Correction

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The authors note that the affiliation for Julien Bacal and Philippe Pasero should instead appear as Institute of Human Genetics, CNRS, 34396 Montpellier, France. The corrected author and affiliation lines appear below. The online version has been corrected.


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Phosphorylation of CMG helicase and Tof1 is required for programmed fork arrest

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Several important physiological transactions, including control of replicative life span (RLS), prevention of collision between replication and transcription, and cellular differentiation, require programmed replication fork arrest (PFA). However, a general mechanism of PFA has remained elusive. We previously showed that the Tof1–Csm3 fork protection complex is essential for PFA by antagonizing the Rrm3 helicase that displaces nonhistone protein barriers that impede fork progression. Here we show that mutations of Dbf4-dependent kinase (DDK) of Saccharomyces cerevisiae, but not other DNA replication factors, greatly reduced PFA at replication fork barriers in the spore regions of the ribosomal DNA array. A key target of DDK is the mini chromosome maintenance (Mcm) 2–7 complex, which is known to require phosphorylation by DDK to form an active CMG helicase. In vivo experiments showed that mutational inactivation of DDK caused release of Tof1 from the chromatin fractions. In vitro binding experiments confirmed that CMG and/or Mcm2–7 had to be phosphorylated for binding to phospho-Tof1–Csm3 but not to its dephosphorylated form. Suppressor mutations that bypass the requirement for phosphorylation by DDK restored PFA in the absence of the kinase. Retention of Tof1 in the chromatin fraction and PFA in vivo was promoted by the suppressor \(mcm5\)-bob1, which bypassed DDK requirement, indicating that under this condition a kinase other than DDK catalyzed the phosphorylation of Tof1. We propose that phosphorylation regulates the recruitment and retention of Tof1–Csm3 by the replisome and that this complex antagonizes the Rrm3 helicase, thereby promoting PFA, by preserving the integrity of the Fob1–Ter complex.

DKK | Ter | Fob1 | Tof1 | programmed fork arrest

Replication fork arrest can either occur at random sites (1) or, in some cases, is physiologically programmed to occur at specific sequences such as the Ter sites of Escherichia coli and Saccharomyces cerevisiae (also called replication fork barriers: RFBs), which bind to specific terminator proteins. The proteins Tus and Fob1 bind to the Ter sites of \(E.\) coli and \(S.\) cerevisiae, respectively, and cause polar replication fork arrest (2, 3) (reviewed in ref. 4). The arrested fork, with the help of DNA polymerase, helicase, and topoisomerase (5), merges with the fork approaching Ter from the opposite direction, and the resulting catenated daughter molecules are resolved by topoisomerase IV in bacteria (6) and topoisomerase II in eukaryotes (7, 8). In budding yeast, the unloading of the replisomal components from the template after replication termination is facilitated by Cdc48 and a ubiquitin ligase (9).

Eukaryotic cells of diverse types arrest and prevent replication fork entry into the highly transcribed ribosomal (r)DNA locus, reviewed in ref. 4. In budding yeast, a single replication terminator protein called Fob1 binds to the Ter sites (RFBs) located in nontranscribed spacer 1 (NTS1) of rDNA to cause polar fork arrest (10, 11), which prevents transcription–replication collision, fork stalling, and genome instability. Fission yeast has three known terminator proteins: Rtf1, which functions at the mating-type switch locus (12, 13), and Reb1 (Sp. Reb1) and Sap1, which act at the Ter sites of rDNA spacers (14–18). Sp. Reb1 also functions at Ter sites located in the other two chromosomes and promotes “chromosome kissing” (19).

Several studies have shown that a complex of three proteins (Tof1, Csm3, and Mrc1) called the fork protection complex (FPC) acts at replication forks to ensure normal fork progression and stabilization of forks stalled at nonhistone protein barriers and at sites of DNA damage (20). We and others have shown that two members of the heterotrimeric FPC are needed for stable fork arrest at Ter sites and at other nonhistone protein barriers. All eukaryotic cells contain homologs of these two proteins, called Tof1 and Csm3 of budding yeast (21–23), Swi1 and Swi3 of fission yeast (17, 24), and TIM and TIPIN of mammalian cells (25, 26). The third member of the complex, called Mrc1 and clasin in budding and fission yeast and mammalian cells, respectively, is dispensable for maintenance of programmed replication fork arrest (PFA) (22, 23). The FPC binds to mini chromosome maintenance (Mcm) 2–7 and inhibits its DNA-dependent ATPase activity and also the helicase activity of the CMG complex (27). We have previously reported that Tof1 and Csm3 promote stable fork arrest by antagonizing the Rrm3 helicase (23, 28), which removes nonhistone protein.

**Significance**

Programmed replication fork arrest (PFA) at specific terminator sites and the proteins that bind to these sites functionally interconnect replication, transcription, and recombination. PFA prevents collision between replication and transcription that can cause genome instability, promotes intrachromatid recombination at ribosomal (r)DNA that controls replicative life span, and maintains rDNA homeostasis. This work reveals the mechanism of PFA by showing that the Tof1 protein of budding yeast remains associated with the replication fork only when it is phosphorylated, and uses the CMG helicase that drives the replication fork as a landing pad. Tof1–Csm3 promotes PFA by preventing the terminator protein Fob1 from getting displaced by other factors such as the Rrm3 helicase by counteracting the latter. This important mechanism appears to be evolutionarily conserved.


