Multi-step Loading of Human Minichromosome Maintenance Proteins in Live Human Cells*

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Aims: To assess MCM loading in live human cells.

Methods: We used FRAP and FLIP experiments to study MCM2 and MCM4 chromatin loading.

Results: We observed MCM2 and MCM4 chromatin loading at telophase and G1 phase.

Conclusion: MCM loading occurs in a multistep process.

Significance: This study reveals the dynamics of MCM loading in live human cells.

Once-per-cell cycle replication is regulated through the assembly onto chromatin of multisubunit protein complexes that license DNA for a further round of replication. Licensing consists of the loading of the hexameric MCM2–7 complex onto chromatin during G1 phase and is dependent on the licensing factor Cdt1. In vitro experiments have suggested a two-step binding mode for minichromosome maintenance (MCM) proteins, with transient initial interactions converted to stable chromatin loading. Here, we assess MCM loading in live human cells using an in vivo licensing assay on the basis of fluorescence recovery after photobleaching of GFP-tagged MCM protein subunits through the cell cycle. We show that, in telophase, MCM2 and MCM4 maintain transient interactions with chromatin, exhibiting kinetics similar to Cdt1. These are converted to stable interactions from early G1 phase. The immobile fraction of MCM2 and MCM4 increases during G1 phase, suggestive of reiterative licensing. In late G1 phase, a large fraction of MCM proteins are loaded onto chromatin, with maximal licensing observed just prior to S phase onset. Fluorescence loss in photobleaching experiments show subnuclear concentrations of MCM-chromatin interactions that differ as G1 phase progresses and do not colocalize with sites of DNA synthesis in S phase.

Faithful DNA replication is a prerequisite for the preservation of genomic information. In eukaryotic cells, DNA replication initiates from multiple replication origins distributed on chromosomes that direct the assembly of multiprotein complexes (known as “replisomes”), which will move along with each replication fork (1–4). The DNA replicated by sister forks from a single origin is called a replicon. It has been proposed that, in eukaryotic cells, the DNA replication machinery is restrained at specific nuclear structures, called “replication factories,” that exhibit dynamic behavior as they assemble and disassemble in space during S phase (Ref. 5–8 and reviewed in Refs. 9, 10). Sister replication forks generated from the same origin are believed to remain associated within a replication factory while the entire replicon is replicated (11). Replication begins in nuclear euchromatin at ~100–300 foci (visualized after BrdU or PCNA5 staining) distributed throughout the nucleus. During mid-S phase, these foci are located in heterochromatic regions near the nuclear periphery and surrounding the nucleoli, whereas in late S phase, only a few intense and larger replication foci are located at the nuclear periphery and within the nucleoli (6–10).

DNA replication must be limited to only once per cell cycle to prevent rereplication and maintain genomic stability (reviewed in Refs. 12–14). The integrity of genomic information is preserved through the periodic assembly and disassembly of essential prereplication complexes at replication origins. This process, described as chromatin "licensing," involves the loading of the heterohexameric MCM2–7 (minichromosome maintenance) protein complex onto chromatin by the origin recognition complex (ORC) and two essential loading factors, Cdc6 (15) and Cdt1 (16, 17). Licensing ensures the faithful regulation of DNA replication in time and space (18). The euchromatic cell cycle is driven by the periodic activation and inactivation of cyclin-dependent kinases that allow prereplication complex formation to occur in a time window between late mitosis through G1 phase during which Cdk/cyclin levels are low because of the anaphase-promoting complex/cyclosome and...
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cyclin-dependent kinase inhibitors (14). In metazoans, licensing is additionally controlled through Geminin (19), a protein that binds to Cdt1 and inhibits it from loading the MCM complex onto chromatin during S and G₂ phases (20–22). The timing and extent of MCM loading onto chromatin must be accurately controlled through the cell cycle and coordinated with S phase onset. Both underlicensing and overlicensing have been linked to DNA replication stress, genomic instability, and malignant transformation (23–25).

MCM2–7 proteins belong to the AAA (ATPases Associated with diverse cellular Activities) ATPase family. They adopt a hexameric ring-like structure (26–28) large enough to accommodate single-stranded DNA or dsDNA (27–30) and are considered the prime candidates for the eukaryotic replicative helicase (31). The spatial localization of MCMs after the onset of DNA replication, during which MCM proteins bind preferentially to unreplicated DNA rather than to replicating or replicated DNA (32, 33), as well as the excess number of MCM2–7 complexes loaded per ORC (34, 35) raise questions regarding MCM function that are put together as the “MCM paradox.” In vitro studies in yeast support a two-step model for the loading of the MCM2–7 complex onto chromatin (36–38). MCM proteins firstly bind transiently onto origin DNA and are then loaded stably in a step requiring ATP hydrolysis by cdc6 (39). The MCM2–7 complex is loaded in an inactive form at origins. As cells enter S phase, the combined action of cyclin-dependent kinases and Cdc7-Dbf4 (reviewed in Ref. 40) leads to the formation of a complex between MCM2–7, Cdc45, and GINS (Go, Ich1, Nii, and San) (CMG complex) (41), that bears processive helicase activity (42) and is part of the replisome (43, 44).

Live cell imaging studies revealed both the ORC (45) and Cdt1 (46, 47) to be highly mobile within the cell nucleus, whereas a recent study showed stable binding of MCM proteins throughout G₁ and S phases in CHO cells (48). Here, we use live-cell imaging to assess MCM chromatin loading at different cell cycle stages of human cancer cells. Our analysis reveals transient interactions of MCM proteins with chromatin in telophase and Transfection, and FACS Analysis. In addition, transient interactions of MCM proteins with chromatin in telophase and Transfection, and FACS Analysis. In addition, transient interactions of MCM proteins with chromatin in telophase and Transfection, and FACS Analysis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Isolation of Stable Cell Lines, Cell Synchronization and Transfection, and FACS Analysis**—MCF7 cells were grown in Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum at 37°C and 5% CO₂. For live-cell experiments, cells were plated either on MatTek dishes (MatTek Corp.) or in ibidi µ-dishes 35-mm high glass-bottom, in phenol red-free, CO₂-independent medium (Invitrogen). Stable GFP-NLS, GFP-MCM2, and GFP-MCM4 cell lines were selected with 500 µg/ml Geneticin (Invitrogen). Stable Cdt1-GFP cell line generation and characterization have been described previously (47). For transient transfection, MCF7 cells plated in 35-mm dishes were transfected with a total of 1 µg of plasmid DNA for 24 h using Lipofectamine 2000 (Invitrogen) or Turbofect (Fermen-tas) according to the instructions of the manufacturer. For Cdt1 silencing, MCF7 stable cell lines were transfected twice with 400 nM of Cdt1 siRNA or control Luciferase siRNA using Lipofectamine 2000 with a time interval of 24 h and were analyzed 48 h after the second transfection.

For mitotic synchronization, cells were treated either with 40 ng/ml nocodazole (Sigma) for 12 h or with 100 µM monastrol (Sigma) for 16 h, collected by mechanical shake-off, and released into fresh medium. For late G₁ phase synchronization, cells were grown in the presence of 0.5 mM mimosine (Sigma) for 24 h. The synchronization in early S phase was performed with a double thymidine block (2.5 mM, Sigma) or with 5 mM hydroxyurea (Sigma) treatment for 24 h.

For FACS analysis, stable GFP-NLS, GFP-MCM2 and GFP-MCM4 cell lines as well as parental MCF7 cells were fixed with 70% ice-cold ethanol and stained with propidium iodide (2 µg/ml) in the presence of 100 µg/ml RNase in PBS. Cellular DNA content was analyzed using a BD Biosciences flow cytometer with Cellquest software and WinMDi software version 2.8.

