DNA damage slows DNA synthesis at replication forks; however, the mechanisms remain unclear. Cdc7 kinase is required for replication origin activation, is a target of the intra-S checkpoint, and is implicated in the response to replication fork stress. Remarkably, we found that replication forks proceed more rapidly in cells lacking Cdc7 function than in wild-type cells. We traced this effect to reduced origin firing, which results in fewer replication forks and a consequent decrease in Rad53 checkpoint signaling. Depletion of Orc1, which acts in origin firing differently than Cdc7, had similar effects as Cdc7 depletion, consistent with decreased origin firing being the source of these defects. In contrast, mecl-100 cells, which initiate excess origins and also are deficient in checkpoint activation, showed slower fork progression, suggesting the number of active forks influences their rate, perhaps as a result of competition for limiting factors.

Introduction

The replication of eukaryotic chromosomes requires the cell cycle–regulated initiation of numerous replication origins on each chromosome. Coordinating much of this process are two highly conserved kinases, S-phase Cdk and Dbf4-dependent kinase (DDK), which become active at the G1–S transition (Labib, 2010). During early G1 phase, before S-phase Cdk and DDK activation, origin recognition complex, Cdc6, and Cdt1 load minichromosome maintenance (MCM) helicase complexes, in an inactive state, onto DNA at potential origin loci. A key step in replication initiation is the conversion of MCM into the active helicase, resulting in DNA unwinding, replication assembly, and DNA synthesis. DDK plays an essential role in MCM activation by phosphorylating MCM, particularly the Mcm4 and Mcm6 subunits. In fact, this is the only essential function of DDK in yeast, as mutations in MCM subunits that mimic the DDK-phosphorylated state or cause conformational changes that activate the helicase, obviate the normal requirement for DDK function for DNA replication and cell viability (Hardy et al., 1997; Fletcher et al., 2003; Sheu and Stillman, 2010).

As the name implies, DDK is composed of a catalytic kinase subunit, Cdc7, whose activity depends on Dbf4 (Masai and Arai, 2002). Dbf4 binds Cdc7, activating the kinase and targeting it to specific substrates, such as Mcm4. Dbf4 also negatively regulates DDK function as a target of the intra-S checkpoint pathway in response to replication stress or DNA damage (Duncker and Brown, 2003). Activated checkpoint kinase Rad53 phosphorylates Dbf4, inhibiting DDK-dependent activation of unfired origins (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). There are conflicting reports as to whether this regulation directly inhibits DDK activity or affects its targeting to substrate, or both (Oshiro et al., 1999; Weinreich and Stillman, 1999; Sheu and Stillman, 2006). Rad53 activity also regulates the rate of replication fork progression through damaged DNA, suggesting that Rad53 might modulate replication fork progression by regulating DDK activity (Szyjka et al., 2008). In this study, we have examined replication fork dynamics in cells depleted of Cdc7 function and find that replication forks progress more rapidly than in wild-type (WT) cells.
Together with analysis of Orc1- and checkpoint-defective cells, we show that replication fork rate is sensitive to the level of origin firing.

**Results and discussion**

**Cdc7 activity regulates replication fork progression**

To address the potential function of DDK at replication forks, we analyzed the rate of DNA synthesis across two long replicons using BrdU immunoprecipitation (IP) analyzed by microarray (BrdU-IP-chip) in cells depleted of Cdc7 function. To deplete Cdc7 function, we used two well-characterized alleles: cdc7-as3 (L120A and V181A), the catalytic activity of which is directly inhibited by binding of ATP analogue PP1 within the ATP binding site (Wan et al., 2006), and cdc7-1, a temperature-sensitive kinase hypomorph, in the presence of the boh1 allele of MCM5, which enables reduced but sufficient origin firing for viability in the absence of Cdc7 kinase function (Hardy et al., 1997; Hoang et al., 2007).