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barriers from in front of moving forks throughout the budding yeast genome (29).

PFA promotes several important physiological functions. In E. coli, two sets of Ter sites of opposite polarity are located at the antipode (with respect to the origin) of the circular chromosome, forming a replication trap. Fork convergence occurs within this trap, at or near the dimer resolution and segregation sites (called ddf) that are necessary for reduction of the daughter chromosomes to monomers and their proper separation preceding cell division. Ter sites also prevent a topological shift from a Cairns-type to a rolling-circle replication, thereby contributing to genome stability (reviewed in ref. 4). In eukaryotes, PFA is required for (i) prevention of collision between a replication fork and an actively transcribing RNA polymerase approaching from the opposite direction, thereby preventing genome instability (30–33), (ii) promoting genetic imprinting and cellular differentiation in fission yeast (12), and (iii) controlling replicative life span in budding yeast (34, 35).

The two cell-cycle kinases called cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) are required for replication initiation (36–39). CDK (40) but not DDK (41, 42) is involved in regulating the S-phase checkpoint. Replication initiation is a two-step process that involves the assembly of a pre-replication complex (RC) through recruitment of Mcm2–7 by an origin-bound Orc1–6 complex with the help of the Cdt1 and Cdc6 proteins. The inactive Mcm2–7 is then phosphorylated by DDK, which initiates a multi-step assembly process involving the heterotetrameric GINS (Go, Ichi, Ni, and San) complex (43) and Cdc45 (cell division cycle gene 45) (44, 45) to generate the CMG complex, which has vigorous helicase activity (46–48). CDK and DDK also restrict DNA replication to only once per cell cycle (49).

In budding yeast, the requirement for DDK can be bypassed by a suppressing point mutation called mcm5-bob1 (50). The N-terminal region of Mcm4 contains an inhibitory domain that is neutralized by DDK-catalyzed phosphorylation. Consequently, deletion of this domain bypasses the need for DDK for CMG assembly (51–53). Recently, it was shown that RIF1, which controls the timing of origin firing in S. cerevisiae and recruitment of the PP1 phosphatase, causes dephosphorylation of the residues of Mcm2–7 that are phosphorylated by DDK. Deletion of RIF1 suppresses the cdc7-1 mutation and allows growth to occur at the semi-permissive temperature of 30 °C (54).

The present work shows that in vitro phosphorylation of not only Tof1 but also Mcm2–7 by DDK is essential for recruitment/retention of Tof1 at the replisome, which is obligatory for PFA. Consistent with this observation, mutational inactivation of DDK in vivo caused either the release of Tof1 or its failure to be retained in the chromatin fractions. The apparent need for DDK to cause PFA was bypassed by suppressor mutations that bypass Mcm2–7/CMG function. Together, these results indicate that in addition to the known role of DDK in CMG assembly and helicase activation, it also contributes to the assembly of the CMG platform that recruits phosphorylated but not dephosphorylated Tof1 to the replisome. Together with our earlier results that Tof1–Csm3 antagonizes the Rrm3 helicase, which displaces Fob1 from Ter sites, we propose that phosphorylation of Tof1 and Mcm2–7 promotes stable association of the FPC with the replisome and is needed for functional PFA.

**Results**

**Inactivation of DDK Caused Significant Reduction of PFA.** Does DDK, besides its roles in replication initiation, limiting replication to once per cell cycle, and checkpoint control, also regulate PFA? We addressed this question by investigating replication fork arrest at the Ter site of the rDNA of budding yeast in temperature-sensitive mutants of DDK called cdc7-1 and dbf4-1 of its two subunits at the permissive (24 °C) and nonpermissive temperatures (37 °C). We used Brewer–Fangman 2D gel electrophoresis (55), as modified (23), to follow fork progression in the spacer regions of rDNA. Our stratagem was to let initiation occur at 24 °C and then allow fork progression at 37 °C, which should prevent any new origins from firing. Asynchronous cultures were grown at 24 °C to midlog phase and then shifted to 37 °C for various time periods (1–2 h) and the cells were harvested. This approach, in our hands, resulted in higher yields of scarce replication intermediates, particularly from the mutant cells. Control experiments were similarly performed at 24 °C but without a shift to 37 °C. FACS analyses of the asynchronous cultures used for the experiments are shown (Fig. S1). It should be noted that although all of the 2D gels showed Y-shaped arc (Y-arc), the pattern corresponding to bubble arc was not observed because the experiments were not designed to capture and display the early replication intermediates. Fig. 1A shows the topography of NTS1 containing a single ars and tandem Ter sites present in each rDNA repeat. Replication fork arrest at the twin Ter sites, as revealed by five independent sets of 2D gels, as expected showed normal fork arrest at Ter in WT cells at both temperatures (Fig. 1B), but there was a significant reduction in the relative intensity of the Ter spots at the
nonpermissive temperature (Fig. 1 C and D) in cdc7-1 and dbf4-1 cells, as determined by quantification of the images using the NIH ImageJ program. We concluded from these experiments that temperature-dependent inactivation of either the cdc7-1 or dbf4-1 component of DDK significantly reduced the magnitude of PFA at the Ter sites. Is unhindered fork progression through a protein barrier a general property of temperature-sensitive (ts) mutations at the nonpermissive temperature in several replication proteins, or is it specific to DDK? Is this caused by direct action of DDK on replication termination, or is it an indirect and relatively trivial consequence of changes in the rate of fork progression as a result of loss of DDK activity that might have caused fewer forks to reach the Ter sites? Was this possibly caused by an increase in the rate of fork progression that somehow overpowered the Fob1–Ter barriers? Alternatively, could this have been caused by the displacement of Fob1 from Ter sites precipitated by the loss of DDK activity? These questions have been systematically addressed below.

