Chromatin assembly controls replication fork stability

Marta Clemente-Ruiz & Félix Prado+
Departamento de Biología Molecular, CABIMER-CSIC, Seville, Spain

During DNA replication, the advance of replication forks is tightly connected with chromatin assembly, a process that can be impaired by the partial depletion of histone H4 leading to recombinogenic DNA damage. Here, we show that the partial depletion of H4 is rapidly followed by the collapse of unperturbed and stalled replication forks, even though the S-phase checkpoints remain functional. This collapse is characterized by a reduction in the amount of replication intermediates, but an increase in single Ys relative to bubbles, defects in the integrity of the replisome and an accumulation of DNA double-strand breaks. This collapse is also associated with an accumulation of Rad52-dependent X-shaped molecules. Consistently, a Rad52-dependent—although Rad51-independent—mechanism is able to rescue these broken replication forks. Our findings reveal that correct nucleosome deposition is required for replication fork stability, and provide molecular evidence for homologous recombination as an efficient mechanism of replication fork restart.

Keywords: chromatin assembly; recombination; replication

INTRODUCTION

A tight control of the DNA replication process is necessary to maintain the integrity of the genome, as impaired replication fork advance is associated with DNA damage and genetic instability. In eukaryotic cells, replicative stress induces a complex DNA damage response in which the S-phase checkpoints aim to maintain the stability of stalled replication forks (Lopes et al., 2001; Tercero & Difley, 2001) and provide the time required for either to repair or tolerate the damage. In some cases, however, replication forks collapse, leading to double-strand breaks (DSBs) that are repaired by homologous recombination (HR).

Advance of the replication fork requires disruption of the chromatin fibre in front of the fork and assembly of the nascent DNA strands onto nucleosomes. DNA and histone synthesis are highly coordinated to ensure the exact supply of histones at the fork. Nucleosome assembly occurs rapidly after the passage of the replication fork and involves the initial deposition of the histone H3–H4 tetramer followed by the binding of two histone H2A–H2B dimers (Polo & Almouzni, 2006). DNA replication and chromatin assembly are tightly coupled by genetic and physical interactions between the DNA polymerase processivity factor proliferating-cell nuclear antigen (PCNA) and the PCNA-loader replication factor C (RFC) with chromatin assembly factor 1 (CAF1) and antisilencing factor 1 (Asf1; Shibahara & Stillman, 1999; Franco et al., 2005), two evolutionarily conserved histone chaperones that form part of the replication-dependent H3–H4 deposition complex (Tyler et al., 1999; Tagami et al., 2004).

The tight connection between chromatin assembly and DNA replication has led to the analysis of the consequences of mutations in genes encoding chromatin assembly factors on genetic instability. These studies have shown that defective histone H3–H4 deposition causes increased sensitivity to genotoxic agents, accumulation of DNA damage, activation of the S-phase checkpoints, and high rates of HR and chromosomal rearrangements (Tyler et al., 1999; Myung et al., 2003; Ye et al., 2003; Prado et al., 2004; Driscoll et al., 2007; Li et al., 2008). Another approach is the analysis of a yeast strain in which histone H4 is expressed from a doxycycline-inducible tet promoter (t::HHF2 cells). Partial depletion of H4 in t::HHF2 cells affects chromatin assembly and leads to an accumulation of recombinogenic DNA damage (Prado & Aguilera, 2005). Taken together, these results support the idea that impaired chromatin assembly affects genome integrity; however the mechanisms by which this genetic instability arises remain unclear.