WT and cdc7-as3 cells were synchronized in late G1 phase with α-factor and treated with PP1 25 min before release into S phase; upon release into S phase, aliquots of each culture were pulse labeled with BrdU for discrete intervals (Fig. 1 A). Analysis of bulk DNA content by FACSscan showed rapid progression of WT cells through S phase, unaffected by the presence of PP1, whereas cdc7-as3 cells were delayed in bulk DNA synthesis, in a PP1-dependent manner (Fig. 1 B). Analysis of BrdU incorporation showed depletion of origin firing in PP1-treated cdc7-as3 cells, both in the number of origins that fired genome wide and in their levels of BrdU incorporation (see Materials and methods). We estimated that 234 origins fired in WT cells, and 157 fired in cdc7-as3 cells; these represent mainly earlier firing origins, as determination of later origins was precluded by possible BrdU signal from converging replication forks. In addition to fewer origins detected to fire, the level of BrdU incorporation was lower at these origins in cdc7-as3 cells, consistent with less efficient activation (Fig. 1 C). Arrangement of the origins’ BrdU incorporation levels according to their replication timing (see Materials and methods) showed that later origins were more diminished than earlier origins in cdc7-as3 cells (Fig. 1 C).

This pattern of origin firing was observed along the chromosome III and VI regions that we analyzed in detail. In PP1-treated WT and cdc7-as3 cells, BrdU-IP-chip at 10–30 min showed similar levels of DNA synthesis occurring at the early origins, ARS306 and ARS607 (Fig. 1 D). However, at 10–30 and 25–45 min, BrdU incorporation at slightly later origins, ARS603.5 and ARS605, was diminished in cdc7-as3 cells, consistent with depletion of Cdc7 function. The activity of the earliest origins may reflect residual activity of Cdc7-as3 resistant to PP1 (perhaps bound to ATP) or instead may reflect the execution of Cdc7 function at these origins before Cdc7 depletion in late G1. We exploited this early origin firing to examine the consequences of Cdc7 inactivation on fork progression. During the 25–45- and 40–60-min intervals, the extent of BrdU incorporation along the ARS306 and ARS607 of cdc7-as3 than WT cells, suggesting a faster rate of replication fork progression in Cdc7-depleted cells. The distal BrdU incorporation apparent in WT cells during the 55–75-min pulse likely reflects subtelomeric origin activity. We also compared fork progression in WT and cdc7-as3 cells by analyzing the cumulative incorporation of BrdU over time (Fig. S1 B). This method yielded similar results as the pulse-labeling approach, showing more rapid progression of replication forks through the ARS306 and ARS607 replicons in cdc7-as3 than in WT cells. Together, these results indicate that Cdc7 is dispensable for replication fork progression and suggest that Cdc7 regulates the rate of replication fork progression along an undamaged DNA template.

Next, we analyzed whether Cdc7 function regulates the progression of replication forks traversing a damaged DNA template. G1-synchronized WT and cdc7-as3 cells were treated with PP1 and released into S phase in the presence of the DNA alkylating agent methyl-methane-sulfonate (MMS). Aliquots of each culture were pulsed with BrdU at defined intervals (Fig. 1 A). FACSscan analysis showed slower progression of WT and cdc7-as3 cells through S phase as expected because of the presence of MMS, with somewhat slower bulk DNA replication in the cdc7-as3 cells, consistent with their reduced efficacy of origin firing (Fig. 1 B). We estimated that 219 origins fired in WT, and 134 fired in cdc7-as3 cells treated with MMS, and the efficiency of origin firing based on the level of BrdU incorporation was lower at most origins in cdc7-as3 cells (Fig. 1 C). The number of origins detected in MMS-treated versus untreated cells was only modestly decreased because the measurement in cells without MMS did not effectively detect later firing origins. However, comparison of WT and checkpoint-defective (mec1-100) cells indicates that >90 origins are detected as checkpoint inhibited by MMS treatment by our analysis (see Fig. 4).

