Continued DNA Synthesis in Replication Checkpoint Mutants Leads to Fork Collapse

Sarah A. Sabatinos, Marc D. Green, and Susan L. Forsburg

Molecular and Computational Biology, University of Southern California, Los Angeles, California, USA

Hydroxyurea (HU) treatment activates the intra-S phase checkpoint proteins Cds1 and Mrc1 to prevent replication fork collapse. We found that prolonged DNA synthesis occurs in *cds1Δ* and *mrc1Δ* checkpoint mutants in the presence of HU and continues after release. This is coincident with increased DNA damage measured by phosphorylated histone H2A in whole cells during release. High-resolution live-cell imaging shows that mutants first accumulate extensive replication protein A (RPA) foci, followed by increased Rad52. Both DNA synthesis and RPA accumulation require the MCM helicase. We propose that a replication fork “collapse point” in HU-treated cells describes the point at which accumulated DNA damage and instability at individual forks prevent further replication. After this point, *cds1Δ* and *mrc1Δ* forks cannot complete genome replication. These observations establish replication fork collapse as a dynamic process that continues after release from HU block.

Fission yeast *Schizosaccharomyces pombe* cells treated with hydroxyurea (HU) suffer nucleotide depletion and activate the intra-S phase checkpoint. This causes reversible cell cycle arrest in early S phase and prevents firing of late replication origins (12, 19, 62). The intra-S phase checkpoint stabilizes existing DNA replication forks and promotes recovery and replication resumption upon release. Loss of the intra-S phase checkpoint corrupts replication forks, leading to DNA damage and cell death. However, the events leading to successful recovery or fork collapse remain poorly defined.

HU-treated replication forks synthesize 5 to 10 kb of DNA before they slow in budding yeast (4, 27, 49). Evidence from several model systems suggests that MCM helicase and polymerases are briefly uncoupled during fork slowing, which leads to increased DNA unwinding and accumulation of replication protein A (RPA) on single-stranded DNA (ssDNA) (37, 39, 42, 43). This promotes Rad3 (ATR, or ScMec1) kinase activation (36, 66), which activates Cds1 kinase (ScRad53) (26). Cds1 inhibits the origin-activating Hsk1 kinase (ScCdc7) to block late origin firing (24, 51, 54), and the Mcm4 subunit of the MCM helicase is a likely Cds1 substrate (5). Cds1 also phosphorylates the Mus81 nuclease, preventing accumulation of toxic structures (7, 22). Finally, Cds1 phosphorylates Cdc25, preventing mitosis in the presence of damage (6, 26, 65).

The mediator protein Mrc1 recruits Cds1 to Rad3 to promote efficient checkpoint activation, but Mrc1 is also a nonessential component of the replication fork complex (56, 57, 63, 64). Mrc1 couples the leading-strand DNA polymerase epsilon (Polε) to the MCM helicase (23, 28, 41) and regulates recombination during replication stalling and restart (1, 22, 47).

The absence of either Cds1/ScRad53 or Rad3/ScMec1 during HU treatment leads to late origin firing, DNA unwinding, and cell death (2, 12, 26, 49, 52). This is accompanied by DNA double-strand breaks (DSBs), histone H2A phosphorylation (phospho-H2A; H2A_x in metazoans), and accumulation of the Rad52 recombination protein in repair foci (1, 30). These markers are a feature of replication fork collapse (5, 14, 27), which results in Chk1 kinase activation (26). Because the G₂-M damage checkpoint is activated, cells do not divide prematurely (8, 26, 55).

Rather, the primary cause of lethality in *cds1Δ* and *mrc1Δ* mutants is a failure to recover, not a failure to arrest the cell cycle.