**Immunofluorescence, Western Blotting, and Immunoprecipitation**—Immunofluorescence was done as described previously (49). Primary antibodies used were as follows: α-MCM2 (BD Transduction Laboratories, 1:500); α-MCM4 (BD Pharmingen, 1:600); and α-Cdt1 (50) (1:250), α-Geminin (47) (1:250), and α-cyclin A (1:100) (Neomarkers). DNA was stained with Hoechst 33258 (Sigma), DAPI (Vector), or TOTO-3 (Molecular Probes).

For Western blotting, total cell lysates were prepared by lysing cell pellets directly in SDS-PAGE loading buffer. Primary antibodies used were as follows: α-MCM2 (BD Transduction Laboratories, 1:1000), α-MCM4 (BD Pharmingen, 1:6000), α-MCM7 (Santa Cruz Biotechnology, 1:500), α-Cdt1 (50) (1:250) and α-GFP (1:6000) (Roche), and α-tubulin (Sigma, 1:20,000).

For immunoprecipitation experiments, total cell lysates from asynchronous MCF7 cells as well as from the GFP-MCM2, GFP-MCM4 and GFP-NLS stable cell lines were prepared by using a lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 5 mM MgCl₂, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and complete EDTA-free protease inhibitor mixture tablets (Roche). GFP-tagged proteins were immunoprecipitated using anti-GFP antibodies (mouse monoclonal anti-GFP, 3E6, catalog no. 1181460001, Roche) bound to protein G-agarose beads (Upstate). Immunoprecipitates were analyzed by Western blotting.

**Chromatin Association Assay**—Approximately 5 × 10⁵ cells were washed with ice-cold PBS buffer and lysed in 0.1 ml of 0.1% TX-100 mCSK buffer (10 mM Pipes (pH 7.9), 100 mM NaCl, 300 mM sucrose, 0.1 (v/v) % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM Na₃VO₄ and 10 mM NaF) for 15 min on ice. After centrifugation at 13,000 rpm for 15 min at 4°C, the supernatant that was isolated represented the soluble fraction (S100). The pellet was washed with an equal volume of ice-cold 0.1% TX-100 mCSK buffer and suspended in SDS sample buffer (chromatin-enriched fraction, P100).
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Plasmids—To fuse GFP to the N termini of MCM2 and MCM4, the open reading frames of MCM2 and MCM4 (provided by Dr. A. Perrakis) were subcloned into pCDNA3.1/EGFP (Invitrogen) between the EcoRI and Xhol sites. PCNA tagged to monomeric red fluorescent protein (mRFP) (PCNA-RFP) was kindly provided by Dr. C. Cardoso (51). A GFP-nls construct was generated by cloning three copies of the SV40 nuclear localization sequences at the C terminus of the GFP sequence in the pEGFP-C1 vector.

Photobleaching Experiments and Analysis—Photobleaching experiments were performed on a Leica TCS SP5 confocal microscope equipped with a ×63 1.4 numerical aperture (NA) oil immersion lens. During experiments, cells were maintained at 37 °C and 5% CO2. For fluorescence recovery after photobleaching (FRAP) experiments, bleaching was accomplished on a defined region of interest of 2 μm diameter within the cell nucleus. Fifty prebleach images were recorded with 3% laser power of the 488-nm line at 70% argon laser intensity, and bleaching was attained by a double bleach pulse of 0.066 s using the 458-, 476-, 488-, and 496-nm laser lines combined at maximum power. Following bleaching, 250 images were recorded at 0.066-s intervals with 3% laser power of the 488-nm line. Raw data were normalized as described previously (47). Immobile fractions and t1⁄2 values were extracted after a double exponential fitting using the program easyFRAP (52) and the FRAPcalcV9j application of Dr. Kota Miura (EMBL).

Fluorescence loss in photobleaching (FLIP) experiments (53–55) were performed by repeatedly bleaching a specified region within the cell nucleus. In Fig. 7A, a circular region of 4 μm in diameter within the cell nucleus was repeatedly bleached with 60 pulses using the 458-, 476-, 488-, and 496-nm laser lines combined at maximum power. In Fig. 7C, a region corresponding to ~1/3 of the nucleus was bleached as above. In Fig. 7B, about 1/3 of the cell nucleus was bleached with 150 pulses using the 476- and 488-nm laser lines combined at 70% laser intensity using a FRAP booster (Leica TCS SP5 confocal microscope). All fluorescent molecules within this region, and molecules that are mobile within the nucleus and move through this region during the bleaching step, become bleached, leading to a gradual depletion of the mobile pool from the nucleus. Following FLIP, confocal images were recorded to visualize immobile structures of GFP-MCM2 within the nonbleached part of the nucleus (53–55). In Fig. 8, the redistribution of fluorescence was monitored by acquiring a series of 10 optical sections along the z axis of each cell at 5-min intervals over a period of 2 h.

Quantification and Image Analysis—siRNA-mediated depletion of Cdt1 was estimated by quantifying mean fluorescence intensities of cells subjected to immunofluorescence with anti-Cdt1 antibody using ImageJ 1.37g (Wayne Rasband, National Institutes of Health, Bethesda, MD).

For each cell subjected to FLIP in Fig. 8, the maximum projection of the z-stacks was quantified, and a linear filter was applied to remove noise and smoothen the image by setting the intensity value of each pixel to the average of the pixels in its 6 × 6 neighborhood. The corresponding data are shown as mesh plots. The analysis was performed using MATLAB R2007b.

RESULTS

A System for Studying MCM Protein Dynamics within Live Human Cells—To study the dynamics of MCM proteins within live human cells, the MCM2 and MCM4 proteins were fused to GFP at their N termini (GFP-MCM2 and GFP-MCM4). MCM4 was chosen because it was shown to interact with the GINS complex (42), and its phosphorylation by Cdc7 facilitates the interaction with Cdc45 on chromatin (56). MCM2, as well as MCM3, carry nuclear localization sequences (NLSs) sequestering the other MCM subunits to the cell nucleus (57). The GFP-MCM2 and GFP-MCM4 constructs, under the control of the constitutive CMV promoter, were used for transient expression in MCF7 human breast cancer cells. GFP-MCM2 localized to the nucleus and was excluded from the nucleoli, similar to endogenous MCM2 (data not shown). GFP-MCM4, transiently expressed, showed both nuclear and occasional cytoplasmic localization (data not shown), consistent with previous results showing that MCM4 lacks a nuclear localization signal and is transported to the nucleus in complex with other subunits (48, 57). Several MCF7 cell lines stably expressing GFP-MCM2, GFP-MCM4 or a nuclear localized construct of GFP (GFP-NLS) were generated and analyzed by GFP fluorescence and Western blot analysis (data not shown). Clonal cell lines expressing the tagged proteins at levels similar to the endogenous MCM2 and MCM4, respectively, were selected for further analysis (Fig. 1A). In Fig. 1, B and C, GFP fluorescence in the stable cell lines is compared with immunofluorescence using anti-MCM2- and anti-MCM4-specific antibodies. Both GFP-MCM2 (Fig. 1B) and GFP-MCM4 (C) exhibited the same subcellular localization as the endogenous proteins, localizing to the nucleus and being excluded from the nucleoli. This analysis further verified the low-level expression of the tagged moieties because no increase in total anti-MCM2 and anti-MCM4 staining was observed in the stable cell lines and indicated correct complex formation of GFP-MCM4 expressed to low levels, allowing its correct localization to the nucleus. To verify that the constitutive expression of the tagged MCM2 and MCM4 moieties, even at low levels, did not interfere with correct cell cycle progression, the cell cycle profiles of the stable cell lines were compared with the parental MCF7 cell line. FACS analysis of the stable cell lines produced cell cycle profiles similar to the parental MCF7 cells (Fig. 1D). This finding was further confirmed by immunofluorescence against different cell cycle markers. The percentage of cells in GFP-MCM2-, GFP-MCM4- and GFP-NLS-expressing cell lines that were positive for Cdt1 (G1 phase marker), Geminin (S-G2 phase marker) and cyclin A (S-G2 phase marker) was similar to those of the control MCF7 cells (Fig. 1E).