RESULTS

Replication forks collapse in cells partly depleted of H4

To determine whether the accumulation of recombinogenic DNA damage in t::HHF2 cells is a consequence of defective DNA replication, we investigated the fate of replication forks by using two-dimensional-gel electrophoresis. Cells were synchronized in G1 with α-factor and released into S-phase, and DNA samples were analysed at various times to follow the progression of replication forks from the early replication origin ARS305 (Fig 1A). As shown in Fig 1B, firing and early elongation from ARS305 can be followed by the formation of a bubble arc; replication fork progression along adjacent restriction fragments by the accumulation of a complete arc of single Y-shaped molecules; and converging forks and Holliday junction (HJ)-like structures by the accumulation of double Y- and X-shaped molecules. The total number of replication intermediates (RIs) at the origin was reduced in t::HHF2 cells compared with the wild type (46 ± 10%; Fig 1C; data not shown) over time, although the proportion of bubbles, single Ys and Xs was not affected (Fig 1D). Similar results were...
obtained by the analysis of RIs from cells collected every 150 s upon G1 release and pooled into four samples to prevent the loss of RIs by asynchronic release from the α-factor arrest (data not shown). As most G1-synchronized t::HHF2 cells replicated their DNA (see below), this decrease cannot be due to reduced replication origin firing, as, in this case, a complete single Y-arc, indicative of passive replication of the ARS305 fragment by forks coming from a neighbouring origin in cells in which ARS305 was not fired, should be detected. We have also eliminated the possibility that RIs break during DNA extraction by collecting and digesting the DNA in agarose plugs to preserve its integrity (supplementary Fig S1 online). Interestingly, RIs in the wild type peaked at 25 min in the origin and at 35 min in fragment B, whereas the peak of RIs in t::HHF2 cells was at 25 min both in the origin and in fragments A and B, suggesting that the forks move faster in the mutant cells (Fig 1C). Consistent with an

Fig 1 | Replication intermediates collapse in cells partly depleted of histone H4. (A) Schematic representation of the telomere-proximal region replicated from the early origin ARS305 (black oval). The position of dormant origins (grey ovals), restriction fragments analysed by two-dimensional-gel electrophoresis and DNA fragments amplified by qPCR for ChIP analysis are shown. (B) Schematic representation of the migration pattern of the bubble-, single Y-, double Y- and X-shaped RIs by two-dimensional-gel electrophoresis. (C) Analysis of RIs at the ARS305 (Or) and two adjacent regions (A and B) from DNA samples digested with EcoRV and HindIII. Quantification of the RIs, taking the total amount of wild-type RIs in the ARS305 fragment at 1 h as 100 is shown. (D) Relative amount of bubbles, single-Y and X-shaped molecules at the EcoRV-HindIII ARS305 fragment. (E) Percentage of cells with Mre11-YFP foci. (F) Determination by Western blot of the amount of total and phosphorylated histone H2A (P-H2A) in cells synchronized in G1 and released into S-phase for 1 h with 0.05% (+) or without (−) MMS. B, BamH1; ChIP, chromatin immunoprecipitation; E, EcoRV; H, HindIII; MMS, methyl-methane sulphonate; qPCR, quantitative PCR; Or, origin; RI, replication intermediate; wt, wild type; YFP, yellow fluorescent protein.
Fig 2 | Partial depletion of H4 causes the collapse of stalled replication forks. (A) Analysis of stalled RIs at the ARS305 (Or) and four adjacent regions from DNA samples digested with EcoRV, HindIII and BamHI. Quantification of the RIs, taking the total amount of wild-type RIs over the whole region at 1 h as 100, is shown. Similar results were obtained by synchronization with cdc15-2 in telophase (supplementary Fig S2B online). (B) Analysis of the amount of H4, plasmid supercoiling and stalled RIs at the EcoRV-HindIII ARS305 following depletion of H4. Ponceau staining is shown as a loading control; r and sc indicate relaxed and supercoiled plasmids, respectively. The percentage of RIs in t:HHF2 cells relative to the wild type grown under the same conditions is shown. (C) Association of Pol ε-3HA to the replication fork in cells synchronized in G1 and released into S-phase in the presence of 0.2 M HU by ChIP analysis. Both input and immunoprecipitated DNA from G1 and HU-treated cells were amplified by real-time PCR with amplicons situated along the region (see Fig 1A). The enrichment in Pol ε-3HA at each zone is graphed relative to the enrichment in the wild type in G1, taken as 1. The inset shows the amount of Pol ε-3HA in wild-type and t:HHF2 cells by Western blot. (D) Replication initiation from ARS305 in HU-treated cells. The amount of DNA at ARS305 relative to a fragment located at 18 kb from the origin, determined with the values obtained in (D) by amplification of the input with amplicons ARS305 and 305+18 kb, is represented. ChIP, chromatin immunoprecipitation; dox, doxycycline; gal, galactose; glu, glucose; HA, haemagglutinin tag; HU, hydroxyurea; Or, origin; RI, replication intermediate; wt, wild type.
accumulation of broken replication forks in t::HHF2 cells, we observed an increase in the amount of both Mre11-yellow fluorescent protein (YFP) foci and phosphorylation of histone H2A (P-H2A; Fig 1E,F, respectively), two of the earliest events in the DSB repair response.