To characterize this recovery defect, we examined *cds1Δ* and *mrc1Δ* mutants following HU treatment and release. We used nucleotide analogue incorporation to label new synthesis specifically and found surprising amounts of incorporation in both *cds1Δ* and *mrc1Δ* cells during both HU block and release. This synthesis does not require new origin firing. This observation suggests that checkpoint-deficient mutants retain fork architecture and catalytic activity to generate extended tracts of DNA synthesis despite the presence of HU. DNA synthesis during HU block and release in the mutants correlates with accumulation of RPA foci, which is followed by Rad52 repair foci. Finally, we show that MCM helicase activity from existing forks is required for DNA synthesis during the HU block and is the major cause of RPA focus formation in *cds1Δ* plus HU. Together, these observations indicate that replication fork collapse is progressive and that fork breakdown is neither immediate nor uniform throughout the genome. Rather, these data imply that loss of replication is stochastic and occurs, not during the initial stages of treatment, but instead during recovery from HU treatment, when cells reach a crisis that we term the “collapse point.”

MATERIALS AND METHODS

Yeast growth and flow cytometry. Fission yeast cells were cultured as described previously (48) in YE5S or Edinburgh minimal medium 2 (EMM) with appropriate supplements. Genotypes are listed in Table S1 in the supplemental material, with additional notes on specific alleles. HU was removed by filtration, and 5-ethynyl-2'-deoxyuridine (EdU) was added to cultures at 10 μM or bromodeoxyuridine (BrdU) was added at

Received 2 August 2012 Returned for modification 23 August 2012

Accepted 1 October 2012

Published ahead of print 8 October 2012

Address correspondence to Sarah A. Sabatinos, sabatino@usc.edu, or Susan L. Forsburg, forsburg@usc.edu.

Supplemental material for this article may be found at <http://mcb.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/MCB.01060-12

0.1 mg/ml. Cells were fixed in cold 70% ethanol before fluorescence-activated cell sorting (FACS) using 1 μ M SytoxGreen (Invitrogen) in 50 mM sodium citrate, 10 μ g/ml RNase A. Samples were sonicated and then run on a FACScan (BD Biosciences) for Sytox (FL1-A) or EdU (FL1-H). Analysis and median peak values were calculated in FlowJo (TreeStar Inc.) and plotted relative to time. The rate of EdU signal labeling was calculated as the slope of incorporation over time during block (0 to 4 h of HU treatment) and release (4 h of HU treatment onward).

DNA damage detection. Soluble lysates were separated by SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) to detect hemagglutinin (HA) and PCNA (see Table S2 in the supplemental material). Whole-cell or nuclear-spread immunofluorescence was determined as described in reference 15 using yeast-specific S129 phospho-H2A antibody (Millipore). Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) in SlowFade Gold mount (Invitrogen). Images were acquired on a Leica DMR epifluorescence microscope (63 $\times$ lens; 1.4 numerical aperture [NA]) and analyzed in ImageJ (46) using a threshold value at least 2 times greater than the cytoplasmic background. Images are presented using the ImageJ "smart" lookup table (LUT) for p-H2A signal (whole cells) or "orange" (spreads). Proportion and confidence intervals (CI) were calculated for p-H2A signals from at least 2 independent experiments.

DNA fiber spreads. DNA fiber spreads were prepared as described previously (5, 15). Cells were treated with 0.2% sodium azide buffer on ice and then spheroplasted and allowed to adhere to poly-L-lysine glass coverslips. Lysis was performed on-slide for 5 min before the slides were tilted at 30 $^\circ$ and fibers extended. The fibers were fixed in 4% paraformaldehyde, rinsed in phosphate-buffered saline (PBS), and heat fixed for 10 min.

EdU incorporation and detection. Cells were treated with 10 μ M EdU for the indicated times, fixed (70% ethanol), and treated with the AlexaFluor 488 Click-iT kit (Invitrogen) using the kit directions. Click-treated cells were washed (1 $\times$ PBS, 1% bovine serum albumin [BSA]) and sonicated in PBS with 10 μ g/ml RNase A for FACS. EdU fiber tracts were treated on slide, mounted in SlowFade Gold with DAPI (Invitrogen), photographed, and processed as for fiber immunofluorescence. Whole-cell EdU samples were stained with DAPI and then analyzed for low and high incorporation (2 times or 4 times higher than the cytoplasmic background).