In WT and cdc7-as3 cells, BrdU incorporation was similar for ARS306 and ARS607 at 10–30 min, whereas BrdU incorporation at the slightly later ARS603.5 and ARS605 was reduced in cdc7-as3 cells (Fig. 1 D). As in the absence of MMS, the rate of BrdU incorporation along the ARS306 and ARS607 replicons was greater in cdc7-as3 than in WT cells. We estimated replication fork rates in MMS using regression analysis based on the leading edge of BrdU incorporation across the ARS607 to chromosome VI right arm region (see Materials and methods). The firing of subtelomeric origins in WT cells precluded unambiguous determination of fork rate in the absence of MMS; however, the presence of MMS facilitated this analysis by inhibiting firing of subtelomeric origins through the intra-S checkpoint (Tercero et al., 2003). This analysis yielded a mean rate of 446 bp/min in MMS-treated WT cells and 1,031 bp/min in MMS-treated cdc7-as3 cells. The more rapid progression of BrdU incorporation in cdc7-as3 cells was dependent on PP1 (Fig. S1 E). Thus, Cdc7 is required for the normal rate of replication fork progression along undamaged and MMS-damaged templates.

To corroborate these unexpected findings, we used the cdc7-1 allele in a similar analysis. G1-synchronized WT and cdc7-1 mec5-bob1 cells were shifted to 32°C for 1 h before release into S phase in the presence of MMS and pulsed with BrdU (Fig. 2 A). Total DNA content analysis showed similar slow rates of DNA synthesis in WT and cdc7-1 mec5-bob1 cells in the presence of MMS (Fig. 2 B), whereas cdc7-1 cells lacking the mec5-bob1 suppressor allele showed tight arrest of DNA
Figure 1. Cdc7 function regulates replication fork progression. (A) WT and cdc7-as3 cells were synchronized with α-factor for 3 h and 35 min, treated with PP1 for 25 min, and released from α-factor with PP1 and with or without 0.033% MMS. (B) DNA content analysis by FACScan. Analysis of PP1 untreated cells is also shown. (C) Heat maps of BrdU incorporation levels at origins are arranged according to each origin’s published replication timing from early to late (left to right). (D) Aliquots were pulsed with BrdU for the indicated intervals and analyzed by BrdU-IP-chip. Results for segments of chromosomes III and VI are plotted, with origin locations indicated above. Data shown are from a single representative experiment out of two replicates, except data in C, and were calculated from both replicates.
Figure 2. Cdc7 functions upstream of Rad53 in fork regulation. (A) WT, cdc7-1 mcm5-bob1, and cdc7-1 mcm5-bob1 pph3Δ cells were synchronized with α-factor for 3 h at 23°C, shifted to 32°C for 1 h, and released from α-factor at 32°C with 0.033% MMS. (B) DNA content analysis by FACSscan. (C) Heat maps of BrdU incorporation levels at origins are arranged according to each origin’s published replication timing from early to late (left to right).
synthesis (Fig. S2 B), demonstrating the effective inhibition of Cdc7-1 function under these conditions, and at least partial restoration of origin firing by mcm5-bob1, as reported previously (Hoang et al., 2007). BrdU incorporation showed similar effects on origin firing of Cdc7 inhibition by cdc7-1 mcm5-bob1 as by cdc7-as3; we estimated that 221 origins fired in WT, and 175 fired in cdc7-1 mcm5-bob1 cells. As in cdc7-as3 cells, the level of BrdU incorporation at most origins was decreased; however, the earliest origins were least affected (Fig. 2 C). This result is consistent with the previous demonstration that the earliest origins are partially resistant to elimination of Cdc7 function in the presence of the mcm5-bob1 allele; the mcm5-bob1 allele alone does not affect origin firing (Hoang et al., 2007). Cdc7-1 function under these conditions, and at least partial restoration of origin firing by mcm5-bob1, as reported previously (Hoang et al., 2007). BrdU incorporation showed similar effects on origin firing of Cdc7 inhibition by cdc7-1 mcm5-bob1 as by cdc7-as3; we estimated that 221 origins fired in WT, and 175 fired in cdc7-1 mcm5-bob1 cells. As in cdc7-as3 cells, the level of BrdU incorporation at most origins was decreased; however, the earliest origins were least affected (Fig. 2 C). This result is consistent with the previous demonstration that the earliest origins are partially resistant to elimination of Cdc7 function in the presence of the mcm5-bob1 allele; the mcm5-bob1 allele alone does not affect origin firing (Hoang et al., 2007). BrdU incorporation at ARS306 and ARS607 was similar between WT and cdc7-1 mcm5-bob1 cells, whereas the firing of the slightly later origins was compromised specifically in the cdc7-1 mcm5-bob1 cells (Fig. 2 D). The incorporation of BrdU along the chromosome III and VI replications progressed more rapidly in cdc7-1 mcm5-bob1 than WT cells, with rates of 1,364 and 541 bp/min, respectively (Fig. 2 D). The more rapid BrdU incorporation in the cdc7-1 mcm5-bob1 strain was accompanied by association of replication protein A, which binds single-stranded DNA at replication forks, consistent with more rapid progression of bona fide replication forks (Fig. 2 E). These observations support the results with the cdc7-as3 allele and indicate a role for DDK in regulating the rate of fork progression.