Abolition of PFA Is Not a General Property of Several Mutant Forms of Proteins Known to Be Defective in Replication Initiation. We investigated whether the loss of fork arrest upon inactivation of DDK was a specific or general defect caused by reduction in the rates of initiation and ongoing replication by ts mutations in other replication proteins. Two-dimensional gel analyses of fork movement using several ts mutants in proteins known to be involved in initiation (e.g., psf1-1, psf2-1, sld2-2, sld5-12, and mcm10-1) showed that PFA at Ter sites in these mutants was not significantly diminished at 37 °C in comparison with that of the WT cells (Fig. 24 and Fig. S2). As an example, the psf-1 mutant is known to be nonleaky (43). Published 2D gel analyses of its replication intermediates clearly show significant reduction of the bubble arc in 2D gels in comparison with that of the WT, clearly indicative of loss of replication initiation at 37 °C (43). Therefore, loss of PFA is not an inevitable consequence of a loss of initiation function.

DNA Combing Showed No Correspondence Between Replication Fork Arrest and the Relative Differences in Fork Movement. To investigate whether a relative difference in the rate of fork progression in the two mutants compared with that of WT cells could cause PFA or its abolition, we performed DNA combing experiments with the WT, cdc7-1, and psf1-1 cells at the nonpermissive temperature (Fig. 2B). The cells of the three genotypes were first grown at 24 °C, shifted to 37 °C, and pulse-labeled with BrdU. The DNA was gently extracted to minimize hydrodynamic shear and spread on silanized coverslips and visualized by antibodies against BrdU (green) and single-stranded DNA (red). Several hundred molecules were recorded under a fluorescence microscope, tract lengths were measured, statistical analyses were performed (Table 1), and the data were plotted as described in Materials and Methods and the legend to Fig. 2. The relative differences in the rates of fork movement were small but consistent, with the magnitudes as follows: psf1-1 > cdc7-1 > WT (Fig. 2 B–D). Keeping in mind that fork arrest at Ter occurred in psf1-1 and WT cells but not in cdc7-1 at 37 °C, the data suggested that there was no detectable correlation between the relative rate of fork progression and fork arrest at Ter. It should be noted that the psf1 and WT tracts are longer in the DNA combing profile because initiation was blocked in cdc7-1 and psf1-1 cells, and those that initiated, presumably because of less competition for existing initiation factors, cause somewhat faster fork movement (56).

Failure to Arrest Forks in cdc7-1 Cells at 37 °C Was Not Due to Displacement and Loss of Fob1 from the Ter Sites. We wished to investigate whether the absence of DDK activity caused Fob1 to dissociate from the Ter sites by measuring the magnitudes of Fob1 occupancy at Ter in the cdc7-1 strains at 24 °C, 30 °C, and 37 °C for 3 h. ChIP analyses of Fob1 binding to Ter in vivo were performed using a segment of DNA from the 35S transcribing region as the control. The data showed that Fob1 remained bound to Ter sites in cdc7-1 cells at all temperatures tested (Fig. 2 E and F).

Key Role for DDK-Catalyzed Phosphorylation in PFA. It is known that the requirement for DDK-catalyzed phosphorylation of Mcm2–7 for cell survival can be bypassed by several different suppressor mutations. For example, cdc7-1 or a cdc7Δ deletion can be suppressed by the mcm5-bob1 suppressor, which maintains cell viability in the absence of DDK (50). We compared and contrasted
PFA at the Ter site of rDNA in strains containing cdc7-1 mcm5-bob1 and cdc7Δ mcm5-bob1 and the WT control. The experiments were repeated twice. The images of the 2D gels showed that PFA was restored in cdc7-1 mcm5 bob1 and cdc7Δ mcm5-bob1 cells at 37 °C (Figs. 3A and 4A, iii) as contrasted with the cdc7-1 single mutant, which failed to arrest forks at Ter at 37 °C (Fig. 1C). The results were consistent with the conclusion that (i) DDK was dispensable for fork arrest in the presence of mcm5-bob1 and (ii) alteration in the Mcm2-7 structure caused by the suppressors controlled PFA, probably by assembling CMG independent of DDK. Additional pathways of suppression of DDK and their effect on fork arrest are described below. It should be noted that although in some of the 2D gels the sizes of the Y-arcs seem to be different, these are caused by image processing and there are no real differences.