**Fork collapse is a direct effect of HY depletion**

To determine the effect of partial depletion of H4 on the stability of stalled replication forks, G1 synchronized cells were released into S-phase in the presence of hydroxyurea (HU), a drug that reduces the pool of dNTPs and causes stalling of the replication forks. As shown by fluorescence activated cell sorter (FACS) analysis, wild-type and t::HHF2 cells arrested at early S-phase to further slow progress as they were maintained in HU (see later). At the molecular level, wild-type replication forks initially stalled and accumulated in the proximity of the ARS305 with a peak at 60 min after G1 release; then, as cells remained in the presence of HU, the signal in this region gradually disappeared and slowly increased in adjacent fragments (Fig 2A; supplementary Fig S2A online). By contrast, the forks appeared earlier and replicated more DNA in the mutant cells than in the wild type. Importantly, stalled RIs in the mutant cells did not accumulate and decreased progressively compared with the wild type (Fig 2A; supplementary Fig S2A online), and this reduction was associated with an increase in the amount of single Y- (twofold) and X-shaped (fivefold) molecules relative to bubbles (see later), further supporting that stalled replication forks collapse in t::HHF2 cells. Consistent with this, the higher bubbles-dependent structural complexity of replicating relative to complete chromosomes was reduced in t::HHF2 cells (supplementary Fig S3 online).

Next, we analysed the immediate effect of depleting H4 on replication fork stability (Fig 2B). For this, t::HHF2 cells were transformed with plasmid pUK421 to increase the initial amount of H4 by expressing it from the inducible GAL1 promoters. Then, the cells were synchronized in G1 and split into two cultures—either expressing H4 or not—that were maintained in HU for 1 h. At this point, t::HHF2 cells in glucose showed a slight decrease in the total amount of H4, which, in turn, caused a rapid reduction in nucleosome density, as determined by a loss of plasmid negative supercoiling (Fig 2B; Kim et al, 1988). Importantly, this defect in nucleosome assembly was accompanied by a six fold reduction in the amount of stalled replication forks (from 17 to 2.7%; Fig 2B), indicating that fork collapse is a direct consequence of defective histone deposition.

**Partial depletion of H4 causes a loss of replisome integrity**

Next, using chromatin immunoprecipitation (ChIP) analysis we determined the association of the polymerase ε (Pol ε–3HA) with the fork in cells synchronized in G1 and released into S-phase in the presence of 0.2 M HU for 50 min (Fig 2C). As shown previously, in HU-treated wild-type cells, pol ε-3HA was enriched in the proximity of the ARS305 (Fig 2C; Franco et al, 2005). By contrast, pol ε-3HA in t::HHF2 cells was barely detected over the whole region, indicating that replication fork collapse is linked to defective replisome integrity. As shown for unperturbed replication forks, this reduction was not due to defective replication initiation, as determined by quantification of the total amount of DNA in the origin relative to an unreplicated fragment located at 18 kb from the ARS305 both in G1 and S-phases with HU (Fig 2D).

**The S-phase checkpoints are functional in t::HHF2 cells**

The stabilization of stalled replication forks by S-phase checkpoints requires recruitment of the heterodimer Ddc2–Mec1 to damaged forks and the subsequent activation by phosphorylation of the Rad53 kinase (Lopes et al, 2001; Tercero & Diffley, 2001). As shown in Fig 3A, Rad53 was phosphorylated by the presence of replicative DNA damage to the same extent and with the same kinetics in t::HHF2 and wild-type cells. In the absence of genotoxic agents, we observed a 3.7-fold increase in the frequency of budded t::HHF2 cells with Ddc2-YFP foci compared with the wild type (Fig 3B). This result is consistent with the accumulation of P-H2A (Fig 1F), which is mediated predominantly
The fact that t::HHF2 cell viability is dependent on Rad52 (Prado & Aguilera, 2005) prompted us to evaluate the importance of HR in the rescue of broken forks. S-phase progression analysis using flow cytometry showed that the absence of Rad52 increased the time required for t::HHF2 cells to complete DNA replication both under unperturbed conditions (Fig 4A) and on removal of the DNA damaging agent (Fig 4B; supplementary Fig S4B online). Interestingly, two-dimensional-gel analysis showed that the absence of Rad52 reduced the amount of Rs at the ARS305 (47 ± 9%), whereas the double mutant t::HHF2 rad52A showed a synergistic decrease in the amount of Rs (8 ± 2%; Fig 5A; data not shown). As shown previously for t::HHF2 cells, the shape of the Y-arc did not change in these mutants (Fig 5A), ruling out defects in replication origin firing. In addition, the absence of Rad52 had a minor effect on the stability of stalled replication forks both in wild-type and t::HHF2 cells (Fig 5B; supplementary Fig S5A online), indicating that Rad52 did not participate in preventing replication fork collapse. Hence, these results suggest that HR is required for the rescue of collapsed forks. Consistent with this, the relative increase in X-shaped molecules was dependent on Rad52 (Fig 5B), suggesting that they represent HJ structures.