Chromatin fiber immunofluorescence. EdU-only detection was performed on PBS-rehydrated fiber slides according to the Click-iT kit (Invitrogen) directions. Dual detection of EdU/BrdU involved first rehydrating slides (1 $\times$ PBS) and then denaturing DNA in 4 N HCl for 20 min. Slides were neutralized in 0.1 M Na₂B₄O₇ (5 min) and then washed extensively in 1 $\times$ PBS before adding Click-iT reagent (as described above). EdU-Alexa Fluor 555-conjugated fibers were blocked (1% BSA, 10% fetal calf serum [FCS], 0.1% Tween 20 in PBS) for 0.5 to 1 h and then incubated with anti-BrdU overnight (see Table S2 in the supplemental material). The MoBU1 antibody (Invitrogen) does not cross-react with EdU. The slides were washed, incubated with secondary antibody (see Table S2 in the supplemental material), and then washed with PBS plus 0.1% Tween 20 before being mounted in SlowFade Gold with DAPI (Invitrogen). Samples were imaged on a DeltaVision Core epifluorescence wide-field microscope (Applied Precision, WA), using a 60 $\times$ lens (1.4 NA), and then deconvolved and projected in softWoRx software (Applied Precision). Images were analyzed by counting total EdU or BrdU tracts (n) in blinded samples, where the number of tract ends (potential replication forks) was $2n$. Categorical data were evaluated for statistical significance using χ^2 and 2-tailed Z statistics.

Live-cell imaging. Live cells were imaged on a DeltaVision Core microscope as described above. Movies were captured at 0.25-h intervals in microfluidic chambers (CellAsic, CA) at constant flow and temperature (WeatherStation; API). Short-term imaging on EMM-agarose pads was at 30 $^\circ$ C. Serial z sections were deconvolved and maximum intensity projected in softWoRx. An RPA heat map was made in ImageJ, using the "Fire" LUT from raw data. Minimally, >300 cells over two separate fields

were imaged in each experiment, and 2 to 4 biological replicates were assessed for consistency and then pooled and presented as proportions $\pm$ 95% CI. Focus induction was calculated by dividing the number of RPA foci at 3 h of HU treatment and 2 h at 36 $^\circ$ C by 1 h of HU treatment (25 $^\circ$ C). Absolute RPA was calculated by subtracting the baseline of multiple RPA foci in asynchronous cells. The 95% CI of induced values was calculated using standard (sum squared) error rules.

RESULTS

Replication fork stalling and resumption are accompanied by DNA damage. Phosphorylation of histone H2A marks sites of replication stress and DSBs in fission yeast; it is seen only sporadically in wild-type cells treated with HU. However, *cds1 Δ* cells accumulate substantial phospho-H2A during HU treatment (5, 35). We monitored phospho-H2A and Chk1 phosphorylation to measure G₂-M checkpoint activation in *cds1 Δ* and *mrc1 Δ* cells during and after HU treatment (Fig. 1A). We observed little phospho-H2A in wild-type cells plus HU and a transient increase after release (Fig. 1B and C). In contrast, phospho-H2A signal was present after 4 h of HU treatment in *cds1 Δ* and *mrc1 Δ* cells and accumulated further during HU release. The HU-induced phospho-H2A signal in *cds1 Δ* and *mrc1 Δ* cells was bright and spread throughout the nucleus (pannuclear) (Fig. 1B and C), while wild-type signal was less intense and occurred in discrete puncta. Chk1 phosphorylation in *cds1 Δ* and *mrc1 Δ* cells also increased after release (Fig. 1D) (30). Thus, phospho-H2A is enhanced in *cds1 Δ* and *mrc1 Δ* cells during HU treatment and release.