Deletion of the Inhibitory Domain in the N-Terminal Region of Mcm4 Restored Fork Arrest in DDK Δ Mutants at 37 °C. The N-terminal region of Mcm4 contains an anchoring domain for Dbf4 recruitment, and residues 2–174 of Mcm4 also contain an inhibitory domain (52). Because the main function of Mcm4 phosphorylation by DDK is believed to be neutralization of the inhibitory domain of Mcm4, a ts dbf4-1 mutation, as expected, is suppressed by the N-terminal deletion (Δ2–174) of Mcm4 (51–53). If PFA is controlled by DDK through phosphorylation of Mcm4, Mcm4 required to form the CMG complex, it should be manifested in stable fork arrest in a dbf4-1 mcm4Δ (Δ2–274) double mutant at both 24 °C and 37 °C for 3 h. Two-dimensional gel analysis confirmed this prediction by showing that, whereas the single mutant dbf4-1 showed significantly reduced fork arrest at 37 °C (Fig. 1D), a dbf4-1 mcm4Δ (Δ2–174) double mutant showed no defect in replication fork arrest at 37 °C (Fig. 3B). Thus, the results provided additional support for the conclusion that although Mcm2-7 phosphorylation by DDK was required for stable fork arrest, the N-terminal deletion of Mcm4 facilitated PFA, most likely by permitting formation of active CMG complex in the absence of DDK activity.

Deletion of Rif1 Suppressed the cdc7-1 Mutation at 30 °C and Restored PFA at the Semipermissive Temperature. It was recently reported that Rif1 can erase Mcm2-7 phosphorylation by recruiting the protein phosphatase PP1 to the replisome. Therefore, rif1Δ suppressed a cdc7-1 mutation at 30 °C and restored cell growth at that temperature (54, 57, 58). To investigate whether there was a correspondence between suppression of DDK mutants by rif1Δ and restoration of PFA, we first performed growth analysis and confirmed that rif1Δ could suppress a cdc7-1 mutation at 30 °C but not at 37 °C (Fig. 3C). We took this result to mean that whereas a moderate defect in cdc7-1 observed at 30 °C was suppressible by rif1Δ, a more severe phenotype manifested at 37 °C was not suppressed. We then performed 2D gel analyses of replication intermediates of a rif1Δ cdc7-1 double mutant and the cdc7-1 single mutant at all three temperatures and observed fork arrest in the double mutant at 24 °C and 30 °C but not at 37 °C for 3 h (Fig. 3 D–H). In contrast, similar analysis using a cdc7-1 single mutant revealed, as expected, a clear reduction in fork arrest at both 30 °C (Fig. 3 D and E) and 37 °C (Fig. 1C). Together, the data from three separate suppressors of DDK mutants support the conclusion that phosphorylated Mcm2-7, but not its dephosphorylated form, plays a critical role in promoting PFA, and that the requirement for Mcm2-7 phosphorylation is dispensable in mutants that can initiate replication in the absence of DDK.

**DDK-Catalyzed Phosphorylation of the Mcm2-7 Complex Was Necessary for the Retention of Tof1 at the Replication Fork.** How does DDK-dependent CMG (Mcm2-7) phosphorylation control fork arrest at Ter? We previously reported that Tof1 and Csm3, but not Mcm1, of the FPC are necessary for stable fork arrest at Ter sites and protein barriers elsewhere in the chromosome and that Tof1-Csm3 protects the arrested fork by counteracting the Rrm3 helicase, which evicts Fob1 protein from the Ter sites in the absence of either Tof1 or Csm3 (23, 28). We hypothesized that a phosphorylated Mcm2-7 complex was necessary for the recruitment and/or retention of Tof1 at the replisome and that inactivation of DDK by a ts mutation at the nonpermissive temperature was predicted to evict Tof1 from, or fail to recruit it to, the replisome. We expected this to cause unhindered fork passage not only through the Fob1–Ter complexes but also past many other DNA–protein barriers. Furthermore, we expected a suppressor such as mcm5-bob1 to restore PFA, presumably by blocking removal of Tof1 from the replisome at 37 °C. We tested these predictions as described below.

First, we wished to determine whether Mcm4 phosphorylation was reduced by inactivation of DDK in vivo. We performed Western blots (WBs) using FLAG-tagged Mcm4 in cdc7-1 rif1Δ mutants to monitor dephosphorylation of Mcm4 by gel electrophoresis at 24 °C and 37 °C. We used a rif1Δ strain to enhance the yield of phosphorylated Mcm4 for easier detection, and the data showed that inactivation of cdc7-1 at 37 °C caused a significant reduction in the ratio of the dephosphorylated to the phosphorylated form of Mcm4 (Fig. 4 A, i and ii).

To follow the degrees of retention of Tof1 protein in the nuclear vs. cytoplasmic fractions in the presence and absence of active DDK, we synchronized the cells of the different genotypes by growing them at 24 °C until reaching log phase and then arrested the cells in G1 phase with α-factor, released the block by proteolyzing the α-factor, and allowed the cells to grow for an additional 30 min at either 24 °C or 37 °C. Spheroplasts were prepared and fractionated into soluble and chromatin fractions, and the presence or absence of Tof1-myc in each was measured by WBs using anti-myc antibodies. Measurement of