**Cdc7 acts upstream of Rad53 in fork regulation**

The aforementioned results show that Cdc7 function controls the rate of replication fork progression on undamaged and MMS-damaged DNA. We showed previously that deactivation of Rad53 promotes progression of forks slowed in response to MMS (Szyjka et al., 2008), and previous studies have shown differences in Rad53 activation in cells lacking Cdc7 activity (Tercero et al., 2003; Dohrmann and Sclafani, 2006; Ogi et al., 2008; Sheu and Stillman, 2010). Thus, reduced Cdc7 activity might permit rapid replication fork progression through damaged DNA by diminishing checkpoint signaling leading to Rad53 activation. To investigate whether Cdc7 depletion affects the level of checkpoint activation, we examined Rad53 activation as reflected in its altered electrophoretic mobility caused by its phosphorylation (Pellicioli et al., 1999). Under the conditions of the aforementioned experiments, Rad53 activation was reduced in cdc7-1 mcm5-bob1 compared with WT cells based on the smaller proportion of slower to faster migrating Rad53 (Fig. 2 F). This result is consistent with previous findings that Cdc7 is required for the normal level of checkpoint activity in cells undergoing replication stress and is consistent with the idea that reduced checkpoint activation resulting from reduced Cdc7 activity affects the rate of fork progression.

A previous study concluded that the requirement of Cdc7 for checkpoint activation in response to DNA damage reflects its function in initiation and the establishment of replication forks (Tercero et al., 2003). Cdc7 activity also is regulated as a direct target of the checkpoint via Rad53-dependent phosphorylation of Dbf4, which inhibits DDK function in origin firing. If Cdc7 is upstream of Rad53 in activation of the checkpoint, fork slowing should occur in response to increased Rad53 activity, even in the absence of Cdc7 function. Conversely, if Cdc7 activity is a downstream target or effector of the checkpoint required for regulation of fork rate, increased Rad53 activity should fail to slow forks in the absence of Cdc7 function. To increase Rad53 activity, we exploited our previous finding that deletion of the Rad53 phosphatase PPH3 results in Rad53 hyperactivity and slower replication fork progression in MMS (O’Neill et al., 2007; Szyjka et al., 2008). As predicted, pph3Δ resulted in increased Rad53 activity in WT and Cdc7-deficient, MMS-treated cells, although the level of Rad53 activity was lower in cells lacking Cdc7 function, consistent with reduced origin activation (Fig. 2 F). The increased Rad53 activity correlated with slower bulk DNA replication and slower fork progression (Fig. 2, B and D). Thus, enhanced Rad53 activity slows forks in the absence of Cdc7 activity, which is consistent with Cdc7 acting upstream of Rad53 in fork slowing in response to replication stress.

