G2 phase chromatin lacks determinants of replication timing

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DNA replication in all eukaryotes follows a defined replication timing program, the molecular mechanism of which remains elusive. Using a *Xenopus laevis* egg extract replication system, we previously demonstrated that replication timing is established during early G1 phase of the cell cycle (timing decision point [TDP]), which is coincident with the repositioning and anchorage of chromatin in the newly formed nucleus. In this study, we use this same system to show that G2 phase chromatin lacks determinants of replication timing but maintains the overall spatial organization of chromatin domains, and we confirm this finding by genome-wide analysis of rereplication in vivo. In contrast, chromatin from quiescent cells retains replication timing but exhibits disrupted spatial organization. These data support a model in which events at the TDP, facilitated by chromatin spatial organization, establish determinants of replication timing that persist independent of spatial organization until the process of chromatin replication during S phase erases those determinants.

Introduction

All eukaryotic organisms replicate their DNA according to a defined replication timing program. The significance of this temporal regulation is not known; however, temporal control of DNA replication is linked to many basic cellular processes that are regulated both during the cell cycle and during development (MacAlpine and Bell, 2005; Farkash-Amar and Simon, 2009; Hiratani et al., 2009; Schwaiger et al., 2009). Unfortunately, very little is known about the mechanisms regulating this program. We have used a cell-free system in which nuclei isolated from mammalian cells at different times during G1 phase are introduced into *Xenopus laevis* egg extracts, which initiate DNA replication rapidly and synchronously in vitro. With nuclei isolated during the first 1–2 h after mitosis, replication does not proceed in any specific temporal order, whereas initiation within nuclei isolated thereafter follows the proper replication timing program. Thus, replication timing is established at a time point during early G1 phase, designated the timing decision point (TDP; Dimitrova and Gilbert, 1999). We further showed that the TDP is coincident with the repositioning of early- and late-replicating segments of the genome to their specific interphase positions (Dimitrova and Gilbert, 1999; Li et al., 2001), and others later demonstrated that this coincided with reduced chromatin mobility or anchorage (Chubb et al., 2002; Walter et al., 2003). A similar phenomenon was also observed in budding yeast (Raghuraman et al., 1997; Heun et al., 2001a). However, it was also found that chromosomal segments can move away from their preestablished subnuclear positions later in the cell cycle but still maintain their replication timing (Bridger et al., 2000; Heun et al., 2001a; Mehta et al., 2007). Together, these studies suggested a model in which anchorage at the TDP could seed the self-assembly of position-specific chromatin architectures that set thresholds for replication, which, once established, persist independent of position until their time of replication in the upcoming S phase (Gilbert, 2002; Hiratani et al., 2009; for review see Gilbert, 2001).

What are the determinants of replication timing that appear at the TDP? We have taken advantage of the narrow cell cycle window of the TDP to search for chromatin changes occurring coincident with the establishment of delayed replication timing

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Abbreviations used in this paper: DHFR, dihydrofolate reductase; Mcm, minichromosome maintenance; RTD, replication timing determinant; TDP, timing decision point.

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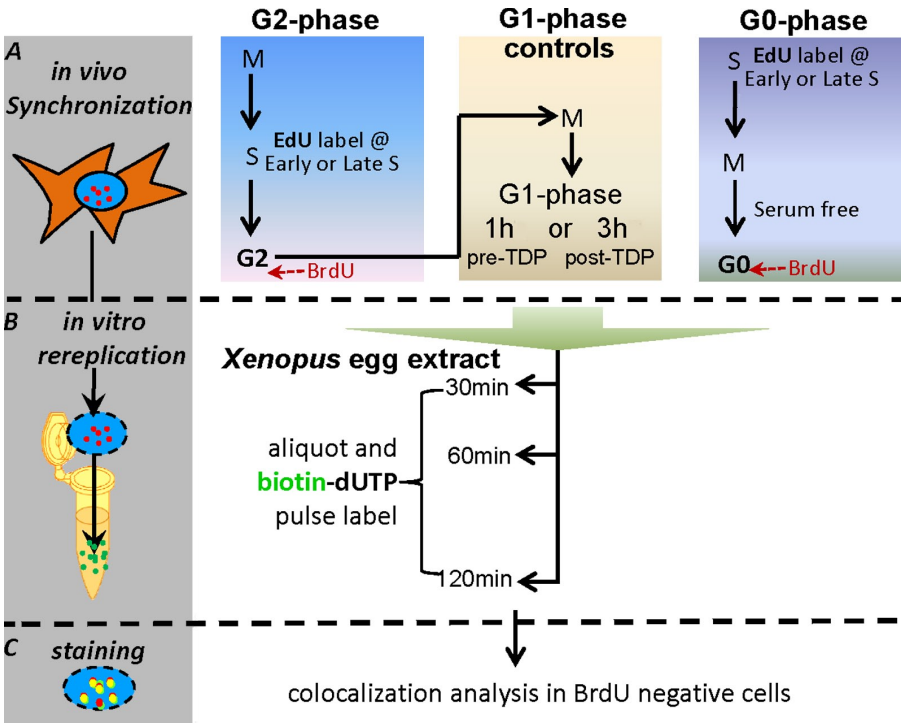

Figure 1. **Generalized protocol.** (A) In vivo synchronization is shown. The protocols used in Figs. 2–5 all begin with the prelabeling of cells in early or late S phase with EdU. To synchronize cells in G2 phase, cells were first synchronized in mitosis by shake off and pulse labeled in either early (~10 h after M phase) or late (~18 h after M phase) S phase with EdU. At ~20 h after mitosis, cells were pulse labeled with BrdU to identify any cells still in late S phase and collected. To make G1 phase controls, aliquots of G2 phase cells were allowed to proceed into mitosis; mitotic cells were then collected by shake off and released into the following G1 phase for 1 (pre-TDP) or 3 h (post-TDP). To prepare cells in G0, asynchronously growing cells were labeled with EdU and chased for 5 (EdU label in late S phase) or 12 h (EdU label in early S phase). Mitotic cells were isolated and released into serum-free medium for 96 h and pulse labeled with BrdU before collection to identify any remaining proliferating cells. (B) In vitro rereplication is shown. Nuclei from each of the cell preparations in A were introduced into a *Xenopus* egg extract. In some reactions, the total level of DNA synthesis per nucleus was monitored by incorporation of digoxigenin-dUTP (not depicted). To evaluate replication timing, reactions were pulse labeled with biotin-dUTP for 5 min at 30 (early), 60 (middle), or 120 min (late) in vitro. (C) Staining is shown. Biotin incorporation in nuclei from the reactions in B was colocalized to either the sites of early and late DNA synthesis labeled in vivo or to specific chromosomal sites of interest.

of heterochromatin. However, chromatin constituents that we have investigated are either constitutively present or associate with chromatin before the TDP (Wu et al., 2006). Similarly, disruption of genes that regulate chromatin structure (Suv39 h1/2, G9a, MII, Eed, Mbd3, Dicer, Dnmt1, and Dnmt3a/3b) has little or no effect on global replication timing, although some modest or localized effects have been observed (Li et al., 2005; Wu et al., 2006; Jørgensen et al., 2007; Goren et al., 2008; Yokochi et al., 2009). Also, transcription of pericentric heterochromatin is cell cycle regulated but is not active until after the TDP (Lu and Gilbert, 2007). We reasoned that further insight into the nature of the replication timing determinants (RTDs) could be gained by investigating when replication timing is lost during the cell cycle. RTDs must be maintained at least until the time of replication during S phase. The two most logical times for the loss of such determinants are at the replication fork, where chromatin is reassembled, or during mitosis when nuclear architecture is dismantled.

In this study, we have distinguished between these two possibilities, demonstrating that G2 phase chromatin lacks the determinants of a normal replication timing program upon rereplication in *Xenopus* egg extracts despite retaining the normal chromatin spatial organization established at the TDP. Rereplication within G2 phase nuclei in cultured cells also did not follow the normal temporal program. In contrast, chromatin within quiescent cell nuclei retained replication timing even though the organization of chromatin within the nucleus was severely altered. Thus, spatial organization is neither necessary nor sufficient to maintain the replication timing program after the TDP. Importantly, our data suggest that the process of replication itself eliminates or dilutes determinants of replication timing, which are not reassembled until early G1 phase of the following cell cycle.