### Table 1. Mean BrdU tract length (kilobases)

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<th>Time, min</th>
<th>WT</th>
<th>cdc7-1</th>
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<tr>
<td>0</td>
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<td>15</td>
<td>28.45</td>
<td>40.21</td>
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<tr>
<td>30</td>
<td>46.19</td>
<td>62.16</td>
<td>79.92</td>
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Orc3 from the same fractions, using anti-Orc3 antibodies, was used as an internal control. In WT cells, Tof1 was associated with the chromatin fractions at both 24 °C and 37 °C (Fig. 4 B). By contrast, in cdc7-1 cells, we consistently observed, in six replicates of the experiment, that Tof1 remained chromatin-bound at 24 °C but was greatly reduced in the chromatin fractions at 37 °C (Fig. 4 C and F, i). Similar experiments were performed in a cdc7-1 mcm5-bob1 strain. As predicted, Tof1-myc was retained in the chromatin fraction even after incubation at 37 °C (Fig. 4 D and F, ii). Surprisingly, in the congenic cdc7-1 strain a myc-tagged Csm3 was retained in the chromatin fraction (Fig. 4 E and F, iii). We propose that trapping of Csm3 by secondary interactions with other proteins, for example with the replication protein A [single-strand DNA-binding protein (59)], might have prevented its release from the chromatin fraction.

In the presence of the mcm5-bob1 suppressor, these cells grew without DDK and also displayed replication fork arrest at Ter at both 24 °C and 37 °C (Figs. 3 A, ii, iii). The data suggested that direct interaction between DDK and Tof1 was not obligatory for PFA when Mcm2–7 was activated in the suppressor mutant. We therefore investigated why Tof1 was not recruited or retained in the chromatin fraction upon inactivation of DDK in the biochemical experiments described below.

**Protein–Protein Interaction Between Tof1 and the Replisome.** We wished to test the hypothesis that, in addition to the known role of phosphorylation of Mcm2–7 by DDK for CMG assembly, Mcm2–7 phosphorylation also contributes to the function of CMG as a landing pad for Tof1 recruitment. We performed in vitro protein–protein interaction experiments using phosphorylated and dephosphorylated CMG and Tof1–Csm3, and also examined the Mcm2–7 complex (both the phosphorylated and dephosphorylated forms) and separately Cdc45 and GINS for binding to Tof1–Csm3. The purity of the various proteins used was determined by Coomassie blue-stained SDS/PAGE profiles (Fig. S3). The experiments provided two significant insights into the regulation of PFA: (i) Tof1 bound to the phosphorylated
CMG and Mcm2–7 but not to their dephosphorylated forms or to Cdc45 or GINS, and (ii) only phospho-Tof1 was competent to interact with CMG and Mcm2–7. The evidence for these conclusions is presented below.

We purified the Tof1–13myc–Csm3 complex, taking care to protect the proteins from possible dephosphorylation during purification by using multiple phosphatase inhibitors (SI Materials and Methods). We tested the purified complex to determine its phosphorylated state by Zn$^{2+}$-Phos-tag PAGE analysis (60), which revealed that ~50% of the purified Tof1 was phosphorylated, as indicated by the upward mobility shift of the protein bands and loss of these shifted bands after dephosphorylation (Fig. 5A, Left). A control SDS/PAGE of the same protein without Phos-tag is shown in Fig. 5A, Right, with actin as the loading control. The Tof1 protein, before and after dephosphorylation, had almost the same mobility, commensurate with its molecular mass, by SDS/PAGE. The gels were replicated three times with nearly identical results. The data suggested that in vitro dephosphorylation did not cause incidental proteolytic degradation of Tof1. To measure the possible effect of dephosphorylation on binding to phospho-CMG, an aliquot of Tof1-myc was pretreated with λ-phosphatase and the enzyme was inactivated by a mixture of phosphatase inhibitors (PhosSTOP).

