig. 3*D*). As expected, these cells were also sensitive to low concentrations of hydroxyurea (Fig. S2*B*). The treatment with Mcm2 siRNA also caused DNA damage, activation of checkpoints, and hypersensitivity to replication inhibitors (Fig. S3).

Increased Chromosome Instability. As shown above, cells proliferating with limited origin licensing accumulated DNA lesions during replication and activated a DNA damage response to prevent mitosis. To estimate the extent of chromosomal damage in the cell population arrested in G_2, caffeine was added to abrogate the checkpoint and drive the cells into mitosis, and chromosome spreads were scored for signs of instability such as breaks, gaps, and aberrant rearrangements (triradial or quatriradial chromosomes). Cells proliferating with reduced MCM concentration displayed all these signs of chromosomal instability, which were further enhanced by the presence of aphidicolin (Fig. 4). It should also be noted that after 5 days of proliferation with low MCM concentration, a significant fraction (12.4%) of interphase cells had micronuclei, indicative of aberrant chromosome segregation and/or cytokinesis. This effect was more acute (29.6%) in the presence of aphidicolin (Fig. S2*C*).

Limited Activation of Backup Replication Origins. Under conditions of limited licensing, cells displayed a reduced tolerance to

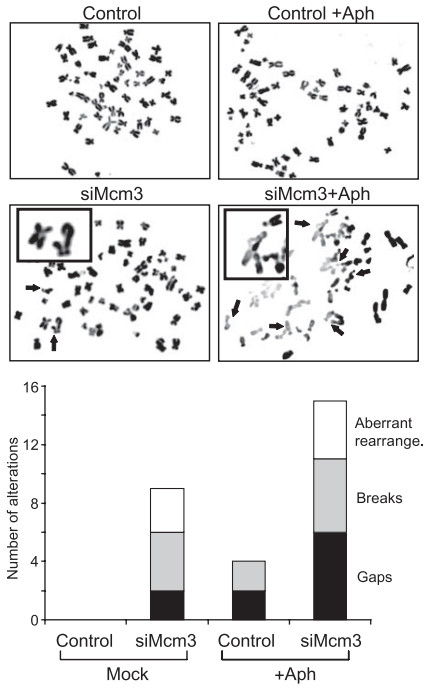

Fig. 4. Increased chromosomal instability under conditions of limited licensing. Metaphase spreads of control or Mcm3 siRNA-treated cells (96 h after siRNA), grown in the absence or in the presence of 0.1 μM aphidicolin, were scored for chromosome gaps (dark gray boxes), breaks (light gray boxes), and aberrant rearrangements (white boxes). Ten metaphases of each cell condition were analyzed.

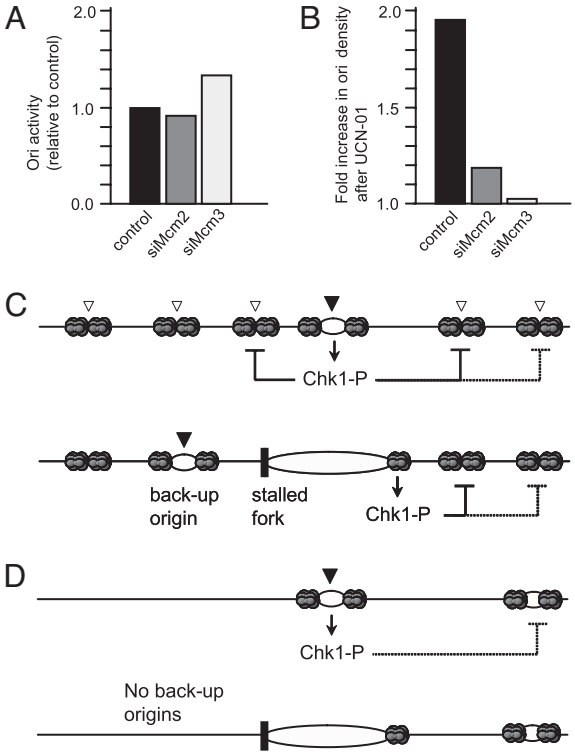

Fig. 5. Loss of backup replication origins. (*A*) Relative frequency of origin activation in control cells or cells treated for 48 h with the indicated siRNA, as determined by single-molecule analyses. (*B*) Fold-increase in origin activation when the same cells were treated with UCN-01 for 5 h. (*C*) Schematic of DNA replication under full licensing conditions: MCM complexes are located in multiple sites within a replicon. The activation of one ''main origin'' (black arrow) sends a signal, mediated by Chk1, to inhibit the activation of nearby origins (open arrows). In the eventuality of a fork collapse, the activation of a previously ''silent'' origin rescues the stretch of nonreplicated DNA. (*D*) DNA replication under limited licensing conditions. Fewer potential origins are licensed than in the control situation, and the rescue of stalled forks is compromised by the lack of backup origins.