Results

In vitro replication of G2 phase chromatin does not follow a specific temporal order

To study replication timing in G2 phase, chromatin needs to be relicensed for replication. In the normal cell cycle, replication is limited to once and only once per cell cycle by permitting licensing only during G1 phase, manifested by the association of minichromosome maintenance (Mcm) proteins with chromatin. This license (along with the Mcm proteins) is then removed during DNA replication, and chromatin is maintained in the unlicensed state by geminin and high Cdk activity, both of which prevent Mcm loading. This process prevents rereplication during S and G2 phase until cells pass through mitosis and chromatin is relicensed (Blow and Dutta, 2005; Arias and Walter, 2007). However, if G2 phase nuclei are gently permeabilized, nuclear geminin and Cdk are lost from these nuclei, and *Xenopus* replication factors can relicense and replicate G2 phase chromatin (Blow and Laskey, 1988; Leno et al., 1992). To obtain populations of G2 phase cells, we synchronized mouse C127 cells in mitosis by mechanical shake off (98% mitotic figures) and released them into fresh medium for a period of time sufficient to reach mid S phase. We added nocodazole to block fast cycling cells in mitosis, which could be eliminated by a second shake off. 10 h after mid S phase,

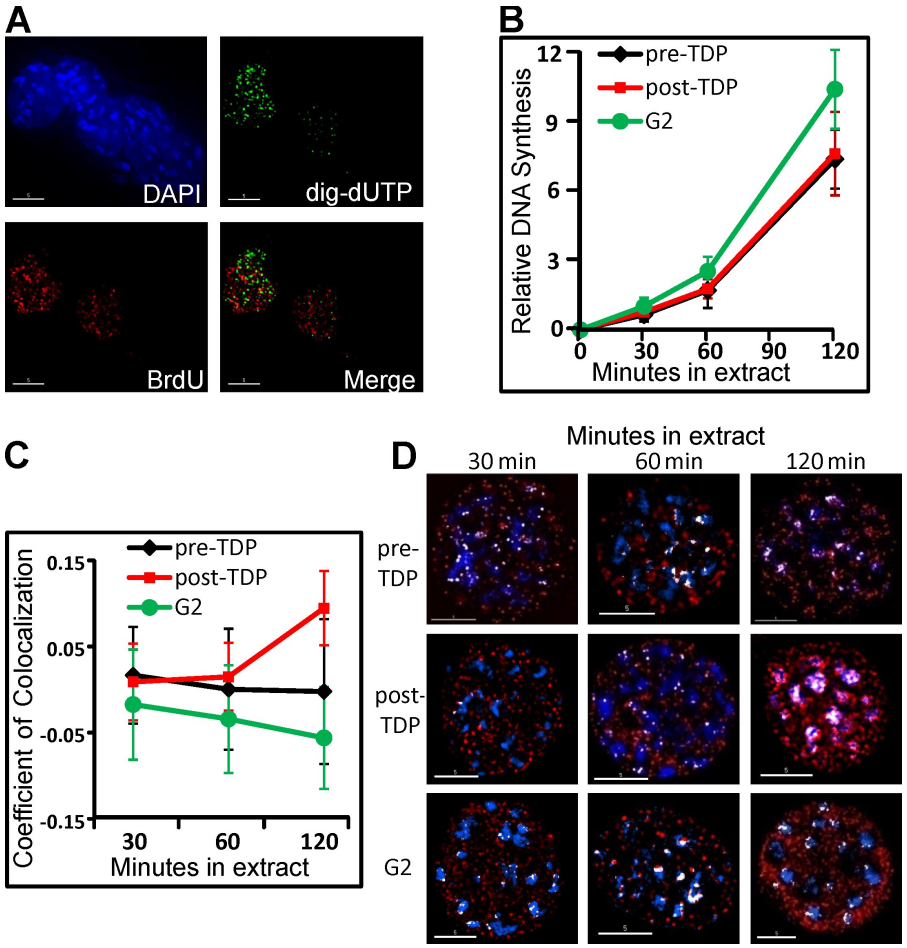

Figure 2. **Asynchronous rereplication of chromocenters in G2 phase.** (A) Geminin prevents in vitro replication of G2 phase chromatin. G2 phase nuclei from mouse C127 cells synchronized and prelabeled with BrdU as described in Fig. 1 were introduced into a *Xenopus* egg extract supplemented with geminin and digoxigenin-dUTP for 120 min. Nuclei were stained with fluorescent anti-BrdU (red) and anti-dig (green) antibodies, and DNA was counterstained with DAPI (blue). BrdU-positive nuclei (still in S phase at the time of collection) incorporated digoxigenin-dUTP as a result of elongation of preexisting forks, but no initiation of replication was observed within G2 phase nuclei. (B) Replication proceeds efficiently within G1 and G2 phase nuclei. Nuclei prepared as described in Fig. 1 A were introduced into a *Xenopus* egg extract supplemented with digoxigenin-dUTP. Aliquots were removed at the indicated times, and nuclei were stained with fluorescent anti-dig (and for G2 phase nuclei anti-BrdU) antibodies. The incorporation of digoxigenin-dUTP per (BrdU negative) nucleus was quantified using an imaging system with softWoRx and normalized to the lowest value for all nuclei in all preparations. $n > 30$ nuclei per condition/time point (arbitrary units). Note that G2 phase nuclei incorporate 40% more digoxigenin-UTP. Because there is twice as much DNA in G2 phase nuclei, the efficiency of replication is ~70% that of G1 phase. (C) Chromocenters are preferentially replicated late only in post-TDP nuclei. Nuclei prepared as described in Fig. 1 A were introduced into a *Xenopus* egg extract, and aliquots were removed and pulse labeled for 5 min with biotin-dUTP at the indicated times. Nuclei were stained with fluorescent avidin to highlight incorporated biotin, and chromocenters were identified as sites of intense DAPI staining. G2 phase nuclei were additionally stained with anti-BrdU antibodies as in A. Colocalization between chromocenters and in vitro biotin labeling was quantified using softWoRx. Pre-TDP and G2 phase nuclei showed a similar amount of colocalization throughout the in vitro reaction, whereas post-TDP nuclei showed significantly more chromocenter replication late in the reaction (120 min). $n > 50$ nuclei/condition/time point. (D) Exemplary images of individual pre-TDP, post-TDP, and G2 phase nuclei from C fixed and stained at different time points during the in vitro reaction. Colocalization between chromocenters (DAPI-dense regions in blue) and in vitro biotin-dUTP pulse labeling (red) are highlighted in white. For G2 phase, only BrdU-negative nuclei were analyzed. Note that the post-TDP 120-min nucleus shows considerably more pixels colocalized with chromocenters than other nuclei. Error bars indicate mean ± SD. Bars, 5 µm.

most (~70–80%) attached cells were in G2 phase. Cells still in late S phase could be identified by pulse labeling with BrdU before harvesting. Control cells were synchronized in G1 phase at either 1 (G1-1h; pre-TDP, no timing program) or 3 h (G1-3h; post-TDP, timing program intact) after mitosis (Fig. 1). After digitonin permeabilization of the nuclear membrane, nuclei were introduced into a *Xenopus* egg extract supplemented with digoxigenin-dUTP. To verify that BrdU-negative nuclei are unlicensed G2 phase nuclei, aliquots of these extracts were supplemented with geminin to prevent *Xenopus* Mcms from licensing chromatin, which revealed DNA synthesis only within the contaminating BrdU-positive S phase nuclei (Fig. 2 A). Without geminin, ~70% of both G2 and G1 phase nuclei initiated replication within 30 min, and this percent increased up to almost 95% by 60 min (unpublished data). By measuring the relative amount of digoxigenin-dUTP incorporation per nucleus, we found the overall rate of replication in G2 phase nuclei to be close (~70%) to that of permeabilized G1 phase nuclei (Fig. 2 B). As previously reported (Wu et al., 1997; Dimitrova and Gilbert, 1998; Thomson et al., 2010), the overall length of S phase was compressed, and late replication began during the second hour (Fig. S1 A). When biotin-dUTP was used to pulse label replication at different times during the reaction and nuclei were stained with fluorescent avidin, the in vitro spatial patterns of DNA synthesis with post-TDP nuclei followed the typical early/late temporal order, whereas pre-TDP and G2 phase nuclei did not display any recognizable spatial patterns for DNA synthesis early (30 min), late (120 min), or even very late (180 min) during in vitro reaction (Fig. S1 A), suggesting the absence of temporal control.

As a model for late-replicating chromatin, we monitored the timing of mouse chromocenter rereplication in this system. Mouse chromocenters are clusters of pericentric chromatin that normally replicate in mid/late–S phase (Wu et al., 2006). Permeabilized G2 phase nuclei were introduced into *Xenopus* egg extract, and in vitro replication was monitored by pulse labeling with biotin-16-dUTP (Fig. 1). Chromocenters could be easily identified as prominent DAPI-dense regions, and their replication could be monitored by colocalization with biotin. Permeabilized nuclei from cells synchronized at G1-1h and G1-3h served as controls. We observed significantly stronger colocalization