cds1 Δ -dependent damage is proposed to come from Mus81 cleavage of stalled replication forks (14). However, we found that phospho-H2A signal occurred in both *mus81 Δ* and *mus81 $^+$* cells (Fig. 1E). Significantly, the signal in *cds1 Δ* or *cds1 Δ mus81 Δ* cells was pannuclear, while wild-type and *mus81 Δ* spreads contained dimmer, discrete foci. Chk1 was activated in *cds1 Δ mus81 Δ* cells, but not in *mus81 Δ* cells alone (Fig. 1F). Thus, Cds1 prevents Mus81-independent lesions that trigger DNA damage response, as well as Mus81-specific DSBs. However, the viability of *cds1 Δ mus81 Δ* cells was higher than that of *cds1 Δ* cells alone, indicating that Mus81 damage is directly responsible for some *cds1 Δ* cell damage causing cell death (Fig. 1G).

Checkpoint-deficient cells synthesize DNA during HU block. To investigate the source of checkpoint activation, its effect on replication, and mechanisms of fork collapse, we looked in detail at DNA synthesis. We reasoned that a fraction of *cds1 Δ* and *mrc1 Δ* cell replication forks might remain functional during HU treatment and then collapse during release. To test this, we used strains that incorporated thymidine analogues (see Fig. S1 in the supplemental material) and added EdU to label newly synthesized DNA during arrest and release (Fig. 2A). We categorized cells as low nuclear EdU (low-threshold) or extensive nuclear EdU (high-threshold) incorporation in all genotypes (see Fig. S2A and B in the supplemental material). Few wild-type cells developed EdU signal during HU treatment (Fig. 2B). This is consistent with current models suggesting that the active intra-S phase checkpoint inhibits replication forks and blocks late origin firing. After release, wild-type cells resumed replication, and 90% of the cells incorporated high levels of EdU as measured by FACS and the high-threshold nuclear signal (Fig. 2B and C; see Fig. S2 in the supplemental material). We performed rate calculations on FACS data to confirm that EdU incorporation was restrained during HU

FIG 1 DNA damage signals are formed after release from HU. (A) Asynchronous (AS) cells (strains FY3858, FY3859, and FY3866) were treated with HU and released to fresh medium to detect DNA damage signals. Refer to Fig. S1 in the supplemental material for strain validation. (B) A heat map of phospho-histone H2A (p-H2A) immunofluorescence at 0.5 h postrelease (bottom) relative to DNA (top) (DAPI/brightfield [BF]) demonstrates intense p-H2A signals in *cds1Δ* and *mrc1Δ* cells (scale, 10 μ m). Nuclei had no p-H2A (asterisks) or punctate (arrows) or pannuclear p-H2A. (C) Proportion of nuclei with punctate phospho-H2A, or pannuclear phospho-H2A signal, quantified from 2 biological replicates of the experiment shown in panel A ($\pm$ 95% CI). (D) Chk1HA activation in wild-type, *mrc1Δ*, and *cds1Δ* cells (FY4611, FY4867, and FY4875) post-HU treatment. The blots were probed for anti-HA (top) or anti-PCNA loading control. The Chk1HA bandshift (asterisk) in *mrc1Δ* and *cds1Δ* cells post-HU treatment indicates Chk1 activation above the unmodified Chk1 band (arrow). (E) (Left) Nuclear spreads of wild-type, *cds1Δ*, *mus81Δ*, and *mus81Δ* *cds1Δ* cells (strains FY3456, FY2190, FY3288, and FY3732) after 4 h of HU treatment. The *mus81Δ* *cds1Δ* nuclei with p-H2A displayed a qualitative pattern similar to that of *cds1Δ* cells, but not *mus81Δ* cells. (Right) p-H2A signal was widespread in *cds1Δ* strains and less common in other strains, as shown by the proportion of p-H2A-positive spreads from a representative experiment ($\pm$ 95% CI). (F) Chk1HA phosphorylation occurs in *mus81Δ* *cds1Δ* (FY4913) but not *mus81Δ* (FY4911) strains after HU release. (G) Relative viability after acute treatment with 12 mM HU (strains FY4611, FY4867, FY4875, FY4911, and FY4913). *mrc1Δ* and *mus81Δ* *cds1Δ* cell viabilities overlap at later points. The average over 3 biological replicates is shown ($\pm$ standard error of the mean [SEM]). wt, wild type.

treatment and resumed after release (see Fig. S2D and E in the supplemental material).