replication stress during S-phase. Because the speed of fork progression was not affected by the reduction of MCM levels (Fig. 2*C*), the problem could emerge from alterations in origin usage. The frequency of origin activation in a cell population was estimated by single-molecule analyses using a IdU/CldU double-labeling technique that unequivocally distinguishes newly fired origins from other replication intermediates (Fig. S4). Interestingly, the density of active origins was altered only slightly by MCM knockdown relative to control cells (0.91-fold and 1.35-fold variation after Mcm2 and Mcm3 siRNAs, respectively; Fig. 5*A*).

Besides the "main" origin activated in each replicon, previous work has suggested that additional origins may fire under conditions of stress, e.g., after the collapse of a fork emerging from the main origin (16). During an unperturbed S-phase, active forks send a signal mediated by Chk1 kinase to prevent the firing of neighboring origins. In fact, inhibition of Chk1 doubled the density of active origins (19), increasing the frequency of DNA breaks (20). We hypothesized that the potential to activate "extra" origins upon Chk1 inhibition might be altered under conditions of limited licensing. Indeed, origin density increased by 2-fold in control cells treated with UCN-01 for 5 h, in agreement with a previous report (19), whereas cells treated with siRNA to Mcm2 or Mcm3 essentially failed to activate additional origins (1.19- and 1.02-fold enrichment, respectively; Fig. 5*B*). Furthermore, Chk1 inhibition in control cells caused a 5-fold increase in the frequency of DNA fibers displaying interspersed labeling, that likely represent stretches of DNA with unchecked origin activation (Fig. S4). This effect was only modest in cells treated with Mcm2 siRNA (1.8-fold) and undetectable in cells treated with Mcm3 siRNA. Therefore, full licensing conditions are necessary to enable a reservoir of "silent" origins that may serve backup functions in situations of stress.

Discussion

The MCM Paradox in Human Cells. The poor definition of mammalian origins of replication has made difficult to quantify with precision the amount of chromatin-bound MCM complexes relative to the number of origins. In any case, there must be a large excess of MCM relative to the number of replication forks, because the majority of chromatin-associated MCM complexes do not localize with sites of DNA synthesis (21). We have created conditions of cell proliferation under limited origin licensing in human cells by reducing the effective concentration of MCM complexes. Interestingly, a global reduction in Mcm2–7 levels was achieved with siRNA molecules targeting a single subunit. Most likely, the stability of the Mcm2–7 complex is compromised to different extents by the down-regulation of any of its individual components. We also observed that those MCM subunits partially resilient to degradation after siRNA (e.g., Mcm4 and Mcm6 after siRNA to Mcm3; Fig. S1*C*) were not detected in the chromatin-enriched fraction (Fig. 1*B*), suggesting that a full Mcm2–7 complex has to exist in soluble form before its engagement with the DNA.

It is remarkable that only the silencing of Mcm2 and Mcm3 expression was compatible with cell proliferation. It could be inferred that Mcm2 or Mcm3 are dispensable for mammalian cell proliferation, but we do not believe this is the case, based on the midterm effects observed in our study. Rather, we favor the idea that the acute down-regulation of Mcm2 and Mcm3 still permitted the formation of a limited but sufficient amount of active Mcm2–7 complexes. The acute down-regulation of Mcm4–7, in contrast, may reduce the number of Mcm2–7 complexes beyond a tolerable threshold. It is also possible that the Mcm4–7 subunits have essential roles outside the MCM complex. For instance, Mcm5 participates in transcription (22), and Mcm7 interacts with Rad17 and ATRIP and is likely required for activation of the DDR (23, 24).

MCMs and the Control of "Excess" Origins of Replication: A Model. We have found that the acute reduction (>95%) of the cellular concentration of MCM proteins in a human cell line allowed DNA replication and cell proliferation for several days (Figs. 1 and 2). However, cells proliferating under limited licensing conditions progressively accumulated DNA damage during S phase, became strongly sensitive to drugs that interfere with DNA replication, activated the DDR pathway, and displayed chromosome instability (Figs. 3 and 4).