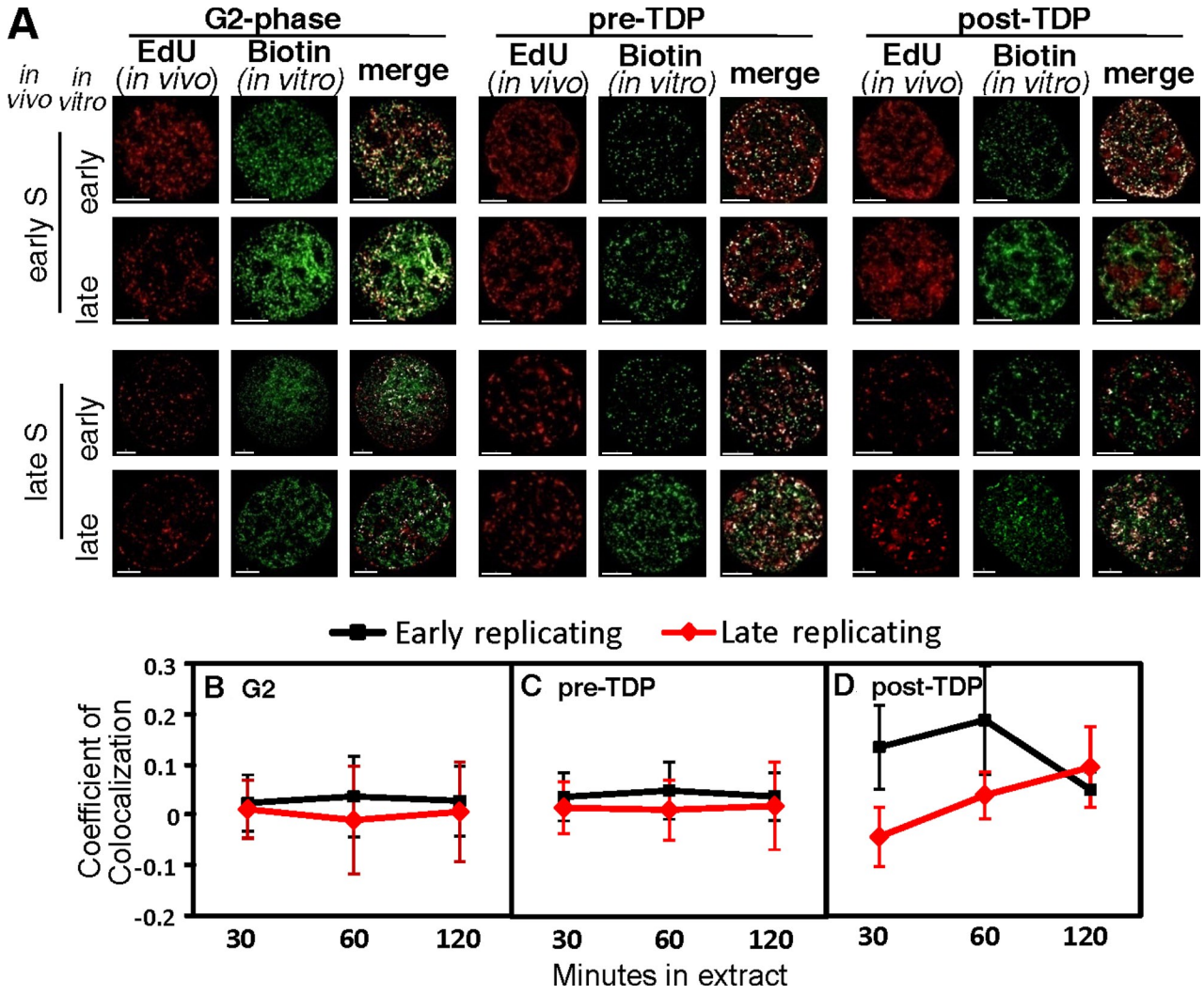

Figure 3. **Global rereplication in G2 phase does not follow a specific temporal order.** (A) Nuclei from mouse C127 cells prelabeled and synchronized in G1 or G2 phase as in Fig. 2 were introduced into a *Xenopus* egg extract and pulse labeled with biotin-dUTP. Nuclei were stained with fluorescent avidin to highlight incorporated biotin, fluorescent azide to highlight sites of early and late replication (EdU) from the prior S phase, and anti-BrdU antibodies to identify contaminating S phase cells. Images were collected and subjected to deconvolution using DeltaVision. Exemplary images of G2 phase (BrdU negative) nuclei or G1 phase control nuclei pulse labeled in vitro at either 30 (in vitro early) or 120 min (in vitro late) are shown. Merged images highlight areas of EdU and biotin colocalization as white pixels. Note the similar level of colocalization among all nuclei for G2 phase and pre-TDP G1 phase, indicating lack of temporal specificity, whereas post-TDP nuclei show colocalization in the proper temporal order (C). (B–D) Quantification of EdU and biotin-dUTP colocalization for G2 phase (B), pre-TDP (C), and post-TDP (D) G1 phase nuclei from the experiments described in A. Colocalization between in vivo early (black) or late (red) S phase EdU label and biotin-dUTP label incorporated at the indicated times during the in vitro reaction was quantified as in Fig. 2. Both pre-TDP and G2 phase nuclei showed similar colocalization throughout the in vitro reaction. Mean ± SD of $n > 50$ nuclei/condition/time point is shown. Bars, 5 µm.

between chromocenters and biotin in post-TDP versus pre-TDP nuclei late during the in vitro reaction (Fig. 2, C and D), confirming that permeabilization did not change the chromocenter replication timing program established at the TDP (Fig. 2, C and D). Chromocenters within G2 phase nuclei showed a low degree of colocalization with biotin throughout the in vitro reaction period (Fig. 2, C and D), which is similar to pre-TDP G1 phase cells, demonstrating a lack of temporal control. With all three preparations of nuclei, replication occurred gradually and on the outer surface of the chromocenters, as occurs in vivo (Guenatri et al., 2004; Wu et al., 2005), indicating that these aspects of chromocenter replication are independent of replication timing. Importantly, although chromocenters were less well clustered in pre-TDP versus post-TDP nuclei, as expected (Wu et al., 2006), they remained well clustered in G2 phase nuclei even after incubation in egg extract (Fig. 2 D). Thus, the late-replicating property of chromocenters is lost in G2 phase despite their compact chromatin.

To confirm these findings, we analyzed the replication timing of globally labeled early- and late-replicating chromatin. To do so, cells were first pulse labeled with EdU (5-ethynyl-2′-deoxyuridine), a novel nucleoside analogue of thymidine (Salic and Mitchison, 2008) for 30 min (in vivo labeling) during early or late S phase, then chased into G2 phase. Nuclei from these cells were permeabilized, introduced into *Xenopus* egg extract, and rereplication was monitored with biotin-dUTP (in vitro labeling). As controls, aliquots of these same prelabeled G2 phase populations were further chased into G1 phase of the next

cell cycle and similarly subjected to in vitro replication. When in vivo (either early or late) and in vitro replication labels were compared (Fig. 3 A), significant colocalization was observed with G2 phase nuclei at all times during the in vitro reaction, indicating a lack of replication timing control (Fig. 3, A and B). Analysis of G1-1h and G1-3h nuclei confirmed that the replication timing program was established at the TDP of the following cell cycle as expected (Fig. 3 C). However, monitoring the in vivo EdU label in the same cells revealed that the overall organization of early- and late-replicating chromatin is reestablished at the TDP and maintained until G2 phase (Fig. 3 A). Moreover, although nuclei swell during incubation in *Xenopus* egg extract, the spatial organization of labeled replication domains in post-TDP or G2 phase nuclei is not disrupted during the course of in vitro replication (Fig. S1 B). Together, these results demonstrate that determinants of the global replication timing program, recognized by *Xenopus* egg cytosol, have been lost in G2 phase cells despite retention of chromatin spatial organization.

Quiescent cells maintain a replication timing program despite altered nuclear organization

One caveat of these experiments is that pre- and post-TDP chromatin are prelicensed in mammalian cells, whereas G2 phase chromatin is licensed in vitro by *Xenopus* licensing factors, which could potentially modify the replication timing program. Because quiescent or G0 cells are also unlicensed but contain prereplicative chromatin, we analyzed replication of chromatin from G0 cells, which similarly must be licensed by the *Xenopus* Mcm proteins to replicate in vitro (Leno and Munshi, 1994). Mitotic C127 cells, prelabeled with EdU during early or late S phase, were released into serum-free medium for up to 4 d until mammalian Mcm proteins were completely released from chromatin (Fig. 4 A). Permeabilized nuclei from these cells were introduced into *Xenopus* egg extract. Similar to G2 phase nuclei, geminin prevented replication of G0 chromatin in *Xenopus* egg extract (Fig. 4 C). The relative level of replication in the absence of geminin was monitored as in Fig. 2 (Fig. 4 B). Colocalization of early or late in vivo EdU labeling with early and late in vitro biotin-dUTP labeling revealed that replication timing was maintained in these cells (Fig. 4, D and E), which is similar to post-TDP G1 phase (Fig. 3 D).

Chromatin is reorganized when cells enter quiescence, which is manifested as changes in subnuclear position (Bridger et al., 2000; Mehta et al., 2010) and an increase in the distance between replication foci (punctuate sites of DNA synthesis) labeled in a previous S phase (Zink et al., 1999). We also noticed that in quiescent C127 cells, replication patterns labeled during the previous S phase became slightly distorted (Fig. 4 D), and pericentric heterochromatin became less compact (Fig. 4 A). We quantified pericentric heterochromatin decondensation by DNA-FISH with a major satellite (pericentric) probe in G0 and G1 phase C127 cells. Major satellite heterochromatin occupied ∼14.8% of nuclear volume in both pre-TDP G1 phase and G0 nuclei but only 10.7% of the nucleus in G1-3h cells (Fig. 4 F). Thus, chromatin reorganization that occurs in quiescent cells does not affect the maintenance of replication timing established at the TDP.