**Decreased initiation from Orc1 depletion also deregulates fork progression**

Our results suggest that the deregulated fork progression of Cdc7-depleted cells derives from Cdc7’s function in replication initiation. To address whether a decreased level of replication initiation is sufficient to deregulate fork progression, we examined fork progression in cells harboring orcl-161, a temperature-sensitive allele of ORC1, which is required for replication initiation; incubation of G1-arrested orcl-161 cells at the nonpermissive temperature reduces MCM occupancy at origins (Aparicio et al., 1997; Gibson et al., 2006). We performed the same temperature shift regimen and release into MMS as we did for the cdc7-1 cells (Fig. 3 A). Total DNA content analysis showed diminished progression through S phase of orcl-161 cells compared with WT, consistent with reduced origin firing in the mutant cells (Fig. 3 B). Rad53 activation also was reduced in orcl-161 cells, only reaching levels comparable to those of WT cells at ~90 min (Fig. 3 C). Interestingly, these higher levels of Rad53 activation coincided with reduced progression of total DNA content in orcl-161 cells at these later times (Fig. 3 B), consistent with checkpoint regulation of fork rates (analysis of fork rates by BrdU-IP is not feasible at these later times). Analysis of BrdU incorporation showed a global reduction in the number of origins that fired and their BrdU incorporation levels in orcl-161 cells, consistent with depletion of Orc1 function. We estimated that 230 origins fired in WT, and 192 fired...
Another feature of reduced Cdc7 and Orc1 activity that we hypothesized might contribute to faster fork rates is the reduced overall number of active forks, which might increase the availability of normally rate-limiting factors to the fewer active forks. To evaluate the effect of the number of active forks, we examined mecl-100 cells, which only weakly activate Rad53 in response to MMS (but sufficiently to maintain fork stability), while activating a larger than normal complement of origins, including late and normally dormant origins (Paciotti et al., 2001; Tercero et al., 2003). Therefore, these cells allow us to test the effect of higher numbers of active forks in combination with low levels of active Rad53.

Checkpoint elimination is not sufficient to deregulate fork rate

We have shown that decreased levels of initiation result in decreased Rad53 activation levels and faster fork rates. However, another feature of reduced Cdc7 and Orc1 activity that we hypothesized might contribute to faster fork rates is the reduced overall number of active forks, which might increase the availability of normally rate-limiting factors to the fewer active forks. To evaluate the effect of the number of active forks, we examined mecl-100 cells, which only weakly activate Rad53 in response to MMS (but sufficiently to maintain fork stability), while activating a larger than normal complement of origins, including late and normally dormant origins (Paciotti et al., 2001; Tercero et al., 2003). Therefore, these cells allow us to test the effect of higher numbers of active forks in combination with low levels of active Rad53. As shown previously, G1-synchronized mecl-100 cells released into MMS (Fig. 4 A) exhibit more rapid progression through S phase as measured by total DNA content (Fig. 4 B) and decreased Rad53 activation (Fig. 4 C; Paciotti et al., 2001). BrdU incorporation analysis showed increased numbers of active forks.
Regulation of replication fork rate

Zhong et al.

including more origins firing and slower forks, in other intra-S checkpoint mutant strains, including rad53Δ and rad53Δ exo1Δ (EXO1 deletion suppresses the MMS sensitivity of rad53Δ cells; Fig. S3; Segurado and Diffley, 2008). A recent study in human cells reported slower fork progression in Ckh1-depleted cells, which was suppressed by additional depletion of Cdc7 activity (Petermann et al., 2010). These findings suggest that increased numbers of replication forks suppress more rapid fork progression, perhaps by depleting essential factors.