We then addressed whether GFP-MCM2 and GFP-MCM4 were able to interact and form complexes with the other endogenous MCM subunits of the MCM2–7 hexamer. Total cell extracts from the GFP-MCM2, GFP-MCM4 and GFP-NLS (as a negative control) stable cell lines were immunoprecipitated using an anti-GFP antibody, and immunoprecipitates were analyzed by Western blotting using specific antibodies for MCM2, MCM4 and MCM7. As shown in Fig. 1F, both GFP-MCM2 and GFP-MCM4 were able to interact and form complexes with the other endogenous MCM subunits of the MCM2–7 hexamer. Total cell extracts from the GFP-MCM2, GFP-MCM4 and GFP-NLS (as a negative control) stable cell lines were immunoprecipitated using an anti-GFP antibody, and immunoprecipitates were analyzed by Western blotting using specific antibodies for MCM2, MCM4 and MCM7. As shown in Fig. 1F, both GFP-
MCM2 and GFP-MCM4 form complexes with the other endogenous MCM subunits.

To assess the ability of GFP-MCM2 and GFP-MCM4 to associate with chromatin during the cell cycle, chromatin association assays were performed in mitotically synchronized cell populations. Cells were arrested in mitosis by nocodazole and mitotic shake-off, and time points were taken following release as cells progressed synchronously in G1 phase. As shown in Fig. 2A, GFP-MCM2, similar to endogenous MCM2, is expressed in mitosis and throughout G1 phase but associates with a chromatin-enriched insoluble fraction following release into G1 phase in parallel to the chromatin association of Cdt1. Note the hypermodification of Cdt1 in mitotically arrested cells, as reported previously (58). Similarly, GFP-MCM4 associated
with chromatin during G1 phase with kinetics similar to the endogenous MCM4 (Fig. 2B). Note the hypermodification of both the endogenous MCM4 and GFP-MCM4 in mitosis (as reported previously (59)) and the apparent decrease in their protein levels at the mitotic block, which point to regulation of MCM4 in mitosis by posttranslational modifications and proteolysis. Note also that Cdt1 chromatin association temporarily precedes MCM2 and MCM4 chromatin association. These experiments confirm that both GFP-tagged proteins associate with chromatin with kinetics similar to the endogenous ones. Taken together, these results show that GFP-MCM2 and GFP-MCM4 functionally mimic the behavior of their endogenous counterparts, allowing their use for the investigation of the spatiotemporal regulation of the endogenous proteins in live human cells.

Fluorescence Recovery after Photobleaching Reveals an Immobile MCM Pool within the Live Cell Nucleus—Our previous work had shown that Cdt1-GFP exhibits a scanning behavior during G1 phase of human cultured cells, maintaining dynamic short-lived interactions with chromatin throughout G1 phase (47). A similar dynamic behavior was also reported for ORC components (45). In contrast, a recent study showed stable binding of MCM proteins to chromatin in CHO cells (48). To investigate the behavior of MCM proteins in live human cells, real-time in vivo confocal fluorescence microscopy was used. Unsyncronized MCF7 cells stably expressing GFP-MCM2, GFP-MCM4, or GFP-NLS were subjected to FRAP. In parallel, MCF7 cells stable for Cdt1-GFP were analyzed for comparison (47). The mobility and kinetic properties of MCM2, MCM4 and Cdt1 were assessed by photobleaching the GFP-tagged proteins in a small circular region (2 μm in diameter) located inside the nucleus and then monitoring the recovery of fluorescence over time. Mean normalized curves derived from 15–50 individual cells are shown in Fig. 3. Curve fitting of all individual recovery curves (52) was used to calculate the half-time ($t_{1/2}$) of the fluorescence recovery and the fraction of molecules that were immobile. Both MCM2 and MCM4 displayed rapid recovery kinetics during the first part of the recovery curve, similar to that of the freely diffusing GFP-NLS, suggesting that a fraction of each protein is free to diffuse within the live cell nucleus. In contrast to GFP-NLS, however, both proteins exhibited substantial immobile fractions that showed no detectable recovery during the time course of the experiment. This indicates that a substantial fraction of both GFP-MCM2 and GFP-MCM4 is immobile within the live cell nucleus. Our previous work had shown that Cdt1-GFP exhibited short-lived interactions with chromatin that delayed initial fluorescence recovery, leading to a longer $t_{1/2}$ for Cdt1 than for the GFP control (47). Consistent with our earlier findings, Cdt1 exhibited a substantially increased $t_{1/2}$ in comparison to GFP-NLS (Fig. 3). Direct comparison of the MCM and Cdt1 FRAP curves shows that MCM proteins exhibit a kinetic behavior that differs significantly from Cdt1. MCM proteins show a fast initial recovery and a substantial immobile fraction, whereas Cdt1 shows a reduced initial recovery and a much smaller immobile fraction. We conclude that MCM proteins maintain long-lived interactions within the nucleus in contrast to Cdt1. This is consistent with findings in CHO cells (48) and indicates that the
majority of the MCM molecules do not move as a complex with Cdt1 within live human cells.

Imaging MCM Dynamics within the Nucleus. An in Vivo Licensing Assay—To verify that the observed immobile MCM pool represented MCM-chromatin association during licensing, we investigated whether the presence of an immobile MCM fraction was dependent on Cdt1, a factor essential for the loading of MCM proteins onto chromatin. We therefore knocked down Cdt1 expression in MCF7 cells stable for GFP-MCM2 and GFP-MCM4 using siRNA. The efficiency of the knockdown was assessed by quantitative immunofluorescence (data not shown). As shown in Fig. 4A, FRAP assays revealed that Cdt1 depletion resulted in significantly reduced immobile fractions for GFP-MCM2 and GFP-MCM4. In contrast, in cells treated with control siRNA, GFP-MCM2 and GFP-MCM4 retained an immobile fraction. We therefore conclude that the presence of an immobile MCM pool is Cdt1-dependent, and it is therefore likely to represent MCM-chromatin interactions during licensing.

We then asked whether the observed immobile fraction of MCM proteins is cell cycle-regulated, consistent with MCM chromatin binding during licensing. To address this question, GFP-MCM2- and GFP-MCM4-expressing MCF7 cell lines were transiently transfected with PCNA tagged with RFP. PCNA has been well established as a marker with characteristic cell cycle-dependent nuclear patterns, exhibiting diffuse nuclear distribution in non-S phase cells and subnuclear focal accumulation in S phase cells (Fig. 4B). By using this method of discrimination (7), cells at different cell cycle stages were identified and classified, according to their pattern of PCNA, as non-S phase, early S phase, middle S phase and late S phase and analyzed by FRAP. Mean FRAP curves for GFP-MCM2 and GFP-MCM4 are shown in Fig. 4, C and D, respectively. In both cases, the recovery half-time (t1/2) among the different cell cycle phases is similar and marginally increased compared with GFP-NLS. However, different immobile fractions were observed for both proteins during the course of the cell cycle. Non-S phase cells demonstrated an immobile fraction of about 22% for GFP-MCM2 and 19% for GFP-MCM4, whereas 30% of GFP-MCM2 and GFP-MCM4 appeared to be immobile in early S phase cells, indicating stable binding of a substantial fraction of MCMs to chromatin at the onset of S phase. In middle to late S phase cells, the immobile fractions were decreased for both GFP-MCM2 and GFP-MCM4, demonstrating that both molecules dissociate from chromatin during the progression through S phase, consistent with licensing loss during S phase.