Massive decondensation of a heterochromatic locus in quiescence with retention of replication timing

Although they do not display chromocenters, CHO cells offer certain advantages over mouse cells in the analysis of replication timing. For example, the spatial patterns of DNA synthesis are more easily distinguished than in mouse (O'Keefe et al., 1992; Dimitrova and Gilbert, 1999). To extend our results to another cell line and species, CHO cells were labeled with EdU in early or late S phase, synchronized in G1, G0, or G2 phase as described for mouse cells, and the global replication timing program within permeabilized nuclei from these cells was evaluated after introduction into *Xenopus* egg extracts. These experiments revealed that CHO cell G2 nuclei also lack a replication timing program, unlike G0 or G1 post-TDP nuclei that retain the program (Fig. S2). Thus, lack of timing program in G2 nuclei is neither cell line specific nor species specific.

Another advantage of CHO cells is that we have identified a prominent late-replicating locus consisting of an ∼3-Mb region containing ∼1,000 copies of a long interspersed repeat sequence (C3) that is confined to a single locus on the long arm of chromosome 1 (Fig. 5 A), making its localization and replication easy to monitor. We previously demonstrated that the replication timing of C3 is determined at the TDP (Dimitrova and Gilbert, 1999). We have now determined by DNA-FISH in synchronized cells that C3 localizes to the nuclear periphery at the TDP (Fig. S3 A) and that the peripheral localization is maintained from post-TDP through G2 phase (Fig. 5 B and Fig. S3). Because there is only one haploid copy of this locus per cell, the timing of C3 replication in *Xenopus* egg extract can be determined as colocalization between C3 DNA-FISH and in vitro biotin-dUTP staining. Using this approach, we found that only post-TDP nuclei retained late replication of C3, whereas in pre-TDP and G2-phase nuclei, C3 replicated randomly (Fig. 5 C).

Unfortunately, we were not able to colocalize biotin-labeled DNA synthesis in vitro with the C3 locus in G0 nuclei because the C3-containing chromatin was dramatically dispersed as cells entered quiescence, rendering it difficult to detect by DNA-FISH (Fig. 5 D). To analyze replication of C3 in G0 cells, we used a previously developed hybridization-based approach (Dimitrova and Gilbert, 1999). In brief, permeabilized G0 CHO nuclei were introduced into *Xenopus* egg extract, and the reaction was pulse labeled with [^{32}P]dATP either early or late during the reaction. Labeled nascent DNA strands were hybridized to the C3 sequence immobilized on nylon filters using a slot blot apparatus. Early-replicating dihydrofolate reductase (DHFR) and middle/late-replicating C1 repetitive sequences (Dimitrova and Gilbert, 1999) were included as controls. Results (Fig. 5 E) revealed that for both post-TDP G1-3h and G0 nuclei, DHFR replicated early, and C3 replicated late during in vitro reaction, whereas C1 replicated at middle/late. When these results were

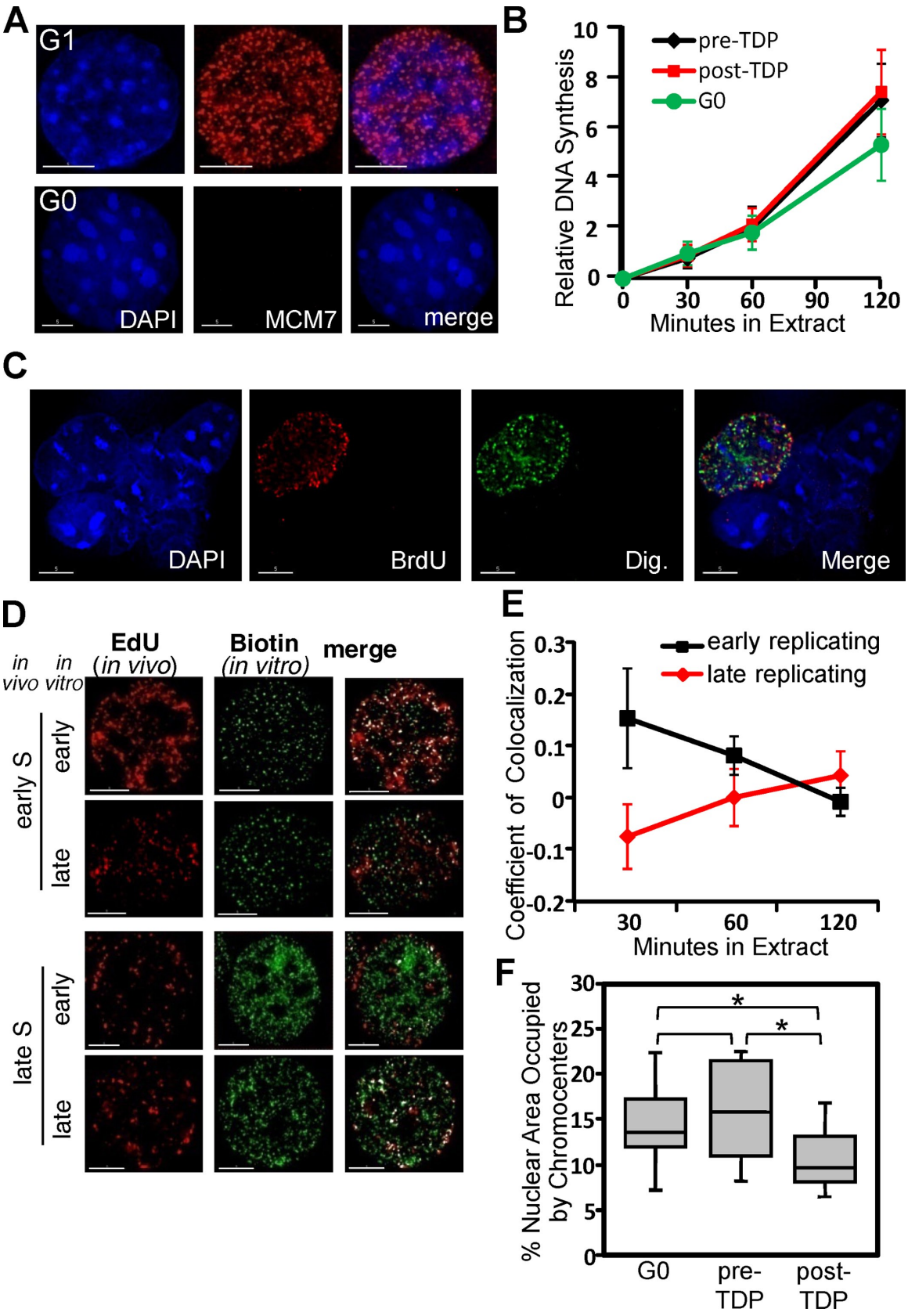

Figure 4. **Quiescent cells retain a replication timing program.** (A) Immunostaining of Mcm7 in normal proliferating G1 phase and quiescent (G0) C127 cells, confirming the loss of Mcm chromatin binding (red, Mcm7; blue, DAPI). (B) Replication proceeds efficiently within G1 and G0 nuclei. Nuclei were introduced into a *Xenopus* egg extract supplemented with digoxigenin-dUTP, and the incorporation of digoxigenin-dUTP per nucleus was quantified as in Fig. 2 A. $n > 30$ nuclei/condition/time point. (C) Geminin prevents in vitro replication of G0 chromatin. G0 nuclei were introduced into a *Xenopus* egg extract supplemented with geminin and digoxigenin-dUTP for 120 min. As in Fig. 2 A, only the few contaminating BrdU-positive nuclei (still proliferating at

quantified, it was obvious that replication timing was very similar between G0 and post-TDP G1 phase nuclei (Fig. 5 F). In contrast, with chromatin from pre-TDP G1 phase nuclei, these sequences were replicated with no temporal preference. This result demonstrated that, despite a dramatic decondensation of C3 locus in G0, which altered both its radial subnuclear position and chromatin compaction, the replication timing program was maintained.

G2 phase rereplication in vivo does not follow a temporal program

To confirm that the aforementioned results could be recapitulated without the use of the in vitro *Xenopus* system, we induced rereplication within live G2 phase C127 cells using a Cdk1 inhibitor, RO3306. RO3306 was previously shown to block HeLa, HCT116, and SW480 cells in G2/M phase by specifically inhibiting Cdk1 activity (Vassilev, 2006; Vassilev et al., 2006). We confirmed that HeLa cells arrest in G2 phase in the presence of RO3306, whereas CHO and mouse L cells did not respond to this drug (unpublished data). Interestingly, we found that C127 cells undergo a complete round of rereplication without an intervening mitosis at a relatively low concentration (~5–12 µM) of RO3306 (Fig. S4 A). This is not unexpected because it has been previously demonstrated that inhibition of Cdk1 activity in G2 phase cells can induce rereplication in some cell lines but not others (Coverley et al., 1998; Diffley, 2004; Hochegger et al., 2007).