origins genomewide in mec1-100 cells, with 219 firing in WT and 310 in mec1-100 cells, the latter including many later origins (Fig. 4 D). Analysis of the chromosome III and VI regions showed similar levels of BrdU incorporation at earlier origins and higher levels at late origins like ARS603 in mec1-100 cells (Fig. 4 E). Replication forks progressed more slowly in mec1-100 cells than in WT cells (Fig. 4 E), with rates of 242 and 517 bp/min, respectively, despite lower levels of Rad53 activation in mec1-100 cells. We have observed similar BrdU incorporation profiles as in mec1-100 cells, including more origins firing and slower forks, in other intra-S checkpoint mutant strains, including rad53Δ and rad53Δ exo1Δ (EXO1 deletion suppresses the MMS sensitivity of rad53Δ cells; Fig. S3; Segurado and Diffley, 2008). A recent study in human cells reported slower fork progression in Ckh1-depleted cells, which was suppressed by additional depletion of Cdc7 activity (Petermann et al., 2010). These findings suggest that increased numbers of replication forks suppress more rapid fork progression, perhaps by depleting essential factors.

Figure 4. Deregulated origin firing in mec1-100 slows replication forks. [A] WT and mec1-100 cells were synchronized with α-factor for 4 h at 23°C and released from α-factor at 23°C with 0.033% MMS. (B) DNA content analysis by FACScan. (C) Immunoblot analysis of phosphorylated Rad53 (Rad53-P); both rows are from the same blot and exposure. Molecular weight markers were not run on this gel; for the migration of size markers relative to the bands detected by this antibody, see Fig. 2 F. (D) Heat maps of BrdU incorporation levels at origins are arranged according to each origin’s published replication timing from early to late (left to right). (E) Aliquots were pulsed with BrdU for the indicated intervals and analyzed by BrdU-IP-chip. Results for entire chromosome VI are plotted, with origin locations indicated above. Data shown are from a single representative experiment out of two replicates, except data in D, and were calculated from both replicates.
number of active replication forks, which is determined by the level of origin firing (Fig. 5). We propose that the number of active forks influences overall fork rate in checkpoint-dependent and -independent ways. Robust checkpoint activation associated with substantial numbers of replication forks encountering.

Replication fork and checkpoint levels regulate replication fork progression
Comparison of origin firing rates and replication fork rates across the experiments in MMS supports a model in which the rate of replication fork progression is inversely related to the number of active replication forks, which is determined by the level of origin firing (Fig. 5). We propose that the number of active forks influences overall fork rate in checkpoint-dependent and -independent ways. Robust checkpoint activation associated with substantial numbers of replication forks encountering.

Figure 5. Replication fork and checkpoint levels regulate replication fork progression. (A) Genome-wide origin firing and local fork rate for the experiments in MMS are plotted; mean and standard deviation are shown (n = 2). Data points are color coded for the experimental group represented. (B) The model depicts fork rate regulation in WT and mutant strains with different levels of origin firing and checkpoint functions. The font intensities and line/arrow thicknesses represent the relative strength of the corresponding pathway or signal. For example, gray fonts indicate a weak or defective function or pathway, and bold fonts indicate a hyperactive function or pathway. The chromosome graphic below each model depicts the levels of origin firing and fork rate in each condition. Open circles represent fired origins, and filled circles represent unfired origins; E, M, and L indicate early, middle, and late firing origins, respectively.
DNA damage slows fork progression. Additionally, large numbers of forks deplete available replication factors or deoxy-nucleoside triphosphates, which limits fork rate even with a reduced or absent checkpoint. However, when fork numbers are reduced, as in *cdc7* and *orc1* mutant cells, reduced checkpoint activation and reduced competition from other forks for limiting factors allow more avid fork progression. In *mec1-100* cells, where deficiency of Rad53 activation is associated with an excess of replication forks, replication factor depletion results in slower fork progression despite the lack of checkpoint activation. This model is based in part on our previous demonstration that suppression of Rad53 activity restores robust fork progression. This model is based in part on our previous demonstration that suppression of Rad53 activity restores robust fork progression, supporting the idea that fork rate is under checkpoint regulation, (Szyjka et al., 2008). Further suppression of Rad53 activity results in slower fork progression despite the lack of checkpoint activation. This model is based in part on our previous demonstration that suppression of Rad53 activity restores robust fork progression, supporting the idea that fork rate is under checkpoint regulation, (Szyjka et al., 2008). Further suppression of Rad53 activity results in slower fork progression despite the lack of checkpoint activation.