These findings show that the presence of an immobile MCM pool, as measured by FRAP, closely mirrors MCM-chromatin association during licensing, consistent with earlier work (48). Our assay can therefore be used to assess licensing in vivo.

MCM-Chromatin Association Kinetics during the Cell Cycle Reveal Increased MCM Loading in Late G1 Phase—We used the in vivo licensing assay to study MCM dynamics during the cell cycle of live human cells. Given that the previously analyzed non-S phase cell population represents a heterogeneous pool of cells in different stages of G1 phase as well as in G2 phase, we investigated MCM kinetic parameters exclusively during G1 phase and examined whether the association of MCM with chromatin changes as G1 phase progresses. To that end, MCF7 cells expressing GFP-MCM2 or GFP-MCM4 were first synchronized in G1/M phase by nocodazole block and mitotic shake-off treatment, released into fresh medium, and analyzed as they progressed synchronously through G1 phase. The efficiency of the synchronization was verified by immunofluorescence using cell cycle markers (data not shown), showing that S phase entry occurred 12–15 h following nocodazole release. Cells were analyzed by FRAP 3 h (early G1 phase) and 9 h (middle to late G1 phase) after release. As depicted in Fig. 5, A and B, both GFP-MCM2 and GFP-MCM4 exhibited a measurable immobile fraction already from early G1 phase (3 h after nocodazole release, observed mean immobile fractions of 8 and 9%, respectively), consistent with stable chromatin association taking place for a small fraction of MCM proteins from early G1 phase. In middle to late G1 phase cells (9 h after nocodazole release), the immobile fractions increased somewhat to a mean of 12 and 19%, respectively. This increasing trend is indicative of reiterative loading of MCMs onto chromatin during the course of G1 phase.

Given the significant immobile fraction observed in early S phase cells through cotransfection with PCNA (Fig. 4, C and D), we wished to directly compare MCM-chromatin association kinetics in late G1 phase with early S phase. For this purpose, MCF7 cell lines expressing either GFP-MCM2 or GFP-MCM4 were arrested in late G1 phase by treatment with mimosine and in early S phase by hydroxyurea or double thymidine block. The efficiency of the arrest and the ability of cells to be released from
each block were verified by immunofluorescence with specific cell cycle markers (PCNA, Geminin, and cyclin A, data not shown). FRAP analysis showed that both GFP-MCM2 and GFP-MCM4 exhibited the largest mean immobile fraction in late G₁ phase (mimosine block, ~25–30%), whereas immobile fractions for both proteins were considerably lower in the early S phase blocks when compared with late G₁ phase cells (Fig. 5, C and D). To verify that maximal loading of MCM proteins takes place in late G₁ phase, MCF7 cells stably expressing GFP-MCM2 or GFP-MCM4 were synchronized in prometaphase by monastrol, which allows reversible mitotic arrest with synchronous entry into the following S phase upon release. BrdU incorporation and staining for Cdt1 was used to characterize progression through the cell cycle and showed that entry into S phase occurred synchronously 15 h post-release from monastrol. Therefore, cells analyzed by FRAP at 3, 7 and 13 h post-release were considered to be in early, middle and late G₁ phase, respectively. As shown in Fig. 5, E and F, both GFP-MCM2 (C) and GFP-MCM4 (D) and GFP-NLS cells were transiently transfected with PCNA-RFP. Cells in early, middle, and late S phase as well as non-S phase cells were identified on the basis of the localization of PCNA and analyzed by FRAP. Recovery of fluorescence in the photobleached region as a function of time is depicted. Mean values are given for the calculated immobile fraction and half-time of recovery for all conditions tested, with corresponding standard deviations. N represents the number of cells analyzed for each condition.

**FIGURE 4.** GFP-MCM2 and GFP-MCM4 bind to chromatin in a Cdt1-dependent manner and exhibit different binding properties through the cell cycle. A, stable GFP-MCM2, GFP-MCM4 and GFP-NLS cells were treated with non-target siRNA (siLuc) or siRNA for Cdt1 (siCdt1) followed by FRAP analysis. Imm. Frac., immobile fraction. N represents the number of cells analyzed for each condition. B, PCNA localization enables discrimination of different cell cycle phases. Stable GFP-MCM4 cells transiently transfected with PCNA-RFP were fixed, and representative images of characteristic non-S phase and S phase patterns (early S phase, middle S phase, and late S phase) were taken. C and D, stable GFP-MCM2 (C), GFP-MCM4 (D) and GFP-NLS cells were transiently transfected with PCNA-RFP. Cells in early, middle, and late S phase as well as non-S phase cells were identified on the basis of the localization of PCNA and analyzed by FRAP. Recovery of fluorescence in the photobleached region as a function of time is depicted. Mean values are given for the calculated immobile fraction and half-time of recovery for all conditions tested, with corresponding standard deviations. N represents the number of cells analyzed for each condition.
MCM proteins are loaded in late G1 phase prior to the onset of DNA replication.

**MCM Proteins Exhibit Dynamic Interactions with Chromatin during Telophase**—Our in vivo data so far indicates that MCM proteins maintain long-lived interactions with chromatin throughout G1 phase, with maximal chromatin association prior to the G1-to-S phase transition.

In vitro studies have shown that MCM protein loading takes place in two phases, with an initial transient association being converted to stable loading accompanied by ATP hydrolysis and dissociation of MCM loading factors. We therefore wished to investigate whether we could detect such a transient MCM-chromatin association early in the cell cycle. Toward this aim, we studied cells in mitosis. We first characterized MCM colocalization with chromatin throughout mitosis. MCF7 cells stable for GFP-MCM4 were synchronized in G2/M phase with nocodazole, fixed at different time points after the release, and counterstained with DAPI to visualize chromatin.

**FIGURE 5.** GFP-MCM2 and GFP-MCM4 display maximal binding to chromatin during late G1 phase. A and B, stable GFP-MCM2 (A) and GFP-MCM4 (B) cells were synchronized in M phase by nocodazole (noc) block and mitotic shake-off. GFP-MCM2/4 cells in early G1 phase (3 h post-mitotic release) and in middle to late G1 phase (9 h post-mitotic release) and unsynchronized (unsynch) stable Cdt1-GFP and GFP-NLS cells were analyzed by FRAP. C and D, stable GFP-MCM2 (C) and GFP-MCM4 (D) cells were synchronized in late G1 phase after mimosine treatment (mimosine), and in early S phase by a double thymidine block (thymidine) or hydroxyurea (HU) treatment and analyzed by FRAP in parallel to unsynchronized GFP-MCM2/4 and GFP-NLS cells. E and F, stable GFP-MCM2 (E) and GFP-MCM4 cells were synchronized in mitosis by monastrol (mon), and time points were taken in early, middle, and late G1 phase (3, 7, and 13 h following release). Recovery of fluorescence in the photobleached region as a function of time is depicted for all conditions. G and H, following curve fitting of individual FRAP curves, immobile fraction (Imm. Frac.) and $t_{1/2}$ of the recovery of the mobile fraction were computed for all cells analyzed in A–F using easyFRAP (52). p.m., postmonastrol; p.n., postnocodazole. N represents the number of cells analyzed for each condition. Data are mean ± S.D.
stages of mitosis were identified. As shown in Fig. 6A, MCM4 appeared to be excluded from chromosomes during prophase, metaphase, and anaphase. In contrast, MCM4 was shown to overlap with DAPI-stained chromatin in cells undergoing telophase as nuclear envelopes are reforming. This is similar to the behavior of Cdt1 during mitosis (47). Similar results were obtained for GFP-MCM2 (data not shown).

To elucidate the chromatin association properties of MCMs during the different stages of mitosis, MCF7 cells stably expressing GFP-MCM2 or GFP-MCM4 were transiently transfected with a plasmid expressing H2B-RFP that marks chromosomes. Cells in the different stages of M phase were identified on the basis of morphology and analyzed by FRAP. Fluorescence recovery is shown as a function of time. Immobile fraction (Imm. Frac.) and $t_{1/2}$ of the recovery of the mobile fraction were computed. $N$ represents the number of cells analyzed for each condition.