Flow cytometry suggested that C127 cells treated asynchronously with RO3306 complete S phase before the onset of rereplication (Fig. S4 A). To verify this, C127 cells were first synchronized in late S phase, pulse labeled with BrdU to label late-replicating DNA, and treated with RO3306 for up to 30 h. Cells were pulse labeled with EdU at various times after drug treatment and stained for BrdU and EdU. Results revealed that cells first completed late replication, which progressed normally as detected by spatial patterns of DNA synthesis, and entered G2 phase during which no DNA synthesis was detected. Subsequently (~3 h later), Mcm was reloaded onto chromatin (determined as in Fig. 4 A), and cells initiated rereplication (Fig. S4 B). During this treatment, the BrdU-labeled late-replicating DNA retained its normal subnuclear spatial pattern, indicating that the chromatin itself was not rearranged (Fig. S4 B). Importantly, although G2 phase cells initiated rereplication asynchronously over the course of several hours, when cells were pulse labeled with EdU at different times during rereplication and EdU was colocalized with late BrdU-labeled DNA, the level of colocalization did not increase with increasing times after reinitiation, as would be expected if the replication timing program was maintained (Fig. S4 C). Furthermore, when cells that were pulse labeled with BrdU in either a normal or a rereplication S phase were flow sorted into four fractions of increasing DNA content and stained for BrdU, the expected spatial patterns were found in each normal S phase fraction, but no recognizable BrdU patterns were found in any rereplication fraction (Fig. S4 D). To verify these results in real time, we constructed a C127 cell line stably expressing GFP-tagged PCNA (Leonhardt et al., 2000) and repeated the aforementioned experimental scheme except that cells were not pulse labeled, but instead, cells in late S phase (based on PCNA spatial patterns) were monitored by live cell imaging from the time of RO3306 addition. These results (Fig. 6) verified that cells completed late S phase PCNA patterns in the presence of RO3306, entered G2 phase, and reinitiated focal PCNA patterns that did not proceed through any recognizable spatiotemporal sequence. Together, these results indicate that rereplication of DNA induced by Cdk1 inhibition does not follow the normal spatiotemporal sequence.

To directly analyze whether rereplication of genomic DNA proceeds according to any specific temporal order, we used a previously described (Hiratani et al., 2008) oligonucleotide array-based genome-wide replication timing profiling method to compare normal S phase progression with RO3306-induced rereplication (Fig. 7 A). In brief, C127 cultures treated with RO3306 were pulse labeled with BrdU, and cells with a DNA content of 4-6C (early rereplication) or 6-8C (late rereplication) were flow sorted, BrdU-substituted DNA was immunoprecipitated with anti-BrdU antibodies, and early- and late-rereplicated DNA was differentially labeled and hybridized to a whole genome oligonucleotide microarray. Early- and late-replicated DNA from the normal S phase of untreated cells were used as a control. Results (Fig. 7 B) revealed that RO3306-induced rereplication had considerably reduced specificity. The residual specificity resembles the normal S phase and can be accounted for by the presence of tetraploid cells within the C127 cell population that have the same DNA content as rereplicating diploid cells (Fig. S5). We conclude that rereplication in living cells induced by Cdk1 inhibition during G2 phase does not follow a defined temporal sequence.

Discussion

In this study, we demonstrate that G2 phase chromatin, when induced to replicate either in *Xenopus* egg extract or in living cells, does not follow the temporal program seen during a normal S phase. Therefore, postreplicative chromatin does not retain

the time of collection) incorporated digoxigenin-dUTP as a result of elongation of preexisting forks, but no initiation of replication was observed within G0 nuclei. (D and E) G0 nuclei were introduced into a *Xenopus* egg extract, pulse labeled for 5 min with biotin-dUTP at the indicated times, and stained as in Fig. 3 (B–D). Colocalization between in vivo early (black) or late (red) S phase EdU label and biotin-dUTP label was quantified as in Fig. 3. Exemplary images (D) and quantification (E) showed that early- and late-replicating sequences labeled in vivo with EdU are replicated in their proper temporal order in vitro. $n > 50$ nuclei per condition/time point. (F) Cells synchronized in G0 or G1 phase were subjected to DNA-FISH using a major satellite probe to specifically label chromocenters. The area occupied by chromocenters was quantified using softWoRx. Box plots of at least 50 nuclei/sample are shown. The boundary of each box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively. Asterisks indicate statistically significant differences (Student-Newman-Keuls method). Error bars indicate mean ± SD. Bars, 5 µm.

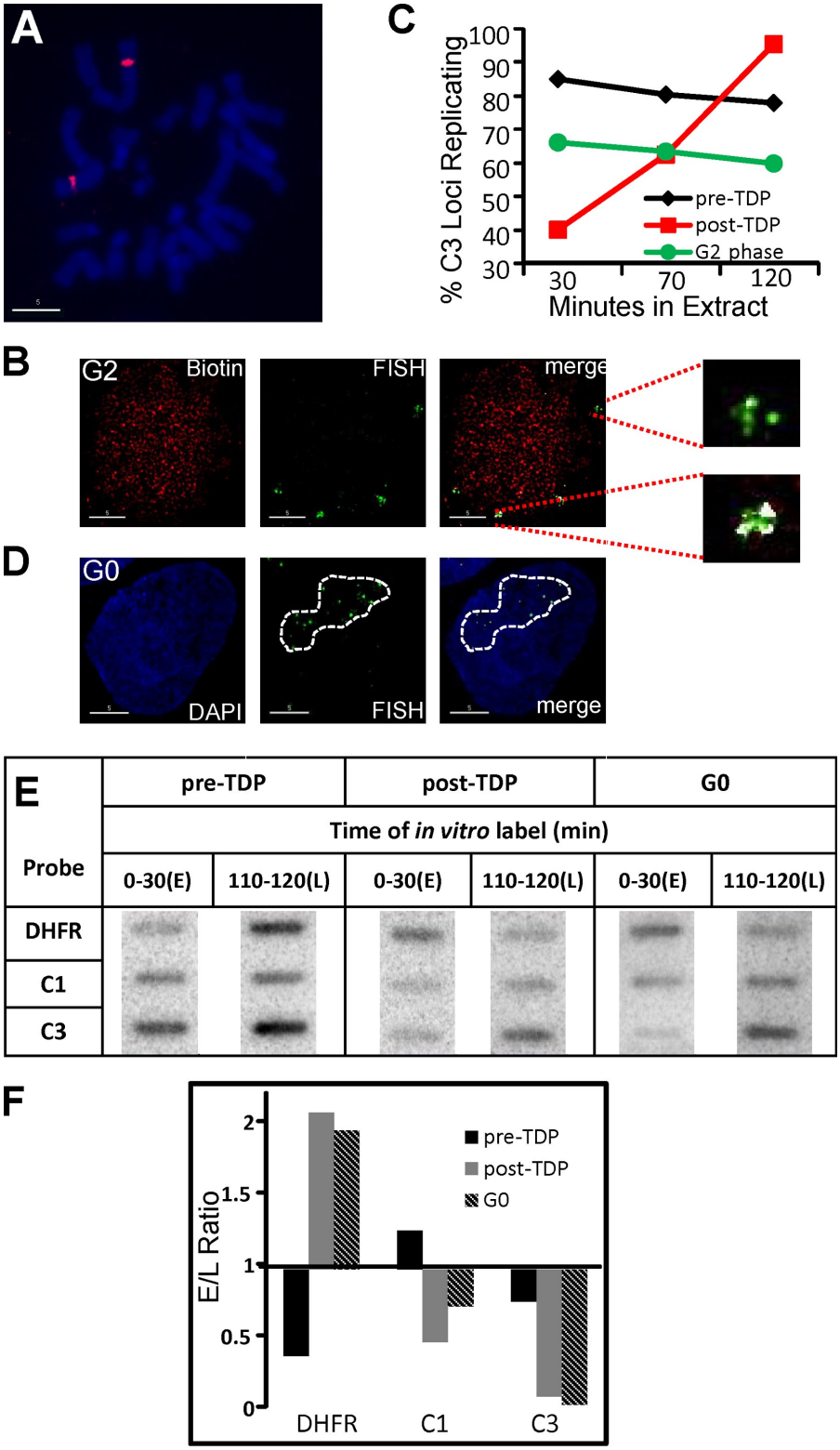

Figure 5. **Massive decondensation of a heterochromatic locus in quiescence with retention of replication timing.** (A) A mitotic spread prepared from CHO cells and subjected to DNA-FISH with C3 probe. C3 was shown to be on chromosome 1 as a single-copy locus. (B) Similar to Fig. 2, nuclei from G2 phase, pre-TDP, and post-TDP G1 phase (not depicted) CHO cells were introduced into a *Xenopus* egg extract and pulse labeled with biotin-dUTP. Nuclei were stained with fluorescent avidin to highlight incorporated biotin and DNA-FISH for C3 locus. Images were collected and processed as in Fig. 2. Images of one G2 phase (BrdU negative) nucleus with four copies of C3 and pulse labeled in vitro at 120 min are shown. Colocalized C3 DNA-FISH signal and in vitro biotin-dUTP pixels are shown in white. Areas covering two of the four C3 loci are magnified (insets) to show the different levels of colocalization. (C) Quantification of C3 in vitro replication. Percentages of C3 DNA-FISH foci engaging in in vitro replication (colocalizing with biotin-dUTP) were quantified from the nuclei prepared in B. Only post-TDP nuclei preferably replicated C3 late in the reaction. $n > 50$ nuclei/condition/time point. Note that in both pre-TDP and G2 phase nuclei, most C3 loci replicate throughout the reaction, indicating that segments of the 3-Mb locus must replicate at different times within the same nuclei. The lower percent for G2 phase nuclei is consistent with a slightly lower efficiency of replication within these nuclei (Fig. 2 B). (D) C3 underwent decondensation in quiescent cells. C3 DNA-FISH to quiescent CHO cells did not detect clear individual signals. Dashed circles denote areas covered by C3 loci in this nucleus. (E) Permeable pre-TDP, post-TDP, or G0 nuclei were introduced into *Xenopus* egg extract. Replication intermediates were pulse labeled with α-[^{32}P]dATP either at early (E; 0–30 min) or late (L; 110–120 min) in vitro reaction. Radiolabeled DNA was purified and hybridized to hamster DNA sequences, including DHFR (early replicating), C1 (middle/late replicating), and C3 (late replicating). Note that this assay is not applicable to G2 phase because of unavoidable S phase contamination in G2 phase population. (F) The relative cpm hybridized to the three probes was quantified. Early to late ratios of each probe were calculated for each cell preparation and plotted, showing similar ratios for post-TDP (gray bars) and G0 (stripe bars) but not pre-TDP (black bars). Bars, 5 µm.

determinants that dictate a replication timing program despite the fact that anchorage and relative subnuclear positions of domains are retained. In contrast, when cells enter quiescence, chromatin retains its predetermined replication timing program despite dramatic changes in chromatin organization. The simplest interpretation of our results is that the replication process itself removes or disrupts RTDs.