### Materials and methods

#### Plasmid and strain constructions

All strains are derived from W303 and are described in Table 1. Gene disruptions were constructed by PCR-based methods (Güldener et al., 1996; Longtine et al., 1998). Plasmid p306-ars305*:BrdU-Inc was constructed by three-way ligation of 620-bp NotI–BglII PCR-amplified fragment 5'-flanking ARS305 and 560-bp BglII–SacI PCR-amplified fragment 3'-flanking ARS305 into NotI–SacI-digested p306-BrdU-Inc (Viggiani and Aparicio, 2008). Plasmid p306-ars305*:BrdU-Inc digested with BglII was used to simultaneously delete ARS305 and integrate the 10.3-kb plasmid with the BrdU-Inc cassette by gene replacement. Correct replacement was confirmed by PCR. The 1.6-kb HindIII–EcoRI fragment of *cdc7-as3* containing the kinase-inactivating mutations L120A and V181A was isolated from pRS551-cdc7-as3 (L120A and V181A; a gift from N. Hollingsworth, Stony Brook University, Stony Brook, NY; Wan et al., 2006) and subcloned into EcoRI–HindIII-digested pRS306. The resulting plasmid, pRS306-cdc7-as3, was linearized with EcoRI and used to exchange *CDC7* with *cdc7-as3* by pop-in/pop-out replacement. All allele replacements were confirmed by DNA sequencing.

#### Yeast methods

Cells were grown in YEPD (yeast extract, peptone, and dextrose) for all experiments. Cells were synchronized in G1 phase by incubation with 5 mM α-factor (T6901; Sigma-Aldrich) for 4 h at 23°C and released by resuspension and gentle sonication in fresh YEPD lacking α-factor and containing 200 µg/ml Pronase E (P83L mutation) in pRS306, to exchange *MCM5* with *mcm5-bob1* by pop-in/pop-out replacement followed by screening for suppression of temperature sensitivity at 37°C. The 7.5-kb SacI–SpeI fragment containing the *mec1-100* allele was isolated from plasmid pM258.51 (a gift from M.P. Longhese, University of Milan, Milan, Italy; Paciotti et al., 2001) and subcloned into SacI–SpeI-digested pRS406. The resulting plasmid, pRS406-mec1-100, was linearized with BstI and used to exchange *MEC1* with *mec1-100* by pop-in/pop-out replacement. All allele replacements were confirmed by DNA sequencing.

### Table 1. List of strains used in this study

| Strain | Genotype |
|--------|----------|
| JPy8   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) RFA1-18Myc (KanMX) |
| JPy9   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) RFA1-18Myc (KanMX) cdc7-1 mcm5-bob1 |
| JY3    | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) sml1Δ::HIS3 |
| JY4    | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) sml1Δ::TRP1 |
| RS1298 | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) cdc7-1 |
| RS1307 | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) cdc7-1 mcm5-bob1 |
| T2y41  | ars608Δ::HIS3 ars305Δ::BrdU-Inc (KanMX) orc1Δ::hisG leu2Δ::ORC1 (LEU2) |
| T2y42  | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (KanMX) orc1Δ::hisG leu2Δ::ORC1 (LEU2) |
| T2y2   | lys2Δ::hisG trp1Δ::BrdU-Inc (TRP1) orc1Δ::hisG leu2Δ::ORC1 (LEU2) |
| T2y3   | lys2Δ::hisG trp1Δ::BrdU-Inc (TRP1) orc1Δ::hisG leu2Δ::ORC1 (LEU2) |
| T2y8   | ars608Δ::HIS3 ars609Δ::TRP1 trp1Δ::BrdU-Inc (LEU2) |
| T2y10  | ars608Δ::HIS3 ars609Δ::TRP1 trp1Δ::BrdU-Inc (LEU2) cdc7-3as |
| YZ78   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) RFA1-18Myc (KanMX) orc1Δ::hisG leu2Δ::ORC1 (LEU2) |
| ZY79   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) cdc7-1 mcm5-bob1 pha3Δ::KanMX |
| ZY34   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) pha3Δ::KanMX |
| ZY35   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) cdc7-1 mcm5-bob1 pha3Δ::KanMX |
| ZY50   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) |
| ZY52   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) mec1-100 |
| ZY61   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) bar1Δ::LEU2 sml1Δ::HIS3 rad53Δ::KanMX exo1Δ::TRP1 |
| ZY61   | ars608Δ::HIS3 ars609Δ::TRP1 ars305Δ::BrdU-Inc (TRP1) bar1Δ::LEU2 sml1Δ::HIS3 rad53Δ::KanMX |