Based on previous studies and our data, we favor a model in which the distribution of MCM proteins along the chromatin sets the limits for total origin availability (Fig. 5 *C* and *D*). In the normal conditions of "complete licensing," sufficient MCM complexes are loaded onto chromatin to mark the positions of many potential origins within the same replicon. After the firing of one of them, a signal is sent from the forks to repress the activity of nearby origins (Fig. 5*C*). Recent work in different systems indicates that this inhibitory signal is mediated by checkpoint kinases, even in the absence of DNA damage. In *Xenopus*, the density of origin firing depends on ATM- and ATR-signaling pathways (25, 26). In fission yeast, loss of Cds1 (the Chk2 homolog) results in an intriguing overlap of early and late DNA replication patterns (27). In mammalian cells, Chk1 participates in the intra-S-phase checkpoint that regulates late origins (28), but, even in an unperturbed S-phase, it represses the

firing of origins located nearby replication forks. These cryptic origins can be activated by inhibition of Chk1 (19, 20) and probably serve an important backup function during replicative stress, e.g., providing new initiation points after the collapse of a replication fork (Fig. 5*C*).

Interestingly, the reduction of MCM levels did not significantly affect the density of "main" origins but impaired the activation of additional ones upon inhibition of Chk1. Therefore, it can be proposed that under conditions of limited licensing, many fewer potential origins are available within each replicon (Fig. 5*D*). All available origins may fire at once (a slight increase in origin density was observed after Mcm3 knockdown), but the cells lack the additional backup origins to rescue collapsed replication forks. This limitation would explain the hypersensitivity to replication inhibitors such as aphidicolin or hydroxyurea (HU). Also, these cells would have a higher chance of leaving genomic fragments underreplicated, explaining the severe chromosomal lesions observed when the checkpoint that prevents mitosis was abrogated with caffeine.

While this manuscript was under review, an interesting study that addresses the same question was published (29). Blow and colleagues reduced the concentration of human Mcm2–7 by targeting Mcm5 with RNAi and found that cells proliferate normally but become hypersensitive to replicative stress as they fail to active "dormant" origins in response to slow fork progression. Our study is in agreement with the conclusions of this report, but it is worth noting that our conditions of Mcm2–7 downregulation were stronger (90–95% suppression vs. 50% in ref. 29), suggesting that human cells may have at least a 10- to 20-fold excess of chromatin-bound Mcm2–7 complexes over the minimum amount compatible with cell survival.

MCM Dosage in Metazoan Organisms. Considering the importance of MCM abundance in tissue culture cells, the loss or malfunction of MCM in an entire organism should have a significant impact in all processes involving cell proliferation, including development and adult tissue homeostasis. Indeed, partial downregulation of MCM7 in *Caenorhabditis elegans* caused hypersensitivity to HU and reduced viability (16). More recently, two mouse models that recapitulate MCM loss-of-function situations have been reported. In the first one, a hypomorphic mutation in Mcm4 caused chromosome instability and increased the susceptibility to mammary adenocarcinomas (30). In the second one, a genetic manipulation of the 3′ UTR in the *MCM2* gene resulted in a mouse strain that expresses only a third of the normal concentration of Mcm2 protein. Mice developed normally and were viable, but their life span was greatly reduced. Young adults displayed proliferation deficiencies in several tissues and started to die after 10–12 weeks, mostly because of lymphomas (31). These results confirm that a full complement of MCM proteins is required *in vivo* to maintain genomic integrity and avoid cancer predisposition.

Is the MCM Paradox Solved?. A simple answer to the classic MCM paradox is that DNA replication can indeed take place with a reduced concentration of MCM proteins, particularly in cell-free systems and tissue culture cells, but a full complement of MCM proteins is necessary to protect cells from the natural replicative stress generated during S-phase and thereby maintain genome integrity. With this model in mind, there is a clear explanation for the deleterious effects of Cyclin E dysregulation in human cells. Premature expression of Cyclin E interferes with the loading of MCM complexes on chromatin but at the same time promotes DNA replication (7). According to our model, an S-phase under limited licensing conditions ensues, increasing DNA damage and eventually leading to chromosomal instability, as reported (8).