Replication timing and spatial organization: establishment versus maintenance of the replication timing program

Recent genome-wide replication timing studies have generated an enormous amount of data regarding replication timing and its dynamics during development in mammalian systems (Farkash-Amar et al., 2008; Hiratani et al., 2008, 2009; Desprat

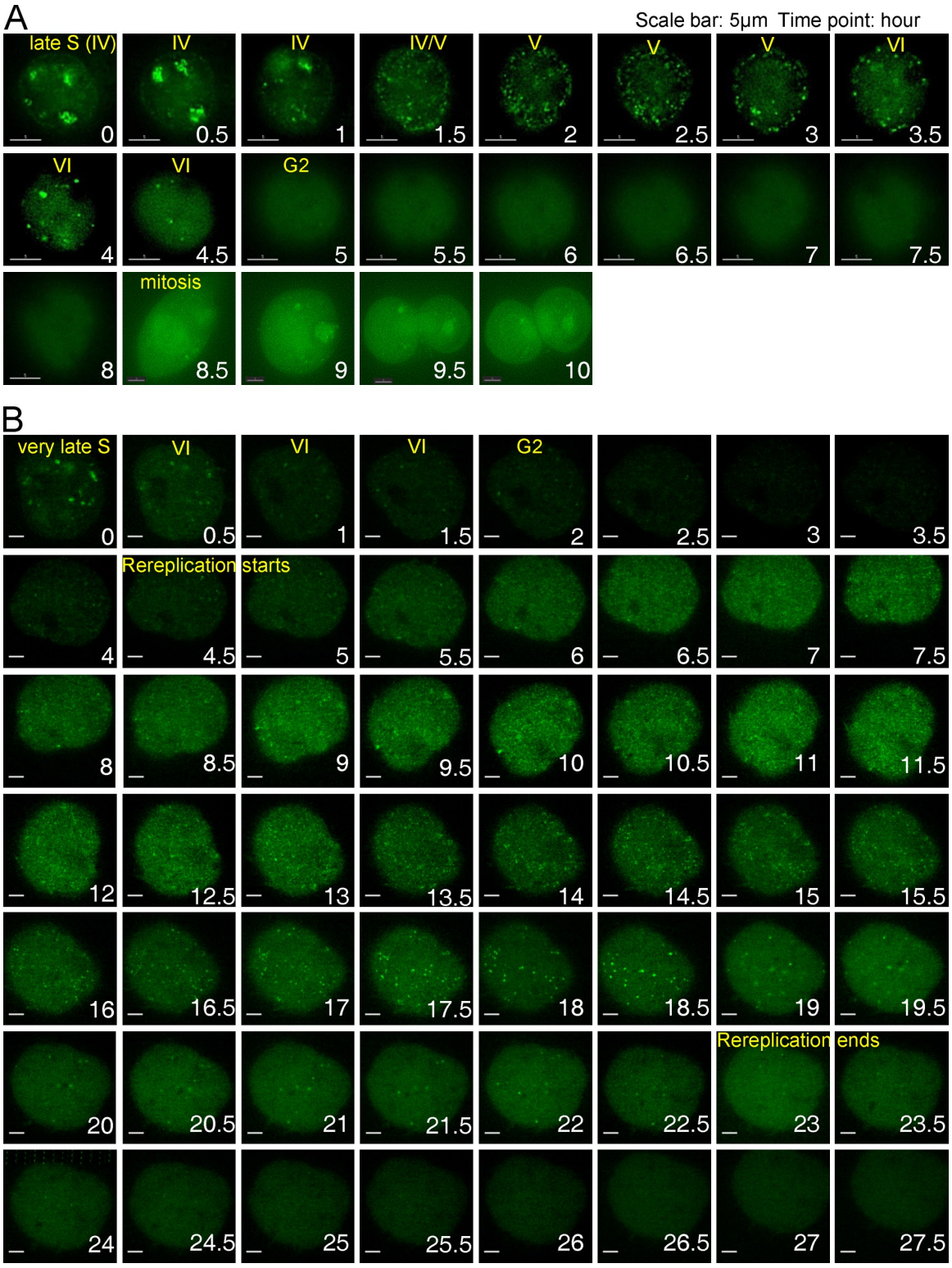

Figure 6. **Live cell imaging of PCNA dynamics during a rereplication S phase.** (A) C127 cells stably expressing GFP-PCNA were followed by a time-lapse live cell imaging microscope every 30 min. Without any drug treatment, GFP-PCNA went through normal spatial–temporal replication patterns. A cell is shown proceeding from late S phase chromocenter replication (pattern IV for C127 cells) to peripheral replication (pattern V), then to a few unfinished regions (pattern VI), finally to G2, M, and early G1 phase of the next cell cycle. PCNA patterns in C127 cells were classified as described previously (Lu and Gilbert, 2007). The contrast was increased for hours 8.5–10 so that the cell morphology is more visible. (B) GFP-PCNA–expressing C127 cells were treated with 6 μM RO3306 and followed for 20–30 h. A cell in very late S phase (pattern VI) is shown at the time of drug addition that entered G2 phase (GFP-PCNA negative) and initiated a second round of DNA replication shortly thereafter. There were no recognizable GFP-PCNA patterns throughout the ~20-h-long rereplication S phase, although the spatial distribution of the few foci at the very end of either normal or rereplication S phase is hard to distinguish. Bars, 5 μm.

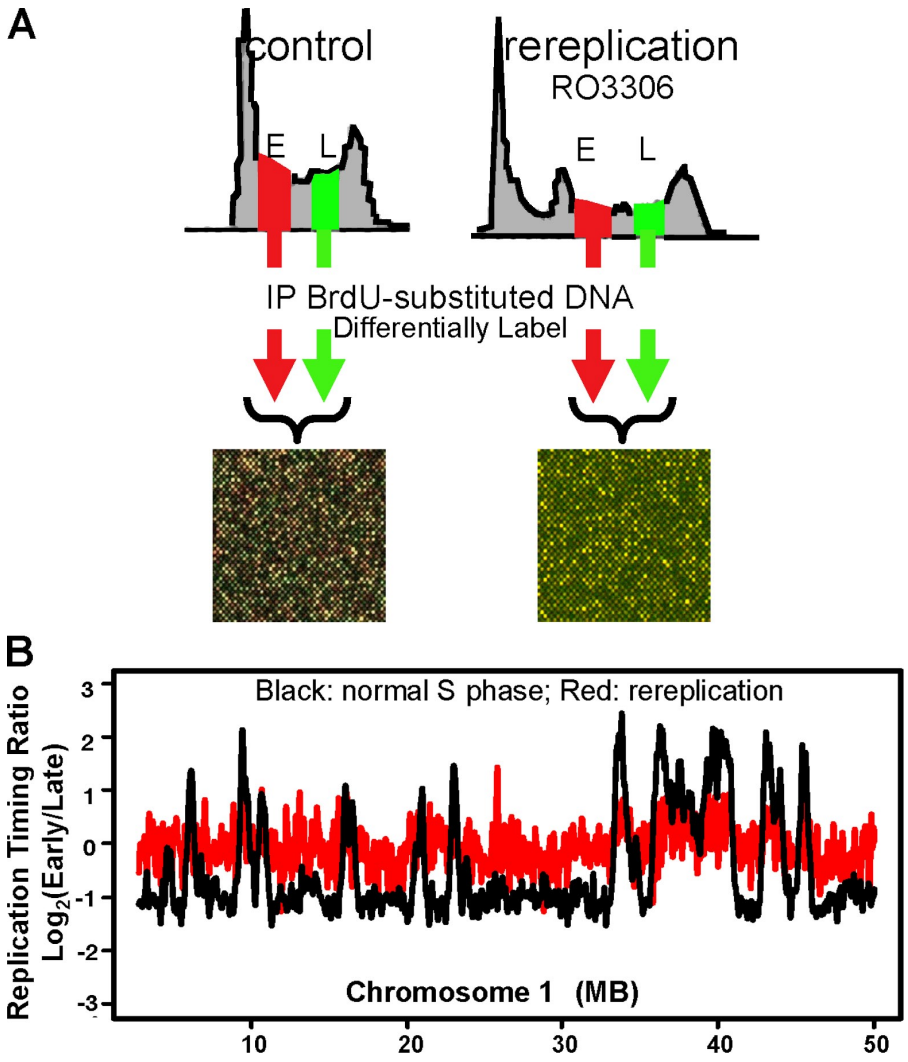

Figure 7. **Genome-wide analysis of replication timing.** (A) Protocol for replication timing profiling. Asynchronously growing control C127 cells or C127 cells treated with 6 µM RO3306 for 20 h were BrdU labeled and FACS sorted into early (E) and late (L) fractions of normal S phase or rereplication S phase. Nascent BrdU-substituted DNA was immunoprecipitated (IP) using BrdU antibodies and hybridized to mouse comparative genomic hybridization arrays with probes every 5.8 kb. (B) Normalized and smoothed replication timing profiles of normal S phase (black) and rereplication (red) were overlayed. A segment of mouse chromosome 1 (3–50 Mb) is shown. Similar results were obtained in two replicate experiments. All datasets are available to view or download at http://www.replicationdomain.org (Weddington et al., 2008).