All strains share the W303a RAD5 genotype MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 bar1-1::hisG except where noted.
The estimate of the fork rate \( x \approx 10^{1.60} \sim 10^{1.67}. \)

Online supplemental material is available at http://www.jcb.org.

Fig. S1 shows analysis of cumulative BrdU incorporation in cdc7-as3. Online supplemental material was published by Rad53 immunoblot analysis was performed with anti-Rad53 at 1:1,000 (C85749; Santa Cruz Biotechnology, Inc.) as described previously (Gibson et al., 2004).

Microarray normalization

BrdU-IP-chip normalization from the Roche arrays was performed as previously described (Knott et al., 2009). In brief, probes from the most dense regions of the corresponding MA plots were isolated, and principle component analysis was performed on their corresponding \( M = \log \frac{P}{1 - P} \) values of the probes within the region. In most cases, there was a single, clearly enriched region of BrdU signal to the right of ARS607. In cases with more than one enriched region, for the purpose of estimating the fork speed, we chose the region with the maximum E score. The sixth column of Table S1 indicates which time intervals were included in the analysis.

Fork rate analysis

For each experiment, we examined the \( \varphi \) values in the single enriched region identified in the previous paragraph. We assumed that the probability of having a fork in the interval defined by the position of a single probe is proportional to the \( \varphi \) value of that probe. To estimate the leading edge of the replication fork, we used \( P = \exp(M) \times \text{Total} \) and \( A = \exp(M) \times \text{Total} \) values. The resultant first and second principle components were then taken to represent each probe’s normalized A and M values, respectively. After this, loess normalization was performed to remove any residual array artifacts (Smyth and Speed, 2003). Analysis of Rfa1 ChIP was performed using MA2C (Song et al., 2007).

Data filtering

We used the values of \( \varphi = \exp(M) \) obtained from the previous step in the subsequent analysis. An enriched probe was defined as one with a \( \varphi \) value of \( > 1 \). An enriched region was defined as a sequence of consecutive enriched probes. Each enriched region was given an enrichment score (E-score), which was the sum of the \( \varphi \) values of the probes within the region. In most cases, there was a single, clearly enriched region of BrdU signal to the right of ARS607. In cases with more than one enriched region, for the purpose of estimating the fork speed, we chose the region with the maximum E score. The sixth column of Table S1 indicates which time intervals were included in the analysis.

Origin firing analysis

Using the piecewise cubic Hermite interpolating polynomial, we interpolated the normalized and smoothed M value of the probes for every 10 bp of the genome. Under the null hypothesis of no enrichment around an origin, the sum of the N interpolated M values of the probes in this region will have the following distribution: \( \sum N \equiv \exp(M) \times \text{Total} \) and \( A = \exp(M) \times \text{Total} \) values. The resultant first and second principle components were then taken to represent each probe’s normalized A and M values, respectively. After this, loess normalization was performed to remove any residual array artifacts (Smyth and Speed, 2003). Analysis of Rfa1 ChIP was performed using MA2C (Song et al., 2007).

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Regulation of replication fork rate • Zhong et al. 383