Surprisingly, we found that both *cds1Δ* and *mrc1Δ* cells incorporated substantial EdU during the HU block, as well as after

release (Fig. 2B and C; see Fig. S2 in the supplemental material). *cds1Δ* cells displayed low-level EdU after only 2 h in HU, with 90% of *cds1Δ* nuclei EdU positive at 4 h of HU treatment (37% high level). Following release, 98% of *cds1Δ* nuclei were EdU positive

FIG 2 Synthesis occurs in checkpoint-deficient cells during HU block and release. (A) Asynchronous (asynch) wild-type, *cds1Δ*, or *mrc1Δ* cells (strains FY3858, FY3866, and FY3859) were blocked in 12 mM HU for 4 h at 32°C. EdU was added at 1 h of HU treatment for continuous labeling during block and release (R). A2 and A4, arrest at 2 h and 4 h. (B) Nuclear EdU signal detected at 2 h in HU (A2), 4 h in HU (A4), or 45 min after release (R). Threshold values 2 times (low) or 4 times (high) greater than in cytoplasm were applied, and >100 nuclei were scored for each sample. The proportions of nuclei with low or high levels of EdU are shown ($\pm 95\%$ CI) over total positive nuclei from one representative experiment. Refer to Fig. S2 in the supplemental material for further details. (C) Flow cytometry comparing new synthesis (EdU) (right; log scale) with total DNA content (SytoxGreen) (left; linear scale) in the continuous-labeling scheme shown in panel A. Samples were fixed at 0 to 4 h in HU, and EdU was added at 1 h of HU treatment, as shown in panel A. Postrelease (R) samples were removed at 0.75 h and 1.5 h after HU was removed while EdU was maintained during release. (D) Asynchronous cells (FY3858, FY3859, and FY3866) were blocked for 4 h in HU and then released in the presence of EdU to label release synthesis, as shown in panel E. (E) Flow cytometry to monitor EdU incorporation (right; log scale), and DNA content (left; Sytox; linear scale) postrelease. All genotypes incorporated EdU similarly by 0.5 h postrelease, but *cds1Δ* and *mrc1Δ* cells did not reach wild-type EdU amounts by 1.5 h. The median peak values of EdU charts are plotted in Fig. S2F in the supplemental material.

(73% high level). Thus, *cds1Δ* cells synthesized substantial DNA during HU block and underwent additional synthesis after release. The rate of *cds1Δ* EdU synthesis (see Fig. S2E in the supplemental material) was higher than that of the wild type in HU treatment (22.7 fluorescent units of EdU/h) and increased further after release (165 EdU/h). Thus, although DNA synthesis in *cds1Δ* cells was partially unregulated, there was still some restraint during HU treatment, leading to an increased rate of synthesis after release from HU treatment.

In *mrc1Δ* nuclei, >50% of cells incorporated low-level EdU

signal after 2 h of HU treatment, with 65% of *mrc1Δ* nuclei showing high-level EdU signal by 4 h of HU treatment. After release, the proportion of *mrc1Δ* nuclei with high-level EdU was constant (70%) (Fig. 2C; see Fig. S2E in the supplemental material). The rate of *mrc1Δ* incorporation was high with HU treatment and showed no further increase after release (see Fig. S2E in the supplemental material). This suggests that the change in the rate of EdU incorporation in wild-type and *cds1Δ* cells that we observed upon HU release is Mrc1 dependent. However, both *mrc1Δ* and *cds1Δ* cells reached a plateau at 2/3

of wild-type EdU signal at 1.5 h postrelease (see Fig. S2D in the supplemental material) and never accumulated a fully duplicated genome content.