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**Fig. 5.** Interactions of phosphorylated and dephosphorylated forms of CMG and Tof1 with each other as revealed by Western blotting. (A, Left) Zn$^{2+}$-Phos-tag gel of phosphorylated and dephosphorylated Tof1. (A, Right) SDS/PAGE profiles of both untreated Tof1-myc and that treated with λ-phosphatase. Actin loading controls are also shown. (B) Interaction of Tof1-myc–Csm3 complex untreated and treated with λ-phosphatase with immobilized CMG-His$_6$. (C) WBs showing binding of Tof1-myc untreated (+) and treated with λ-phosphatase (−) to immobilized FLAG-tagged Mcm2–7. (D) Interaction of Tof1-myc with immobilized untreated CMG-His$_6$ (Left) and dephosphorylated immobilized CMG-His$_6$ (Right). (E) Interaction of Tof1-myc with untreated immobilized Mcm2–7–FLAG and with dephosphorylated and immobilized Mcm2–7–FLAG. (F) Quantification of binding of Tof1-myc untreated (+) and dephosphorylated (−) to immobilized CMG. (G) Binding of untreated (+) and dephosphorylated Tof1-myc to immobilized Mcm2–7–FLAG (not dephosphorylated with λ-phosphatase). (H) Binding of untreated Tof1-myc to immobilized untreated CMG-His$_6$ and to the same after dephosphorylation with λ-phosphatase. (I) Quantification of binding of Tof1-myc to untreated immobilized Mcm2–7–FLAG and to the same after dephosphorylation with λ-phosphatase. (J) Quantification of binding of Cdc45 in solution to untreated immobilized Tof1-myc or to the same after treatment with λ-phosphatase. (K) Quantification of the binding of immobilized GINS complex to Tof1-myc in solution before (+) and after (−) treatment with λ-phosphatase. Error bars represent standard error.
before loading onto a CMG affinity column. We made sure that pretreatment of Tof1-myc with the phosphatase followed by addition of inhibitors was effective in inactivating the enzyme by carrying out standard phosphatase assays, with little or no residual phosphatase activity detectable by standard colorimetric phosphatase assays (SI Materials and Methods and Fig. S4). It should be noted that Csm3 is also a phosphoprotein (PhosphoGRID, www.phosphogrid.org), and could also be contributing to TofI–Csm3 interaction(s) with CMG. Approximately equal picomoles of phosphoryto Tof1 and the dephosphorylated form were applied to the CMG affinity columns, each containing the same amount of the affinity matrix. Control binding to the affinity matrix showed only low background levels of retention of Tof1 (Fig. 5B). The Tof1-myc that bound to the immobilized CMG columns (as measured in seven replicates of WBs using three different protein preparations) was quantified as a percentage of the input protein bound to immobilized CMG. The data consistently revealed an approximately eightfold increase in binding of phosphorylated (untreated) Tof1 to the CMG matrix compared with the dephosphorylated Tof1 protein (Fig. 5B; see quantification in Fig. 5F). Does Tof1–Csm3 in the phosphorylated form bind to the separate components of the CMG complex? We separately purified Mcm2–7, Cdc45, and GINS as described in SI Materials and Methods. Mcm2–7 included a FLAG-tagged Mcm5 subunit. The complex was extensively treated on anti-FLAG beads and challenged with phosphorylated (untreated) or λ-phosphatase treated with (treated with 1 unit λ-phosphatase at 30 °C for 1 h) Tof1 protein. WBs of the bound Tof1 protein visualized with anti-Myc antibodies revealed that whereas phosphorylated Tof1–Csm3 readily bound to the immobilized phospho-Mcm2–7, the dephosphorylated form of Tof1 showed significantly reduced binding to the affinity matrix (Fig. 5 C and G; + and - indicate no treatment or dephosphorylation with λ phosphatase, respectively). Similar binding experiments using Cdc45 and GINS complex showed 15–20% binding by either phospho-Tof1–Csm3 or its dephosphorylated form (Fig. 5 J and K). We conclude from the data that phospho-Tof1 specifically binds to Mcm2–7 but not to Cdc45 and GINS.

Do CMG and Mcm2–7 require phosphorylation to bind to phosho-Tof1? To investigate this question, purified CMG was extensively treated with λ-phosphatase followed by removal of the phosphatase by a gel-filtration step. Aliquots of the dephosphorylated CMG and separately the same amounts of the phosphorylated CMG were immobilized on Ni-NTA columns. Equal amounts of Tof1-myc–Csm3 complex were applied to each affinity column, and the proteins were eluted, resolved by SDS/PAGE, and visualized by WBs using anti-Myc Ab. The results showed that CMG, after dephosphorylation, showed a significant reduction in binding to Tof1-GST (Fig. 5 D and H). Therefore, a continued phosphorylated state of CMG, even after complex formation, is required for optimal binding to and retention of Tof1–Csm3 at the replisome. Similarly, we determined that immobilized phospho-Mcm2–7 but not its dephosphorylated form was able to bind to Tof1-myc–Csm3 complex (Fig. 5 E and I).

Independent support for the aforementioned conclusion is provided by the expression and purification of Mcm2–7 subunits in E. coli reconstitution into an Mcm2–7 complex (that had limited helicase activity (61)). The rationale was that Mcm2–7 expressed in E. coli is not expected to be phosphorylated because of a lack of a DDK or DDK-like kinase. Therefore, Mcm2–7ΔClb could should not bind to Tof1. We confirmed this expectation by attempting to bind phosphorylated Tof1 to immobilized Mcm2–7ΔClb but observed only low background levels of binding with both phosphorylated and dephosphorylated Tof1 (Fig. S5).

The proposed mechanism of PFA, supported by these data, is schematically shown in Fig. 6. The scheme takes into consideration our previous discovery that the Tof1–Csm3 complex counteracts the Rrm3 “sweepase” from displacing Fob1 from Ter, thereby maintaining stable fork arrest (Fig. 6A) (23, 28). In summary, the model posits that DDK phosphorylates Mcm2–7, which, along with Cdc45 and GINS, forms the CMG helicase. The CMG complex then recruits the phosphorylated but not the dephosphorylated form of Tof1–Csm3 to the replisome. Although Tof1 also requires phosphorylation for its recruitment by CMG, the kinase responsible for Tof1 phosphorylation remains unclear at this time. We propose that inactivation of DDK prevents the CMG complex from recruiting or retaining Tof1, thereby allowing Rrm3 to displace Fob1, resulting in unhindered fork progression past Ter (Fig. 6B). Why does PFA still work in cdc7-1 mcm5-bob1 cells at the nonpermissive temperature? We speculate that in the absence of DDK, CMG assembles in an alternative conformation that, as has been suggested, may be dependent on Cdc28, Clb2, and Clb5 (62).