In conclusion, our results reveal the process of HR as an efficient mechanism for assisting collapsed replication forks. Interestingly, this mechanism does not require the DNA strand-exchange protein Rad51, as shown by the fact that t::HHF2 viability was not affected by rad51Δ and the proportion of budded cells with Rad51-YFP foci was similar in t::HHF2 and wild-type cells (Fig 5C).

**DISCUSSION**

The relevance of chromatin assembly in genetic instability has been evidenced from yeast to humans; however, the molecular mechanisms by which this instability takes place remains unknown. By using a yeast strain in which chromatin assembly can be impaired by the partial depletion of H4, we provide evidence that supports for the first time, to our knowledge, the idea that correct nucleosome assembly is required to maintain replication fork stability. Partial depletion of H4 causes a collapse of both unperturbed and stalled replication forks that is accompanied by a loss of integrity of the replisome, accumulation of DSBs and single Ys relative to bubbles, activation of the S-phase checkpoints and accumulation of Rad52-dependent X molecules. In addition, replication fork instability is increased in the absence of HR, further supporting the accumulation of broken replication forks in t::HHF2 cells. This loss of fork stability is not due to defective S-phase checkpoints in t::HHF2 cells. An alternative possibility is that defective histone deposition has a direct effect on the stability of the replication fork, as suggested by the fact that H4 depletion is rapidly followed by defects in nucleosome assembly and replication fork collapse. Consistent with this view, the binding of RFC, PCNA and polymerase ε to stalled replication forks (Franco et al, 2005; Han et al, 2007), and the stability of unperturbed and stalled Rs (M.C-R., and F.P., unpublished data) are affected in mutants defective in H3K56 acetylation—asf1Δ, rtt109Δ and H3K56R—a modification required for histone H3–H4 deposition during replication-dependent nucleosome assembly (Li et al, 2008).

As DNA synthesis and histone deposition are physically and genetically connected, it is possible that a reduction in the amount of available H4 uncouples both processes, exposing the forks to DNA breaks (Fig 5D). This possibility might explain the apparently faster advance of the forks in the mutant cells, which, in turn, could facilitate their collapse. Replication fork breakage would not

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**Fig 4** Homologous recombination is required for DNA replication in t::HHF2 cells. Cell-cycle progression by FACS analysis of cells synchronized in G1 and released into S-phase in (A) the absence of genotoxic agents, in (B) the presence of 0.2 M HU for 1 h and then released into fresh media, and in (C) the presence of 0.2 M HU. NCD was added upon the G1 (A) or the HU arrest (B) to prevent cells from re-entering a new cell cycle. FACS, fluorescence activated cell sorter; HU, hydroxyurea; NCD, nocodazole; wt, wild type.

by Mec1 (Downs et al, 2000). Therefore, replication fork collapse in t::HHF2 cells is not due to defective S-phase checkpoints.

**HR participates in the rescue of collapsed replication forks**

It is noted that t::HHF2 cells progressed through S-phase with only a slight delay compared with the wild type, as determined by DNA content analysis (Fig 4A; Prado & Aguilera, 2005). Furthermore, t::HHF2 cells were able to resume DNA replication upon HU arrest (Fig 4B), and to adapt to a prolonged presence of HU (Fig 4C) without the loss of viability (supplementary Fig S4A online; Prado & Aguilera, 2005). Similar results were obtained with methyl-methane sulphonate (MMS; supplementary Fig S4B online). These data indicate that t::HHF2 cells are endowed with efficient mechanisms to repair and restart collapsed forks.