**FIGURE 6. GFP-MCM2/4 display transient binding during telophase.** A, GFP-MCM4 is absent from chromatin during prophase, metaphase, and anaphase but colocalizes with chromatin during telophase. Stable GFP-MCM4 cells were synchronized in M phase after nocodazole block, fixed, and then DNA was stained with DAPI. B–D, stable GFP-MCM2 (B), GFP-MCM4 (C) and control GFP-NLS cells (D) were transiently transfected with a plasmid expressing H2B-RFP that marks chromosomes. Cells in the different stages of M phase were identified on the basis of morphology and analyzed by FRAP. Fluorescence recovery is shown as a function of time. Immobile fraction (Imm. Frac.) and $t_{1/2}$ of the recovery of the mobile fraction were computed. $N$ represents the number of cells analyzed for each condition.
the drug. On the basis of the pattern of H2B-RFP, cells were classified into different mitotic phases and subjected to FRAP analysis. Qualitative analysis of the obtained data revealed no immobile fractions and fast fluorescence recovery in the bleached region for both MCM2 and MCM4 in cells in prophase, metaphase and anaphase. On the contrary, the kinetic behavior of MCM2 and MCM4 was markedly different in cells in telophase (Figs. 6, B and C). In these cells, although no immobile fraction was observed, the initial recovery was much slower (MCM2 $t_{1/2} = 0.98 \pm 0.09$ s and MCM4 $t_{1/2} = 0.84 \pm 0.2$ s). The kinetic behavior of MCM proteins in telophase is similar to Cdt1 and differs from MCM kinetics during G1 phase. As shown in Fig. 6D, the mobility of GFP-NLS used as a control is largely unchanged during mitosis, with only a marginal decrease in mobility in telophase, in contrast to the pronounced retardation observed for GFP-MCM2 and GFP-MCM4. Taken together, these data indicate that, in late mitosis, MCM proteins maintain transient interactions with chromatin that are converted to stable interactions from early $G_1$ phase. We conclude that the two steps of MCM loading observed in vitro, transient association and stable loading, are separable by FRAP in live cells and that transient interactions are only observable for a short time during telophase.

**FLIP Reveals the Spatial Distribution of MCM Proteins Bound to Chromatin—**FRAP allowed an assessment of the timing and dynamics of MCM-chromatin association in live human cells. To address where within the nucleus chromatin association takes place and whether the topology of MCM-chromatin binding changes during the cell cycle, we employed FLIP (53–55). MCF7 cells expressing GFP-MCM2 were arrested either in mitosis by nocodazole treatment and then released to enter $G_1$ phase or in late $G_1$ phase by mimosine. Cells in early $G_1$ phase (3 h after nocodazole release), middle $G_1$ phase (7 h after nocodazole release) or late $G_1$ phase (mimosine block) were analyzed by FLIP. During FLIP, a region in the cell nucleus was continuously exposed to a bleaching laser, leading to the bleaching of fluorescent molecules that reside in this region or pass through this region during the bleaching step. This erases the fluorescence of the mobile fraction of GFP-MCM2 in the cell nucleus. Upon completion of the bleaching pulses, cells were imaged and examined for immobile structures of GFP-MCM2 in the unbleached part of the nucleus (53–55). Representative images are shown in Fig. 7A. Inspection of the post-FLIP cells revealed discrete GFP-MCM2 assemblies within the non-bleached part of the nucleus. In early $G_1$ phase, GFP-MCM2 immobile molecules were concentrated in the nuclear periphery and around the nucleoli, reminiscent of heterochromatin localization. The same pattern was retained in middle $G_1$ phase, but in that case the foci were more intense, indicating that a higher percentage of GFP-MCM2 molecules were bound to chromatin. Finally, in late $G_1$ phase (mimosine block), the relative proportion of GFP-MCM2-bound molecules was even higher, and immobile structures were also observed throughout the nucleoplasm (Fig. 7A). To repeat our analysis using a different synchronization method, cells expressing GFP-MCM2 were arrested in mitosis with monastrol and released to progress synchronously through $G_1$ phase. Cells were analyzed by FLIP 3 h (early $G_1$ phase), 8 h (middle $G_1$ phase), and 13 h (late $G_1$ phase) after the removal of monastrol. As depicted in Fig. 7B, analysis of the post-FLIP images showed that, in early and middle $G_1$ phase, there is a small fraction of GFP-MCM2 molecules that remains bound to chromatin and is predominantly concentrated in the heterochromatic regions. However, in late $G_1$ phase, the immobile fraction of the GFP-MCM2 molecules is substantially higher, and immobile structures are observed throughout the nucleus as well as in heterochromatin (Fig. 7B), verifying our initial observations. To quantify the fraction of GFP-MCM2 molecules that remained bound to chromatin in early, middle and late $G_1$ phase cells, the integrated intensity of the non-bleached part of the nucleus was measured before and after the bleach pulse in 10, 12, and 13 individual cells analyzed by FLIP in early, middle, and late $G_1$ phase, respectively (data not shown). The post-bleach over the prebleach value was calculated to determine the immobile fractions of the GFP-MCM2 molecules. The immobile GFP-MCM2 fraction is larger in late $G_1$ phase cells (mean immobile fraction = 0.53 ± 0.23) than in early and middle $G_1$ phase cells (mean immobile fraction = 0.22 ± 0.16, data not shown). Statistical analysis of the results showed that this difference is highly statistically significant (Student’s $t$ test, $p < 0.001$). These data further support maximal loading of MCM proteins in late $G_1$ phase, as observed previously by FRAP. They also indicate that MCM-chromatin association is not homogeneous through the nucleus but shows subnuclear concentrations throughout $G_1$ phase.

**MCM Foci Do Not Colocalize with Replication Factories—**We next monitored changes in MCM chromatin binding at the subnuclear level through $S$ phase by FLIP. To this end, MCF7 cells expressing GFP-MCM2 were transiently transfected with PCNA-RFP, which marks sites of active DNA replication. Using this approach, cells in early, middle or late $S$ phase were identified and subjected to FLIP (Fig. 7C). Under these conditions, the fluorescence of the mobile pool of GFP-MCM2 was depleted, whereas GFP-MCM2 molecules that were tightly anchored to chromatin remained unbleached and, thus, visible. Post-FLIP images revealed that bound GFP-MCM2 molecules were concentrated in subnuclear foci predominantly in early $S$ phase cells and, to a lesser extent, in middle $S$ phase cells. In early $S$ phase, large MCM2 foci were restricted in heterochromatic regions around the nucleus and at the nucleolar periphery. In middle $S$ phase, although these structures retained their localization, they were fewer and smaller in size. On the contrary, no immobile fraction of GFP-MCM2 was detected during late $S$ phase. Similar results were obtained for GFP-MCM4 (data not shown). These observations are in line with the FRAP results of Fig. 4, C and D, providing further support for the gradual loss of the MCM immobile fraction during the course of $S$ phase.