**Discussion**

The biochemical mechanism of control of PFA in eukaryotes and its mode of regulation are poorly understood (4). This mechanism is of considerable general interest because of the many important physiological transactions mediated by the process. The cell-cycle kinases CDK and DDK are required for replication initiation, and CDK is involved in limiting replication to only once per cell cycle and in S-phase checkpoint control (37, 39, 40, 49, 63). The work reported here demonstrates that DDK is also required for PFA at a nonhistone protein barrier. This mechanism has been illuminated by our observation that inactivation of cdc7-1 not only caused abolition of PFA at Ter sites of rDNA but that the phosphorylated...
form of Mcm2–7, besides its well-known function in the assembly of the CMG complex, was also necessary for binding to Tof1 in vitro and its recruitment in vivo. Furthermore, phospho-Tof1 but not its dephosphorylated form bound to phospho-CMG and phospho-Mcm2–7 but not to the dephosphorylated protein complexes. Consistent with the in vitro binding data, Tof1 in vivo was retained in the chromatin fraction only in the presence of active DDK in the cell milieu.

Which kinase phosphorylates Tof1–Csm3? The data in Fig. 4 show that Tof1 is still recruited to chromatin in a cdc7–1 mutant in the presence of the mem5–boh1 suppressor at the nonpermissive temperature, which suggests that DDK-catalyzed Tof1 phosphorylation is not necessary in this genetic background and probably another kinase phosphorylates Tof1. It is also possible that a phosphorylated Tof1 is not needed in this genetic background. Nevertheless, several lines of evidence support the possibility that DDK is important for Tof1 function. First, the Schizosaccharomyces pombe homolog of Tof1, called Sw1, interacts with the homologous DDK complex (Hsk1–Dfp1) in the two-hybrid system and the proteins are also shown to be a form of complex by coimmunoprecipitation (64). Second, Tof1–Csm3 and DDK from S. cerevisiae also form a complex, as shown by coimmunoprecipitation, and Tof1–Csm3 is believed to recruit DDK to the replication fork during meiotic DNA replication (65). The requirement of Tof1–Csm3 for subsequent meiotic double-strand break formation could be bypassed by physically connecting DDK to Cdc45/CMG within the replication fork, indicating that Tof1–Csm3 serves as a platform for recruitment of DDK. Whether this interaction also leads to DDK phosphorylation of Tof1 remains to be determined. It is possible that in WT yeast, DDK is recruited by Mcm2–7 and not only phosphorylates Mcm2–7 but also Tof1, but that in the absence of DDK the mem5–boh1 mutant form of Mcm2–7 is able to recruit another kinase, perhaps Cdc28-Chb5 (62), that helps carry out PFA and replication. Further work is needed to resolve this issue.

Tof1 along with Csm3 plays a critical role in stabilizing stalled replication forks at protein barriers even in the absence of S-phase checkpoint activation, and this is central to its function in PFA, although DDK is not necessary for S-phase checkpoint activations (41, 42). However, unlike most instances, in which replication forks stall transiently while awaiting resolution of an impediment to fork progression, fork stalling in PFA is physiologically programmed and longer-lasting. This raises the question of how PFA differs mechanistically from transient fork stalling. We showed previously that Tof1 plays an additional role in PFA by counteracting the effects of the Rrm3 helicase. Rrm3 is known to act as a sweeper for its function in removing nonhistone DNA-binding proteins, thereby allowing unrestrained replication fork movement through protein-bound DNA. In the absence of Rrm3 in yeast, replication fork stalling is observed at over 1,000 sites that are not observed in wild-type cells (66). Many of these sites are known to be densely populated with DNA-binding proteins, including centromeres and tRNA-coding genes, among many others. However, in contrast with PFA, the data from the mem3Δ strain suggest that these DNA-bound proteins are readily removed by the action of the Rrm3 sweeper, so how does Tof1 antagonize the action of Rrm3 in the rDNA array? In this context, it should be kept in mind that a deletion of mem3Δ only partially rescues PFA in cells harboring either tof1Δ or csm3Δ (23). This suggests that besides Rrm3, other factors are also involved in the abolition of PFA in tof1Δ and csm3Δ cells.

As with most regulatory functions in biology, the answer appears to lie in a balancing act between positive and negative regulators and also in the evolutionary tradeoff between genome stability and genome adaptability. The DNA at stalled replication forks is subject to breakage followed by recombination that can lead to genome rearrangements, particularly in the case of repeated DNA sequences. At the same time, collisions between DNA replication and DNA transcription can also lead to replication fork collapse.

The Fob1-mediated DNA replication barrier in the rDNA of S. cerevisiae strikes a balance between these imperatives by preventing collisions between RNA and DNA synthesis and by using the components of the replication fork protection complex to prevent collapse of the replication fork stalled at the barrier. Although other organisms, as well as human cells, appear to use various mechanisms to prevent collisions between replication and transcription in the highly transcribed ribosomal RNA genes, they all use Tof1–Csm3 or the homologous human Tim/Tipin components of the fork protection complex to prevent collapse of the paused replication forks in the rDNA locus (reviewed in ref. 67).

Finally, although it is well-established that DDK-catalyzed phosphorylation of Mcm2–7 is needed for CMC assembly in WT cells, it is not known whether continued phosphorylation of the CMG complex is necessary for fork progression. Our data showing that even after a CMG complex has been formed its dephosphorylation causes failure to recruit the fork protection complex in vitro indicate that continued maintenance of the phosphorylated state of CMG seems to be necessary for error-free fork progression. It is known that under genotoxic stress, in the absence of Tof1 or Mrc1, the helicase and polymerase of yeast dissociate from each other in the replisome, causing fork collapse (68). Therefore, one would infer that continued maintenance of the phosphorylated state of CMG in the postinitiation step would be critical for genome stability.