The efficiency of repair and restart of replication forks is a matter of growing interest. Indeed, it has been shown that HU induces the arrest of cells in G1, with re-entry into the cell cycle determined by reactivation of the Y-arc (Downs et al, 2000). Therefore, HR is likely to be involved in the repair of HU-induced DNA damage in yeast. The fact that HU arrests cells in G1 or S-phase is not due to defective S-phase checkpoints. As DNA synthesis and histone deposition are physically and genetically connected, it is possible that a reduction in the amount of available H4 uncouples both processes, exposing the forks to DNA breaks (Fig 5D). This possibility might explain the apparently faster advance of the forks in the mutant cells, which, in turn, could facilitate their collapse. Replication fork breakage would not
only convert bubbles into single Ys, but also explain the accumulation of DSBs and the activation of the S-phase checkpoints.

We have shown that HR is required for the repair of broken forks in t::HHF2 cells. In principle, the substrate for HR could be the one-ended DSB generated by collapse of the fork or, alternatively, the two-ended DSB generated on the arrival of the oncoming replication fork. The latter possibility seems to be unlikely because the progressive appearance of RIs in our kinetics indicates that replication forks progressed only from the parental 3'-end to prime new DNA synthesis and reinitiate replication, which could be associated or not with the assembly of a true replisome. Finally, the t::HHF2 rad52Δ mutant is sick but still viable and is not affected by the absence of the DNA annealing protein Rad59 (data not shown), indicating that other mechanisms are also able to deal with broken forks in the absence of HR.

An interesting implication of the proposed mechanism is that the break has to occur at the lagging chain of the fork to provide a parental 3'-end (Fig 5D), suggesting that this chain might be more sensitive to defects in histone deposition. Whether this is related to the fact that some inverted DNA repeats form hairpin structures at the lagging template, which cause checkpoint-blind fork stalling (Voineagu et al., 2009), is an interesting possibility that remains to be addressed. In any case, mutations that affect the lagging strand polymerase (Pol δ) but not the leading strand polymerase (Pol ε) result in an accumulation of Rad52-dependent, Rad51-independent HJs (Zou & Rothstein, 1997). This result also supports our observation that the rescue of broken forks by HR does not require Rad51. We hypothesize that the more open chromatin structure at the fork might bypass the requirement for strand exchange proteins, as supported by the fact that Rad51 is dispensable for HR when chromatin of the donor is in an open conformation (Sugawara et al., 1995).
The fact that repression of histone genes causes DNA synthesis inhibition in human cells (Nelson et al., 2002) suggests that the effect of defective histone deposition on replication fork stability might be conserved, even though this inhibition is in contrast with the efficiency of ::HHF2 cells to complete DNA replication. This could be related to a higher efficiency of HR in yeast, but might also reflect the more demanding structural and topological package of DNA into chromatin in mammalian cells.

**METHODS**

Yeast strains, growth conditions, plasmids, and standard methods for DNA extraction and detection, plasmid supercycling, flow cytometry, detection of YFP constructs, Western blot and in situ kinase assay are included in the supplementary information online.

**Analysis of RIs.** Cell cultures were arrested with sodium azide (0.1% final concentration) and cooled down on ice. Total DNA was extracted using the CTAB protocol at the indicated times—except for Fig 5B and supplementary Fig S1 online, which were extracted using the G2/CTAB protocol and in agarose plugs, respectively—digested with restriction enzymes, resolved by neutral/neutral two-dimensional-gel electrophoresis as described previously (Brewer & Fangman, 1987) and analysed by sequential hybridization of the same membrane with various probes. Quantification of the RIs was normalized to the total amount of DNA, including linear monomers, to the size of the restriction fragment and to the percentage of cells synchronized either in G1 (α-factor) or in telophase (cdc15-2). Kinetics in Figs 1C, 2A and 5A were repeated 2–4 times with similar results. The average and standard deviation of 3–5 independent experiments are shown in Figs 1D, 5B and supplementary Fig S5B online.

**Chromatin immunoprecipitation.** Chip assays were performed as described previously (Hecht & Grunstein, 1999) with the HA mouse monoclonal antibody 12CA5 (Roche; www.roche.com). Oligonucleotide sequences for the real-time PCR amplifications performed on purified DNA before (input) or after (immunoprecipitated) immunoprecipitation are shown in supplementary Table SII online. Pol ε-3HA enrichment at each specific region was calculated as the ratio between the immunoprecipitated and the input relative to the same ratio in the wild type arrested in G1, taken as 1. The average and standard deviation of three independent experiments are shown.

**Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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