Several studies have shown that MCMs do not colocalize with sites of DNA synthesis (32, 33, 48, 60–62). We directly compared the localization of GFP-MCM2 structures and PCNA foci after photobleaching soluble molecules by FLIP. As shown in Fig. 7B, GFP-MCM2 foci failed to coincide spatially with PCNA, which labels sites of DNA replication, consistent with previous studies (32, 33, 61). Moreover, PCNA appeared to be in close proximity to GFP-MCM2 foci.
To determine the relative time that MCMs proteins reside on chromatin, the localization of chromatin-bound MCM proteins was imaged for different times following FLIP. MCF7 cells expressing GFP-MCM2 were transiently transfected with PCNA-RFP. Subsequently, cells in G1 phase and early and middle S phase were selected and subjected to FLIP. Following FLIP, a series of 10 images along the z axis (z-stacks) were acquired for each cell. The z-stacks, which were collected every 5 min over a 2-h period, were collapsed into maximum intensity projection images, and two-dimensional fluorescent intensity versus space mesh plots were generated (Fig. 8A). A subnuclear area surrounding GFP-MCM2 foci was defined in each cell, and

**FIGURE 7. Subnuclear distribution of chromatin-bound GFP-MCM2.** A, stable GFP-MCM2 cells were either synchronized in M phase after nocodazole (noc) block and mitotic shake-off and released into G1 phase or synchronized in late G1 phase by mimosine treatment. After 3 h (Post noc 3h) and 7 h (Post noc 7h) following nocodazole release and in late G1 phase (Mimosine block), cells were subjected to FLIP analysis, and representative images are shown before and after the bleaching step. B, stable GFP-MCM2 cells were synchronized in mitosis by monastrol and released into a synchronous G1 phase. 3, 7 and 13 h post-release (early, middle, and late G1 phase, respectively), cells were analyzed by FLIP. Pre- and post-bleach images are shown for representative cells. C, GFP-MCM2 does not colocalize with PCNA foci in the various stages of S phase. Stable GFP-MCM2 cells were transiently transfected with PCNA-RFP. Cells were discriminated according to PCNA patterning. FLIP was carried out, and representative images were taken. Cells before bleaching are displayed on the left (Pre bleach), and cells after bleaching are displayed on the right (Post bleach). In all panels, the bleached regions are marked by a red line in the post-bleached images. In A and B, red arrowheads indicate immobile structures of GFP-MCM2. In C, part of the post-bleach images in early and middle S phase cells has been enlarged (red squares) to highlight the lack of colocalization between PCNA and GFP-MCM2 foci. The difference in fluorescence intensity between A and B is due to differences in recording and bleaching conditions.
the mean intensity of the specified region was plotted over time to ensure that the total intensity remained constant throughout the experiment and that the repeated imaging caused no significant recording bleaching (Fig. 8B). The residence time of GFP-MCM2 molecules on chromatin was estimated by plotting the standard deviation of the per-pixel fluorescence intensity within the defined region as a function of time (Fig. 8C). As shown in Fig. 8C, following FLIP, the high standard deviation of
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per-pixel fluorescence intensity observed arises from regions with high levels of bound GFP-MCM2. The standard deviation decreased over time, reflecting the dissociation of GFP-MCM2 molecules from chromatin, which move and diffuse within the cell nucleus. As shown in Fig. 8C, the reduction in standard deviation of the intensity reached a minimum value within 60–80 min after FLIP. This observation indicates that GFP-MCM2 resides on chromatin for approximately 1 h. No significant difference was detected in the residence time of GFP-MCM2 molecules in cells in G₁ and early and middle S phases (Fig. 8C).

DISCUSSION

Here we use a real-time in vivo licensing assay to study in time and space the dynamic behavior of MCM proteins within live human cancer cells. Our analysis reveals distinct modes of MCM interactions with chromatin through the cell cycle. Transient interactions in late mitosis are converted to stable binding during G₁ phase, whereas significantly increased loading is apparent close to the G₁-to-S phase transition, followed by loss of interactions during S phase.

A Licensing Assay in Live Human Cells—Functional imaging was used to assess the dynamics of MCM proteins within live human cells. The suitability of the approach to accurately report on the interactions of MCM proteins within cells was verified by several observations. Correct localization and expression levels comparable with the respective endogenous proteins were shown for the GFP-tagged MCM proteins used, whereas cell lines stably expressing the GFP-tagged MCM proteins exhibited similar cell cycle profiles to that of parental MCF7 cells. MCM2 and MCM4 GFP-tagged proteins cofractionated with chromatin in close match to the endogenous proteins and formed complexes with endogenous MCM subunits. FRAP data analysis revealed an immobile fraction for MCM proteins at cell cycle stages when chromatin is licensed for replication (G₀ and early S phase). Importantly, siRNA-mediated depletion of Cdt1, a central licensing factor required for the loading of MCMs onto chromatin during G₁ phase, resulted in decreased immobile fractions for both GFP-MCM2 and GFP-MCM4 as compared with control siRNA-treated cells. Taken together, these observations illustrate that mobility assessed by FRAP indeed reflects the binding of MCMs to chromatin in human cultured cells. Thus, live cell imaging analysis permits the study of the binding properties of MCM proteins with native chromatin in intact living human cells, serving as a molecular tool for the measurement of DNA licensing with high spatiotemporal resolution.

Multistep Loading of MCM Proteins to Chromatin—Our analysis showed that MCM interactions with chromatin within live cells take place in multiple distinct steps. In early mitosis and up to anaphase, no association of MCM proteins with chromatin is observed, and MCM proteins apparently freely diffuse within the cell nucleus with kinetics comparable with free GFP. During telophase and as a nuclear envelope reforms, retardation in GFP-MCM2 and GFP-MCM4 recovery becomes apparent, indicating a transient association with chromatin. Recovery is however complete within a few seconds, suggesting that there is no stably bound component. This is paralleled by the behavior of Cdt1, which appears free to diffuse and excluded from chromatin up to anaphase and exhibits transient interactions with chromatin in telophase (47). In contrast to Cdt1, however, which maintains transient interactions with chromatin throughout G₁ phase, both GFP-MCM2 and GFP-MCM4 exhibit an immobile fraction and elevated rate of recovery of the mobile fraction during G₁ and S phases, with the time of 50% fluorescence recovery (t₁⁄₂) ranging from 0.2–0.3 s compared with 0.8–1.0 s in telophase, respectively. We speculate that, during telophase, MCM proteins exchange dynamically on and off chromatin, probing the genome continuously to find appropriate binding sites. Upon entering G₁ phase, MCM proteins alter kinetic properties, and a fraction of them becomes stably bound to chromatin. The stably bound fraction of MCM proteins increases throughout G₁ phase, suggestive of reiterative licensing. Maximal loading is observed at the end of the G₁ phase, just prior to the G₁-to-S phase transition. The bound fraction of MCM proteins becomes gradually lost during S phase, consistent with loss of licensing as DNA is replicated. The binding of MCM proteins to chromatin during G₁ phase appears to be highly stable, consistent with previous studies (48). We have estimated an apparent residence time of 1–2 h on the basis of FLIP analysis, whereas earlier work indicated even longer residence times for MCM proteins in Chinese hamster ovary cells (48). In contrast, other components of the prereplication complex, such as ORC (45) and Cdt1 (46, 47), dissociate completely from chromatin within seconds or minutes. This is consistent with in vitro work in Xenopus egg (63) and yeast extracts (64, 65) that showed that the MCM hexamer can be stably loaded on chromatin, whereas ORC and Cdt1 can be subsequently washed away.