Materials and Methods

Strain Construction. Yeast strains used in this study are listed in Table S1. All strains are from either the A364A or W303 background unless otherwise noted. Strains were constructed by standard genetic and molecular genetic techniques. Epitope tagging was performed using standard PCR-based insertion procedures (69). Incorporation of BrdU cassettes into strains was done as described elsewhere using the plasmids p4050–BrdU–Inc and p4050–BrdU–Inc (70). To disrupt the Bar1 locus by Ura3 incorporation, the pbar1:Ura3 plasmid was digested with BamHI and BglII and then transformed into the appropriate strain and selected on Ura dropout plates.

Oligonucleotides. Oligonucleotides are listed in Table S2.

Analysis of Mcm4 Phosphorylation. Chromatin fractions were used to analyze in vivo Mcm4 phosphorylation (54, 58). Phenylmethylsulfonyl fluoride (PMSF), sodium fluoride (NaF), and sodium orthovanadate (Na3VO4) were added directly to cultures of MCM4-6His-3FLAG cells to a final concentration of 1, 1, and 0.5 mM, respectively. We collected cells by a brief centrifugation (Sorvall 5C Plus; rotor # HS-4) at 5,000 rpm for 5 min and washed them with an ice-cold solution of 1 mM PMSF, 2 mM NaF, and 1 mM Na3VO4. Cells were collected by centrifugation and protein extracts were prepared as described above for the chromatin fractionation assay (51). Proteins were analyzed by electrophoresis on 6% SDS/PAGE, followed by Western blotting using monoclonal anti-FLAG antibody (M2; Sigma-Aldrich) as the primary antibody.

ChIP Assays. ChIP assays were performed in the cdc7–1 strain (YBS556) in which Fob1 was tagged with TAP at the C terminus (primers 2F, 2R, 3F, and 3R), using cells grown at 24 °C and 37 °C (22, 71). Each step has been described before (22, 71), except that cells grown at 37 °C were quickly chilled for 5 min on ice before adding 1% formaldehyde. PCR was performed using the primers 4F, 4R, 5F, and 5R (Table S2).

Two-Dimensional Agarose Gel Electrophoresis. Brewer–Fangman 2D gel electrophoresis was performed to visualize replication intermediates within cells obtained from cultures grown at 24 °C and 37 °C as previously described (53). Primers 1F and 1R were used to amplify the 1.4-kb region encompassing the rDNA NTS region, which was used as a probe to visualize replication intermediates.

Overnight starter cultures were inoculated into yeast, peptone, dextrose (YPD) medium at a starting OD600 of 0.2 and then grown for 2 h at 24 °C. Half of this culture was poured into another flask and swirled for 5 min in a water bath at 37 °C and then grown a further 2–3 h at 37 °C before being harvested. The culture was grown to an OD600 of 0.2 and then harvested. Genomic DNA was prepared and digested with EcoRV and BglII restriction enzymes to yield a 4.5-kb region of rDNA that included the replication origin.
Chromatin fractionation. Chromatin fractionation was performed to determine the level of chromatin-bound Tof1 or Csm3 in cells at 24 °C or 37 °C. The detailed method is described in SI Materials and Methods. Briefly, cells were harvested after Pronase treatment. Proteins from the chromatin and soluble fractions were prepared and resolved by SDS/PAGE and blotted to Nytran membranes, and the membranes were developed with anti-myc and anti-ORC3 antibodies.

DNA Combing. DNA combing was performed as described (72). BrdU was detected with a rat monoclonal antibody (AbCys; clone BU1/75) and goat anti-rat coupled to Alexa 488 (Invitrogen). DNA fibers were counterstained using anti-mouse MAB 3034 (Millipore) and goat anti-mouse coupled to Alexa 546. Images were recorded on a Leica DM6000 microscope equipped with a 40× objective and a CoolSNAP HQ CCD camera (Roper Scientific). Images were processed as described (73). BrdU tracts were measured with MetaMorph (Molecular Devices). Statistical analyses of differences between samples were performed with GraphPad Prism 6.0 using the Mann–Whitney rank-sum test. More details of data collection and statistical analysis are provided in the legend to Fig. 2.

Protein Purification. CMG, Mmc2–7, and Cdc45 were purified by expression in yeast and confirmed as described in greater detail in SI Materials and Methods. Tof1–Csm3 was purified as follows. Tof1 ORF expressed with 13c-Myc was cloned into the vector pJB842 (74). Tof1 was expressed in S. cerevisiae as a GST fusion protein in this vector and purified on a glutathione-agarose column as described in detail in SI Materials and Methods.

Protein–Protein Interaction in Vitro. Details are described in SI Materials and Methods.

Phos-Tag Gels. Phosphorylated Tof1 protein was detected by Zn2+-Phos-tag gels as described in detail in SI Materials and Methods following a published procedure (60, 75).

Phosphatase Assay. The phosphatase assay was performed as described to not only detect phosphatase activity of γ-phosphatase at different dilutions but to check the effectiveness of the phosphatase inhibitor (PhosSTOP; Roche). A standard solution (1 tablet per mL) was prepared and 1 μL of reaction mixture was added to stop dephosphorylation. For the colorimetric assay, 4-nitrophenyl phosphate was used as substrate and color was detected with an ELISA plate reader (76).

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