Materials and Methods

Cell Culture and Chemicals. HeLa cells were cultured in DMEM supplemented with 10% FBS plus streptomycin and penicillin. To knock down gene expression, Stealth siRNA oligonucleotides were designed by using BLOCK-IT designer software and purchased from Invitrogen. Target sequences can be found in *SI Text*. Two rounds of transfection, separated by 24 h, were done by using oligofectamine (Invitrogen) and 100 nM siRNA. Control transfections were done in identical conditions omitting the siRNA. For cell proliferation assays, 1.2×10^5 cells (at an initial concentration of 0.6×10^5 cells per milliliter) were transfected with the indicated siRNA and split 1:4 onto a multiwell plate. Every 24 h, for the duration of the experiment, the cells in one well were counted in a hemocytometer. To perform similar assays under conditions of replicative stress, 0,1 μM aphidicolin or 0,03 mM hydroxyurea was added to the medium. To abrogate the checkpoint-dependent G_2–M arrest, 5 mM caffeine was added to the medium for 5 h. To inhibit Chk1 activation, 10 nM UCN-01 was added for 5 h before cell collection. Except when noted otherwise, all chemicals were from Sigma.

Antibodies. Antibodies anti-Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7 have been described (32) and are available from BD Biosciences PharMingen. Anti-Orc2 and anti-RPA were kindly provided by B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The following commercial antibodies were used: Chk1 P-S345 and Chk2 P-T68 (Cell Signaling Technology); H2AX P-S139 and H3 P-S10 (Upstate Biotechnology); Mek2 (BD Biosciences PharMingen); PARP-p85 (Promega); and 53BP1 (NOVUS Biologicals).

Total Cell Extracts, Cell Fractionation, and Immunofluorescence. Total cell extracts were prepared in 1× Laemmli buffer and sonicated for 15 sec at 15% amplitude in a Digital Sonifier (Branson). The biochemical fractionation to separate soluble and chromatin-bound proteins was performed as described (32). For indirect immunofluorescence, cells were fixed in 2% paraformaldehyde in PBS for 10 min at room temperature (RT) and permeabilized with 0.1% sodium citrate, 0.1% Triton X-100 for 5 min at RT. Cells were overlaid with the indicated primary antibody for 1 h at RT, washed three times with PBS, and overlaid with the corresponding Texas red- or FITC-conjugated secondary antibodies. Nuclear DNA was stained with 1 μg/ml DAPI. Immunostaining after preextraction of soluble proteins was carried out as described (7).

Flow Cytometry. For DNA content analysis, cells were fixed in 70% ethanol, washed with PBS–0.05% Tween 20, and stained with PBS containing 20 μg/ml propidium iodide and 100 μg/ml RNase A. The percentage of cells in each phase of the cell cycle was estimated with ModFit (Verity Software House). For the detection of mitotic cells, anti-H3 P-S10 was added to the cells for 1.5 h, followed by Alexa Fluor 488-conjugated anti-rabbit IgG for 1 h. For analysis of BrdU incorporation, 10 μM BrdU was added to the culture for 20 min before cell fixation. DNA was denatured with 5 M HCl/0,5% Triton X-100 for 20 min, and BrdU was detected with FITC-conjugated anti-BrdU (BD Biosciences PharMingen). All samples were analyzed on a FACScalibur cytometer (BD Biosciences).

Chromosome Fragility Analyses. Cells were treated with 0.5 μg/ml colchicine for 4 h at 37°C before collection. To prepare metaphase spreads, cells were resuspended in 75 mM KCl, incubated for 15 min at 37°C, centrifuged, and resuspended in fixation solution (3:1 vol/vol methanol/acetic acid). One hundred microliters of cell suspension were dropped onto precleaned microscope slides and dried overnight. Metaphase chromosomes were visualized by Giemsa staining. Images were recorded with an Olympus BX microscope and CytoVision 3.1 software (Applied Imaging Corporation).

Fork Progression Rate and Origin Density Estimation. To calculate fork progression rates, cells were first pulsed with 100 μM BrdU for 30 min. To estimate origin activity, cells were pulsed sequentially with 50 μM IdU for 15 min and 100 μM CldU for 25 min, with or without a previous treatment with 10 nM UCN-01 for 5 h. DNA combing was performed as described (33). Image collection and analyses were done with MetaMorph 7 (Molecular Devices). See *SI Text* for a detailed protocol.