MCM Recruitment Can Be Separated from Loading during Late Mitosis—In vitro studies in yeast have shown MCM loading onto chromatin to take place in two steps. Initial recruitment is converted to stable loading following ATP hydrolysis (36–38). We show here that, in human cells, MCM proteins interact transiently with chromatin during telophase, exhibiting kinetics similar to Cdt1. This is consistent with MCM recruitment taking place during late mitosis. A transiently interacting MCM component was not detected during G₁ phase. A likely explanation for our inability to detect a transiently interacting MCM component during G₁ phase, despite indications for reiterative licensing, is that during the G₁ phase, the recruitment of MCM proteins may be a brief intermediate step that quickly converts to stable loading, and, therefore, a transiently interacting fraction is not detectable. In contrast, during late mitosis, stable loading of MCM proteins onto chromatin may not yet be permitted, and MCM proteins may be trapped in a transiently interacting state for a brief window of time, allowing dynamic associations to be revealed. Posttranslational modifications of MCM proteins or other prereplicative complex subunits could result in trapping the MCM complex in a recruited, but not yet loaded, state during telophase. It is interesting to note that both MCM4 and Cdt1 appear to be hyperphosphorylated in mitosis and early G₁ phase (Ref. 58 and Fig. 2). It is also intriguing that, during telophase and early G₁ phase, chromosome territories within the nucleus are estab-
lished in parallel with establishment of the timing of origin firing in the next S phase (10). Work in fission yeast suggested that the temporal pattern of DNA licensing may determine and reflect the replication timing of DNA origins. MCM proteins were shown to bind early firing origins earlier in G1 phase compared with late-firing origins (66). Using FLIP, we observed the accumulation of MCMs at late-replicating heterochromatic regions from early G1 phase in human cells (3 h post-mitotic release) and throughout G1 phase, in line with previous studies on fixed cells (67). This suggests that if such a differential timing of MCM association with late replicating regions takes place in human cells, then it is confined to mitotic exit and very early G1 phase.

The Majority of MCM Proteins Are Loaded onto Chromatin in Late G1 Phase—Maximal binding of MCM proteins with chromatin was observed late in G1 phase, close to the G1-to-S phase transition by both FRAP and FLIP analysis. Although a small fraction of MCM proteins is stably bound to chromatin from early G1 phase (around 15%), 30–50% of the molecules are bound just prior to S phase onset. Although we cannot exclude that maximal binding is due to a gradual loading of MCM proteins during the course of G1 phase, our data are more consistent with a wave of MCM chromatin loading or stabilization of MCM proteins on chromatin, preceding S phase entry. A wave of MCM loading could, for example, be enhanced through cyclin E-mediated protection of cdc6 from proteolysis (68) after the restriction point, whereas a stabilization of MCM proteins on chromatin could be linked to a decrease in Cdt1 protein levels close to the G1-to-S phase transition (50). Our data suggest that, although licensing initiates at the end of mitosis, chromatin remains underlicensed until late in G1 phase, close to the G1-to-S phase transition. Because premature entry into S phase and under licensing have been linked to DNA replication stress (24), gaining insight into the events that control multistep MCM loading is important and will be facilitated by the functional assays presented here.

During S phase, MCM proteins appear to mark unreplicated DNA regions, consistent with previous experiments with fixed (32, 33, 61) and live (48) cells. The gradual loss of MCM foci as S phase progresses supports the notion that, during the process of DNA replication, the DNA replication machinery destabilizes and displaces MCM hexamers from chromatin. Moreover, we observed that the distribution of MCM foci in each phase resembles the characteristic pattern of PCNA of the following phase, in agreement with previous studies performed on fixed cells. In middle S phase, as heterochromatic regions around the nucleoli and at the nuclear periphery were being replicated, MCM foci were distinct from, but in close proximity to, PCNA foci. Previous analyses showed that new replication factories tend to form adjacent to the ones that recently disassembled (8). Close proximity of active replication factories to high concentrations of licensed origins could contribute to recruitment of released replication factors to these adjacent regions, consistent with a “domino” model of replication factory propagation (9).

In addition to providing insight into the molecular mechanisms involved and the dynamics of DNA licensing within the mammalian cell nucleus, the assays described here are of potential practical utility. For example, the combination of an in vivo licensing assay with depletion or overexpression of specific factors may allow the identification of novel factors that block or enhance DNA licensing. Moreover, given that Cdt1/Cdc6 overexpression and, thus, overlicensing of DNA has been associated with malignant transformation and tumorigenesis, the licensing system has been proposed as a novel molecular target for anticancer drug design (23, 25). An in vivo licensing assay may offer a new approach for cell-based screens for the identification and validation of antitumor compounds and drugs under development that will block DNA licensing and, thus, DNA replication in cancer cells.

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REFERENCES

1. Baker, T. A., and Bell, S. P. (1998) Polymerases and the replisome. Machines within machines. Cell 92, 295–305
2. Waga, S., and Stillman, B. (1998) The DNA replication fork in eukaryotic cells. Annu. Rev. Biochem. 67, 721–751
3. Johnson, A., and O’Donnell, M. (2005) Cellular DNA replicases. Components and dynamics at the replication fork. Annu. Rev. Biochem. 74, 283–315
4. Syrneondiou, I. E., Taraviras, S., and Lygerou, Z. (2012) Control over DNA replication in time and space. FEBS Lett. 586, 2803–2812
5. Hozák, P., Hassan, A. B., Jackson, D. A., and Cook, P. R. (1993) Visualization of replication factories attached to nucleoskeleton. Cell 73, 361–373
6. Leonhardt, H., Rahn, H. P., Weinzierl, P., Sporbert, A., Cremer, T., Zink, D., and Cardoso, M. C. (2000) Dynamics of DNA replication factories in living cells. J. Cell Biol. 149, 271–280
7. Somananth, S., Suchyn, T. M., Siegel, A. J., and Berezney, R. (2001) Targeting of PCNA to sites of DNA replication in the mammalian cell nucleus. J. Cell Biol. 81, 56–67
8. Sporbert, A., Gahl, A., Ankerhold, R., Leonhardt, H., and Cardoso, M. C. (2002) DNA polymerase clamp shows little turnover at established replication sites but sequential de novo assembly at adjacent origin clusters. Mol. Cell 10, 1355–1365
9. Chagin, V. O., Stear, J. H., and Cardoso, M. C. (2010) Organization of DNA replication. Cold Spring Harb. Perspect. Biol. 2, a00737
10. Rhind, N., and Gilbert, D. M. (2013) DNA replication timing. Cold Spring Harb. Perspect. Biol. 5, a010132
11. Kitamura, E., Blow, J. J., and Tanaka, T. U. (2006) Live-cell imaging reveals replication of individual replicons in eukaryotic replication factories. Cell 125, 1297–1308
12. Blow, J. J., and Dutta, A. (2005) Preventing re-replication of chromosomal DNA. Nat. Rev. Mol. Cell Biol. 6, 476–486
13. Nishitani, H., and Lygerou, Z. (2004) DNA replication licensing. Front. Biosci. 9, 2115–2132
14. Siddiqui, K., On, K. F., and Diffley, J. F. (2013) Regulating DNA replication in eukaryota. Cold Spring Harb. Perspect. Biol. 5, a012930
15. Cocker, J. H., Piatti, S., Santocanale, C., Nishimoto, T., and Nurse, P. (2000) The Cdt1 protein is required to license DNA for replication in fission yeast. Nature 404, 622–625
16. Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000) The Cdt1 protein is required to license DNA for replication in fission yeast. Nature 404, 625–628
17. Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000) The Cdt1 protein is required to license DNA for replication in fission yeast. Nature 404, 625–628
18. Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N., and Oda, M. (2010) Eukaryotic chromosome DNA replication. Where, when, and how?
Maximal Loading of MCM2/4 in Late G, Phase

Annu. Rev. Biochem. 79, 89–130

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

20. 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

21. Lygerou, Z., and Nurse, P. (2000) Cell cycle. License withheld. Geminin blocks DNA replication. Science 290, 2271–2273

22. Tada, S., Li, A., Maiorano, D., Méchali, 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

23. Blow, J. J., and Gillespie, P. I. (2008) Replication licensing and cancer. A fatal entanglement. Nat. Rev. Cancer 8, 799–806

24. McIntosh, D., and Blow, J. J. (2012) Dormant origins, the licensing checkpoint, and the response to replicative stresses. Cold Spring Harb. Perspect. Biol. 4, a012955

25. Petropoulou, C., Kotantaki, P., Karamitros, D., and Taraviras, S. (2008) Cdt1 and Geminin in cancer. Markers or triggers of malignant transformation? Front. Biosci. 13, 4485–4494