et al., 2009; Pope et al., 2010). Many of these results suggest links between replication timing and chromatin structure, and a few correlations between replication timing and epigenetic marks are quite strong (Hiratani et al., 2008, 2010; Schwaiger et al., 2009). However, to date, chromatin modification enzymes regulating many of these epigenetic marks and some noncoding heterochromatic RNAs are not required to maintain replication timing (Wu et al., 2006; Jørgensen et al., 2007; Lu and Gilbert, 2007; Hiratani et al., 2009). For example, late replication correlates well with H3K9me2, but deletion of the histone methyltransferase responsible for this modification has almost no effect on replication timing (Yokochi et al., 2009). The strongest correlation to replication timing that has arisen from genome-wide studies is its relationship to recent Hi-C chromatin interaction maps (Ryba et al., 2010), which are generated by cross-linking closely juxtaposed sequences within the nucleus (Lieberman-Aiden et al., 2009). This is compelling evidence that replication timing is a reflection of 3D chromatin architecture and is consistent with our previous demonstration that anchorage and positioning of chromatin is reestablished during each cell cycle at the same brief time after mitosis as the establishment of a replication timing program; i.e., the TDP (for reviews see Cimbora and Groudine, 2001; Gilbert, 2001).

In fact, studies in budding yeast have demonstrated that chromosomal context is essential for the late replication of certain replication origins that are positioned near the nuclear periphery during early G1 phase (Raghuraman et al., 1997; Heun et al., 2001a). However, these same studies demonstrated that, once established, late origins can be separated from their chromosomal context and wander away from nuclear periphery but still replicate late during S phase. These results suggest the existence of an epigenetic mark that is established during early G1 phase in a context or position-dependent manner and is subsequently stable at least until the time of replication. Some earlier evidence has suggested that mammalian chromatin can also maintain replication timing after disruption of chromosome organization; when cells enter quiescence, chromosomes 18 and 19 alter their relative radial repositions but maintain their overall early- and late-replication timing, respectively (Bridger et al., 2000). In this study, we confirm that quiescent cells maintain their normal replication timing program despite evident changes in chromatin architecture. These chromatin changes are not uniform. We find only partial decondensation of chromocenters and partial retention in the organization of early- and late-replicating chromatin. Others have also shown detectable but modest or possibly chromosome-specific chromatin changes during

quiescence (Zink et al., 1999; Bridger et al., 2000; Mehta et al., 2007, 2010). However, we also observed massive decondensation of a large segment of heterochromatin that nonetheless retained its replication timing. Therefore, although replication timing appears to reflect the spatial organization of chromatin established at the TDP, that spatial organization is neither sufficient (G2 phase) nor necessary (G0) to maintain the replication timing program. Although at present we do not know the role of subnuclear position in replication timing, these results are consistent with a model in which subnuclear chromatin architecture established during early G1 phase leaves marks on chromatin that influence replication timing and are then stably maintained despite subsequent changes in architecture (for review see Gilbert, 2001). In this model, it is the molecular determinants of these marks rather than spatial organization by itself that is more important for replication timing. This could explain the reported variable effects of tethering to the nuclear periphery on replication timing (Heun et al., 2001a; Ebrahimi and Donaldson, 2008; Ebrahimi et al., 2010).

The replication fork as an eraser of RTDs

RTDs established at the TDP must be maintained until replication during S phase and must be lost before metaphase (Dimitrova and Gilbert, 1999). The two most disruptive times during the cell cycle are disassembly and reassembly of chromatin at the replication fork and the dismantling of the nucleus during mitosis, which disrupts many chromatin interactions and the spatial architecture of chromosomes. In this study, we show that G2 phase chromatin lacks determinants of replication timing. Consistent with extensive earlier studies (Abney et al., 1997; Li et al., 1998; Zink et al., 1998; Heun et al., 2001b; Kimura and Cook, 2001; Walter et al., 2003; Sadoni et al., 2004; Essers et al., 2005; Kumaran and Spector, 2008), we also find that the subnuclear positions and relative spatial arrangements of chromatin domains are maintained in G2 phase nuclei. These results demonstrate that subnuclear position by itself is not sufficient to dictate the replication timing program, further supporting a model in which the marks established at the TDP are more important for maintenance of the replication program than the architecture that may help to establish those marks.

The simplest interpretation of our results is that determinants of replication timing that are acquired at the TDP are lost at the replication fork. A formal alternative possibility is that some factor that is capable of making all chromatin regions equally likely to replicate at any time during S phase is lost at the TDP and gained at the fork. For example, Cdk1 activity does not appear until the middle of S phase, potentially to promote the firing of late origins (Katsuno et al., 2009) and could render late chromatin equally likely to replicate early. This specific scenario is unlikely because our live cell G2 phase experiments were performed in the present of a Cdk1 inhibitor, and others have shown that the temporal order of DNA replication is independent of the level of Cdk activity (Thomson et al., 2010). Generally speaking, such "timing erasure" factors would need to remain tightly associated with all late-replicating chromatin because the same results were obtained with permeabilized nuclei washed free of soluble proteins, as for intact nuclei in vivo. Furthermore, cell fusion experiments have shown that late S phase cells could induce early S phase replication patterns in G1 phase nuclei (Yanishevsky and Prescott, 1978), supporting the interpretation that the timing program is determined by components of G1 phase chromatin, not soluble factors. Certainly, a timing erasure model is more complicated; such factors would have to remain tightly bound to chromatin through mitosis and into early G1 phase to maintain a "nontiming program" that would seemingly serve no purpose. However, a determinant that is acquired at the TDP and lost at the replication fork makes good sense because once DNA is replicated, there is no need to maintain those determinants until the next G1 phase (Fig. 8).

Unfortunately, we cannot directly demonstrate loss of replication timing at the fork because the massive number of preexisting replication forks within S phase nuclei precludes the ability to assay replication timing immediately before and after a specific locus replicates. Direct evidence for this conclusion will require the identification of the determinants and demonstration of their removal at the fork. Replication dilutes histone modifications, and some of them do not mature until the next cell cycle (Aoto et al., 2008; Scharf et al., 2009), so it will be interesting to determine whether these modifications are reestablished at the TDP. Furthermore, given the strong correlation between chromatin interactions as measured by Hi-C (Lieberman-Aiden et al., 2009) and replication timing (Ryba et al., 2010), it will be interesting to know whether Hi-C chromatin interactions are established at the TDP and disrupted during S phase. For example, it is possible that the close proximity of two identical sister chromatids after DNA replication interferes with long-range intrachromosomal interactions. Regardless, the conclusion that determinants of replication timing are lost at the replication fork is a conceptual step toward deciphering the mechanism of replication timing program that focuses our attention on determinants of chromatin and 3D architecture, more so than subnuclear position by itself.

Materials and methods

Cell synchronization and in vivo labeling

Mouse C127 cells or CHOC400 cells were synchronized at mitosis by mechanical shake off after a brief and fully reversible nocodazole treatment as described previously (Wu et al., 1997). For G2 synchronization, 50 ng/ml nocodazole was added 11–12 h after release from mitosis for an additional 10–12 h when ~75% attaching cells are in G2 phase. Cells were pulsed labeled with BrdU before harvesting to distinguish any still in late S phase. Some of these G2 phase populations were further synchronized in the next G1 phase after being released to mitosis and shaken off. For G0 synchronization, mitotic cells were plated directly into serum-free medium for up to 4 d with fresh serum-free medium every day. For in vivo EdU or BrdU pulse labeling, 10 µM EdU or 5 µg/ml BrdU was added to medium for 30 min. RO3306 was provided by L. Vassilev (Hoffmann-La Roche, Inc., Nutley, NJ; Vassilev et al., 2006). For mitotic spread preparation, mitotic cells were treated in hypotonic condition for 15 min, fixed, and dropped onto slides for DAPI staining and chromosome counting.