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1. Mendez J, Stillman B (2003) Perpetuating the double helix: Molecular machines at eukaryotic DNA replication origins. *BioEssays* 25:1158–1167.
2. Sclafani RA, Holzen TM (2007) Cell cycle regulation of DNA replication. *Annu Rev Genet* 41:237–280.
3. Aparicio T, Ibarra A, Mendez J (2006) Cdc45-MCM-GINS, a new power player for DNA replication. *Cell Div* 1:18.
4. Moyer SE, Lewis PW, Botchan MR (2006) Isolation of the Cdc45/Mcm2–7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. *Proc Natl Acad Sci USA* 103:10236–10241.
5. Lengronne A, Schwob E (2002) The yeast CDK inhibitor Sic1 prevents genomic instability by promoting replication origin licensing in late G(1). *Mol Cell* 9:1067–1078.
6. Tanaka S, Diffley JF (2002) Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. *Genes Dev* 16:2639–2649.
7. Ekholm-Reed S, *et al.* (2004) Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *J Cell Biol* 165:789–800.
8. Spruck CH, Won KA, Reed SI (1999) Deregulated cyclin E induces chromosome instability. *Nature* 401:297–300.
9. Malumbres M, Barbacid M (2001) To cycle or not to cycle: A critical decision in cancer. *Nat Rev Cancer* 1:222–231.
10. Di Micco R, *et al.* (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444:638–642.
11. Crevel G, *et al.* (2007) Differential requirements for MCM proteins in DNA replication in *Drosophila* S2 cells. *PLoS ONE* 2:e833.
12. Lei M, Kawasaki Y, Tye BK (1996) Physical interactions among MCM proteins and effects of MCM dosage on DNA replication in *Saccharomyces cerevisiae*. *Mol Cell Biol* 16:5081–5090.
13. Oehlmann M, Score AJ, Blow JJ (2004) The role of Cdc6 in ensuring complete genome licensing and S phase checkpoint activation. *J Cell Biol* 165:181–190.
14. Hyrien O, Marheineke K, Goldar A (2003) Paradoxes of eukaryotic DNA replication: MCM proteins and the random completion problem. *BioEssays* 25:116–125.
15. Takahashi TS, Wigley DB, Walter JC (2005) Pumps, paradoxes and ploughshares: Mechanism of the MCM2–7 DNA helicase. *Trends Biochem Sci* 30:437–444.
16. Woodward AM, *et al.* (2006) Excess MCM2–7 license dormant origins of replication that can be used under conditions of replicative stress. *J Cell Biol* 173:673–683.
17. Feng D, Tu Z, Wu W, Liang C (2003) Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. *Cancer Res* 63:7356–7364.
18. Zou L, Liu D, Elledge SJ (2003) Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc Natl Acad Sci USA* 100:13827–13832.
19. Maya-Mendoza A, *et al.* (2007) Chk1 regulates the density of active replication origins during the vertebrate S phase. *EMBO J* 26:2719–2731.
20. Syljuasen RG, *et al.* (2005) Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage. *Mol Cell Biol* 25:3553–3562.
21. Laskey RA, Madine MA (2003) A rotary pumping model for helicase function of MCM proteins at a distance from replication forks. *EMBO Rep* 4:26–30.
22. Snyder M, He W, Zhang JJ (2005) The DNA replication factor MCM5 is essential for Stat1-mediated transcriptional activation. *Proc Natl Acad Sci USA* 102:14539–14544.
23. Cortez D, Glick G, Elledge SJ (2004) Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proc Natl Acad Sci USA* 101:10078–10083.
24. Tsao CC, Geisen C, Abraham RT (2004) Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling. *EMBO J* 23:4660–4669.
25. Marheineke K, Hyrien O (2004) Control of replication origin density and firing time in *Xenopus* egg extracts: role of a caffeine-sensitive, ATR-dependent checkpoint. *J Biol Chem* 279:28071–28081.
26. Shechter D, Costanzo V, Gautier J (2004) ATR and ATM regulate the timing of DNA replication origin firing. *Nat Cell Biol* 6:648–655.
27. Meister P, *et al.* (2007) Replication foci dynamics: replication patterns are modulated by S-phase checkpoint kinases in fission yeast. *EMBO J* 26:1315–1326.
28. Feijoo C, *et al.* (2001) Activation of mammalian Chk1 during DNA replication arrest: A role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. *J Cell Biol* 154:913–923.
29. Ge XG, Jackson DA, Blow JJ (2008) Dormant origins licensed by excess Mcm2–7 are required for human cells to survive replicative stress. *Genes Dev* 21:3331–3341.
30. Shima N, *et al.* (2007) A viable allele of Mcm4 causes chromosome instability and mammary adenocarcinomas in mice. *Nat Genet* 39:93–98.
31. Pruitt SC, Bailey KJ, Freeland A (2007) Reduced MCM2 expression results in severe stem/progenitor cell deficiency and cancer. *Stem Cells* 25:3121–3132.
32. Mendez J, Stillman B (2000) Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: Assembly of prereplication complexes in late mitosis. *Mol Cell Biol* 20:8602–8612.
33. Michalet X, *et al.* (1997) Dynamic molecular combing: Stretching the whole human genome for high-resolution studies. *Science* 277:1518–1523.