26. Adachi, Y., Usukura, J., and Yanagida, M. (1997) A globular complex formation by Nda1 and the other five members of the MCM protein family in fission yeast. Genes Cells 2, 467–479

27. Ervin, C., Clarke, P., Zech, J., Lurz, R., Sun, J., Uhle, S., Li, H., Stillman, B., and Speck, C. (2009) A double-hexameric MCM2–7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. Proc. Natl. Acad. Sci. U.S.A. 106, 20240–20245

28. Remus, D., Beuron, F., Tolun, G., Griffith, J. D., Morris, E. P., and Diffley, J. F. (2009) Concerted loading of Mcm2–7 double hexamers around DNA during DNA replication origin licensing. Cell 139, 719–730

29. Fletcher, R. J., Bishop, B. E., Leon, R. P., Sclafani, R. A., Ogata, C. M., and Ogata, C. M. (1998) Activation of the MCM2–7 helicase by association with Cdc45 and GINS proteins. Mol. Cell 37, 247–258

30. Fu, Y. V., Yardimci, H., Long, D. T., Ho, T. V., Guainazzi, A., Bermudez, V. P., Hurwitz, J., van Oijen, A., Schärer, O. D., and Walter, J. C. (2011) Selective bypass of a lagging strand roadblock by the eukaryotic replicative DNA helicase. Cell 146, 931–941

31. Bell, S. D., and Botchan, M. R. (2013) The minichromosome maintenance replication helicase. Cold Spring Harb. Perspect. Biol. 5, a012807

32. Dimitrova, D. S., Todorov, I. T., Melendy, T., and Gilbert, D. M. (1999) Mcm2, but not RPA, is a component of the mammalian early G1-phase prereplication complex. J. Cell Biol. 146, 709–722

33. Kruze, T., Musahl, C., Laskey, R. A., and Knüppers, R. (1996) Human replication proteins hCdc21, hCdc6e and P1Mcm3 bind chromatin uniformly before S-phase and are displaced locally during DNA replication. J. Cell Sci. 109, 309–318

34. Edwards, M. C., Tutter, A. V., Cvetic, C., Gilbert, C. H., Prokhorova, T. A., and Walter, J. C. (2002) Activation of the MCM2–7 helicase by association with Cdc45 and GINS proteins. EMBO J. 21, 358–366

35. Pacek, M., Tutter, A. V., Kubota, Y., Takisawa, H., and Walter, J. C. (2006) Localization of MCM2–7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Mol. Cell 21, 581–587

36. McNairn, A. J., Okuno, Y., Misteli, T., and Gilbert, D. M. (2008) Chinese hamster ORC subunits dynamically associate with chromatin throughout the cell-cycle. Exp. Cell Res. 308, 345–356

37. Xouri, G., Dimaki, M., Bastiaens, P. I., and Lygerou, Z. (2007) Cdt1 interacts in the licensing process. A model for dynamic spatiotemporal control of licensing. Cell Cycle 6, 1549–1553

38. Xouri, G., Squire, A., Dimaki, M., Geverts, B., Verveer, P. J., Taraviras, S., Nishitani, H., Houtsmostull, A. B., Bastiaens, P. I., and Lygerou, Z. (2007) Cdt1 associates dynamically with chromatin throughout G1 and recruits Geminin onto chromatin. EMBO J. 26, 1303–1314

39. Kuipers, M. A., Stasevich, T. J., Sasaki, T., Wilson, K. A., Hazelwood, K. L., McNally, J. G., Davidson, M. W., and Gilbert, D. M. (2011) Stable loading of Mcm proteins onto chromatin in living cells requires replication to unload. J. Cell Biol. 192, 29–41

40. Pefani, D. E., Dimaki, M., Spell, M., Karantzelis, N., Mitsiki, E., Kyrousi, C., Symeonidou, I. E., Perrakis, A., Taraviras, S., and Lygerou, Z. (2011) Idas, a novel phylogenetically conserved geminin-related protein, binds to geminin and is required for cell cycle progression. J. Biol. Chem. 286, 23234–23246

41. Nishitani, H., Taraviras, S., Lygerou, Z., and Nishimoto, T. (2001) The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. J. Biol. Chem. 276, 44905–44911

42. Mortusewicz, O., Schermell, L., Walter, J., Cardoso, M. C., and Leonardhardt, H. (2005) Recruitment of DNA methyltransferase 1 to DNA repair sites. Proc. Natl. Acad. Sci. U.S.A. 102, 8905–8909

43. Rapsomaniki, M. A., Kotsantis, P., Symeonidou, I. E., Giakoumakis, N. N., Taraviras, S., and Lygerou, Z. (2012) easyFRAP. An interactive, easy-to-use tool for qualitative and quantitative analysis of FRAP data. Bioinformatics 28, 1800–1801

44. Bancaud, A., Huet, S., Rabut, G., and Ellenberg, J. (2010) Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells. FRAP, photobleaching, photoconversion, and FRIP. Cold Spring Harb. Protoc. 2010, p90–p99

45. Körner, M., Frahm, T., and Hauser, H. (2005) Nucleocytoplasmic shuttling revealed by FRAP and FRIP technologies. Curr. Opin. Biotechnol. 16, 28–34

46. Lippincott-Schwartz, J., and Patterson, G. H. (2003) Development and use of fluorescent protein markers in living cells. Science 300, 87–91

47. Masai, H., Taniyama, C., Ogino, K., Matsui, E., Kazukou, N., Mutsumoto, S., Kim, J. M., Ishii, A., Tanaka, T., Kobayashi, T., Takai, K., Ohtani, K., and Arai, K. (2006) Phosphorylation of MCM4 by Cdk7 kinase facilitates its interaction with Cdc45 on the chromatin. J. Biol. Chem. 281, 39249–39261

48. Kimura, H., Ohtomo, T., Yamaguchi, M., Ishii, A., and Sugimoto, K. (1996) Localization of MCM2–7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Mol. Cell 21, 581–587

49. Nishitani, H., Lygerou, Z., and Nishimoto, T. (2004) Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region. J. Biol. Chem. 279, 30807–30816

50. Ishimi, Y., and Komamura-Kohno, Y. (2001) Phosphorylation of MCM4 at specific sites by cyclin-dependent kinase leads to loss of MCM4 helicase activity. J. Biol. Chem. 276, 34428–34433

51. Laskey, R. A., and Madine, M. A. (2003) A rotary pumping model for DNA replication fork helicase. Proc. Natl. Acad. Sci. U.S.A. 103, 10236–10241
Maximal Loading of MCM2/4 in Late G1 Phase

62. Todorov, I. T., Attaran, A., and Kearsey, S. E. (1995) BM28, a human member of the MCM2-3-5 family, is displaced from chromatin during DNA replication. *J. Cell Biol.* **129**, 1433–1445

63. Rowles, A., Tada, S., and Blow, J. J. (1999) Changes in association of the *Xenopus* origin recognition complex with chromatin on licensing of replication origins. *J. Cell Sci.* **112**, 2011–2018

64. Bowers, J. L., Randell, J. C., Chen, S., and Bell, S. P. (2004) ATP hydrolysis by ORC catalyzes reiterative Mcm2–7 assembly at a defined origin of replication. *Mol. Cell* **16**, 967–978

65. Donovan, S., Harwood, J., Drury, L. S., and Diffley, J. F. (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5611–5616

66. Wu, P. Y., and Nurse, P. (2009) Establishing the program of origin firing during S phase in fission yeast. *Cell* **136**, 852–864

67. Dimitrova, D. S., Giacca, M., Demarchi, F., Biamonti, G., Riva, S., and Falaschi, A. (1996) *In vivo* protein-DNA interactions at human DNA replication origin. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1498–1503

68. Mailand, N., and Diffley, J. F. (2005) CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. *Cell* **122**, 915–926