In vitro DNA replication and labeling in *Xenopus* egg extract

Permeable nuclei were prepared from synchronized cells and introduced into *Xenopus* egg extract as described previously (Wu et al., 1997). The nuclei/extract ratio used in this study was 10,000 nuclei/µl extract. In vitro DNA replication was either pulse labeled with 50 µM biotin-16-dUTP for 5 min or continuously labeled with 10 µM digoxigenin-dUTP to follow relative DNA synthesis. Where indicated, a degradation-resistant recombinant geminin was added at 40 nM as described previously (Okuno et al., 2001). For hybridization of [^{32}P]dATP-labeled DNA to C3 probes (Fig. 5 E), procedures from Dimitrova and Gilbert (1999) were followed. Basically, early- or

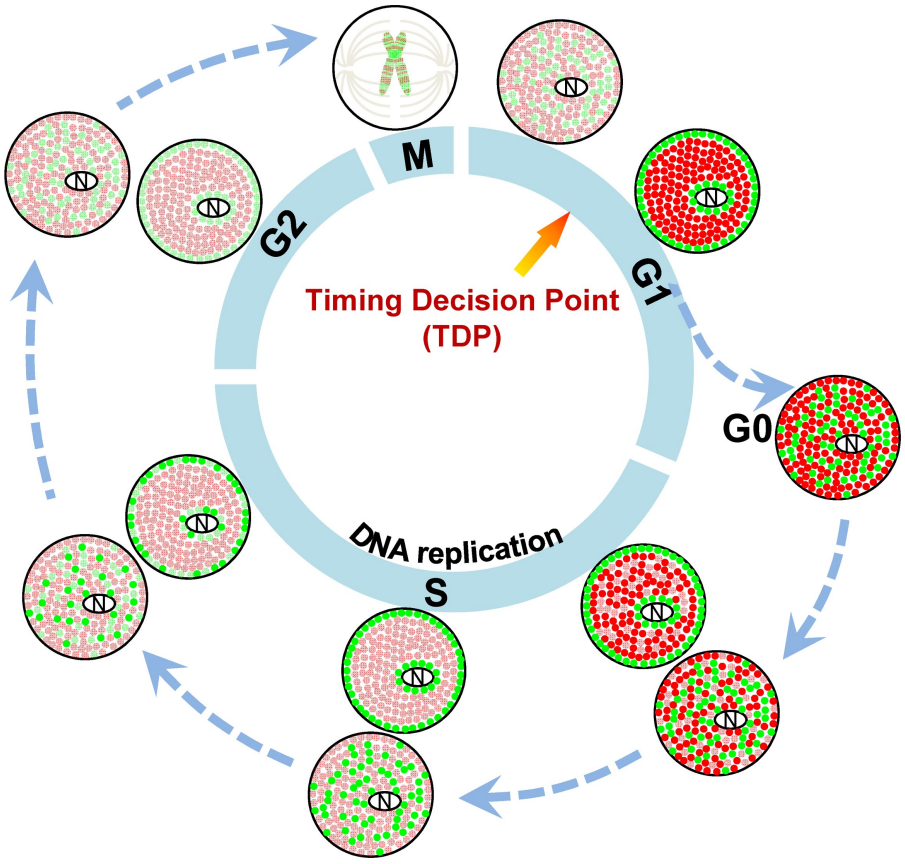

Figure 8. **A working model for initiation and maintenance of RTDs during cell cycle.** In this model, early- and late-replicating chromatin domains are labeled as red or green, respectively, with light colors representing lack of RTDs and bright colors representing the presence of RTDs. Because replication timing and spatial organization of chromatin are established simultaneously during early G1 phase at the TDP (Dimitrova and Gilbert, 1999) and because there is a strong genome-wide correlation between 3D chromosome architecture and replication timing (Ryba et al., 2010), it is hypothesized that spatial reorganization at the TDP drives the assembly of RTDs potentially by creating subnuclear compartments that set thresholds for initiation of replication (for review see Gilbert, 2001). These RTDs are maintained until the time of replication in S phase. During replication, the potential RTDs are modified or removed at the replication fork, indicated by early-replicating domains changing to light colors first, followed by late-replicating domains. In G2 phase, there are no RTDs on chromatin, but the general spatial organization is maintained until being disrupted during mitosis. If cells withdraw from the cell cycle and enter quiescence, the spatial organization of chromatin changes, but RTDs remain intact, and upon return to the cell cycle, replication proceeds in the normal temporal order despite spatial disruption. Because replication timing and presumably 3D architecture are maintained from one cell cycle to the next, some memory must persist through mitosis to re-establish this program at each TDP. However, dismantling and reassembling higher order chromosome architecture could also provide a window of opportunity in which to reprogram 3D architecture and replication timing to influence cellular identity in response to extracellular cues during differentiation. N, nucleolus; S, S phase.

late-replication intermediates were labeled with 100 µCi [^{32}P]dATP during either the first 30 min or between 110–120 min of the in vitro reaction. Labeled genomic DNA was purified, and an equal number of cpm from each sample was hybridized to a panel of DNA plasmids containing DHFR, C1, and C3 sequences. Relative hybridization signals were obtained by phosphorimaging analysis (GE Healthcare) and quantified using Quantity One software (Bio-Rad Laboratories).

DNA-FISH

DNA-FISH for pericentric heterochromatin (mouse chromocenters) and C3 locus was performed with a major satellite probe (Wu et al., 2006) or C3 probe labeled by nick translation with digoxigenin-dUTP (Roche). FISH procedure was previously described (Li et al., 2001).

Immunostaining

EdU was visualized with Alexa Fluor 594–conjugated azide according to the manufacturer's instruction (Invitrogen). BrdU staining was performed as described previously (Wu et al., 2006) with anti-BrdU antibody (Invitrogen) after HCl denaturation of nuclear DNA. For Mcm7 staining, cells were first extracted with CSK buffer with 0.5% Triton X-100 to remove free Mcm fraction before anti-Mcm antibodies were applied as described previously (Dimitrova et al., 1999). Biotin and digoxigenin were stained with FITC-conjugated avidin or anti-digoxigenin antibodies, respectively. Secondary antibodies were conjugated with Alexa Fluor 488, 594, or 647 (Invitrogen).

Microscopy and image analysis

Most images were captured with an image restoration microscope system (DeltaVision; Applied Precision) attached to a fluorescence microscope (IX-71; Olympus) equipped with a Plan Apo 100× 1.40 NA oil objective lens (Olympus) and charge-coupled device camera (CoolSNAP HQ; Photometrics) at room temperature. Approximately 50 optical sections (with 0.2-µm spacing) were taken for each nucleus and enhanced using the softWoRx (Applied Precision) constrained iterative deconvolution process. The softWoRx program was used to calculate Pearson's coefficient of colocalization between two selected channels. Colocalized pixels were shown in white. The coefficient is the ratio between the covariance of the channels and the product of their standard deviations. It ranges from −1 to 1. A value of 1 shows that a linear equation describes the relationship perfectly and positively, whereas a value of −1 shows a perfect negative relationship. The same software was used to quantify digoxigenin signal intensity and chromocenter sizes as needed.

Some images (Fig. S3) on C3 were collected with confocal microscopy (MRC 1024ES; Bio-Rad Laboratories). Colocalization analysis was performed with LaserSharp software (Bio-Rad Laboratories).

For live cell imaging, cells were cultured at 37°C in Delta T dishes (Bioptechs) and imaged using a spinning-disk unit (Olympus) with a UPlan S Apo 100× 1.40 NA objective (Olympus) or an imaging system (DeltaVision), both equipped with the Delta T live cell imaging system (Bioptechs).

Replication timing profiling

Genome-wide replication timing profiles were constructed and analyzed as described previously (Hiratani et al., 2008). In brief, cells pulsed labeled with BrdU for 2 h were fixed and FACS sorted into designed fractions based on propidium iodide staining profile. BrdU-substituted nascent DNA was immunoprecipitated with anti-BrdU antibodies. Samples were amplified, labeled, and hybridized to mouse whole genome microarray with one probe every 5.8 kb (Nimblegen Systems; Roche) according to the manufacturer's standard procedure.

Data were analyzed with the R/Bioconductor (http://www.r-project.org) package. Raw datasets were loess normalized and scaled to have the same median absolute deviation using limma package (R/Bioconductor).

Such normalized data were used to generate a smoothed profile using local polynomial smoothing (loess) for each chromosome (span = 300,000/chromosome size). Such smoothed profiles were shown in Fig. 7.

Online supplemental material

Fig. S1 shows that only post-TDP G1-3h nuclei follow spatial–temporal replication patterns in *Xenopus* egg extracts, and chromatin spatial organization is not changed during the in vitro reaction. Fig. S2 shows that results analyzing the overall replication timing program of chromatin from CHO cells synchronized in G1, G2, or G0 are consistent with those of mouse C127 cells. Fig. S3 shows that replication timing and peripheral localization of C3 are established at the TDP and maintained during S phase. Fig. S4 shows that RO3306 induces a complete round of rereplication in G2 phase C127 cells. Fig. S5 shows that the residual temporal specificity observed during rereplication is accounted for by tetraploid cells within C127 population. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201002002/DC1.

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