Synthesis resumes in *cds1Δ* and *mrc1Δ* cells following HU treatment. Current models propose that replication forks in checkpoint mutants collapse during HU treatment, which prevents replication after release. Given our unexpected observation that DNA synthesis continues during HU treatment, we asked how much *cds1Δ* and *mrc1Δ* synthesis occurs specifically during release by adding nucleotide analogue only after HU removal (Fig. 2D). We found that all strains incorporated EdU after release from HU treatment (Fig. 2E), and the levels were similar in all genotypes at 0.5 h postrelease. However, wild-type cells reached a maximum level of EdU incorporation by 1.5 h post-HU treatment, while checkpoint-deficient cells never reached the same maximum. Rather, they acquired only 30 to 60% of the wild-type level of EdU (Fig. 2E; see Fig. S2F in the supplemental material). Thus, checkpoint-deficient cells not only undergo significant DNA synthesis during HU treatment, they successfully continue DNA synthesis during early stages of release.

Intriguingly, the DNA synthesis measured by EdU incorporation in mutant cells was not obvious in the total DNA content flow cytometry measured by FACS using SytoxGreen staining, the common method for measuring DNA synthesis (Fig. 2C and E). Wild-type DNA content began at 2C, shifted to 1C during HU treatment, and returned to 2C by 1.5 h postrelease (see Fig. S2C in the supplemental material). *cds1Δ* and *mrc1Δ* cells both shifted to 1C DNA during HU treatment, and acquired less than 1.5C during release (approximately 40% of the wild type). This appeared paradoxical, given the extensive EdU incorporation we detected during block and release (almost 70% of the wild type). When plotted as FACS profiles, differences were not obvious, especially since the Sytox peaks were broader. Further, since Sytox fluorescence depends on double-stranded DNA (dsDNA) structure, we asked whether ssDNA accumulation affected signal. Heat denaturation of DNA in fixed cells caused a drop in the apparent Sytox signal (see Fig. S2G in the supplemental material). Thus, standard FACS methods may obscure the remaining DNA synthesis in checkpoint mutants during HU block and release in the presence of extensive ssDNA.

Synthesis occurs in replicated tracts during block and following release. We next asked whether checkpoint-deficient synthesis differs from the wild type using spread chromatin fibers to examine tracts of replicated DNA. Fibers from continuously labeled cells (Fig. 2A) were harvested after 3.5 h in HU or 0.75 h following release and spread to an average of 2 to 3 kb/μm (20) (see Fig. S3 in the supplemental material). In wild-type cells during HU treatment, median EdU tracts were short (~4 μm; 8 to 12 kb) and occurred either as single blocks or in symmetrical tracts surrounding a small gap (Fig. 3A; see Fig. S3 in the supplemental material). Based on other work (20), we inferred that continuous tracts represent origin firing during EdU treatment, and gapped tracts are bidirectionally replicated from origins that fired before EdU addition. This is consistent with replication forks traveling a short distance during HU treatment (10 ± 5 kb) before slowing (24, 44, 49). Following release from HU treatment, wild-type EdU tracts became very long.

In contrast, tracts from *cds1Δ* cells were longer than wild type during HU treatment (~6 μm; 12 to 18 kb) and occurred over a broader size distribution (Fig. 3A). We saw many symmetrical

tracts around gaps (Fig. 3B), which would be consistent with origin firing and replication during HU treatment. After release, *cds1Δ* tract length increased further (~7 μm), again over a large size distribution (see Fig. S3 in the supplemental material). The profile of *mrc1Δ* cells was somewhat different. Tracts from HU-treated *mrc1Δ* cells were abundant but shorter than those from either the wild-type or *cds1Δ* cells. Many pairs of tracts in *mrc1Δ* cells flanked a central gap, but the flanking tracts were often asymmetric in length. Median *mrc1Δ* cell length during HU treatment (3 μm; 6 to 9 kb) increased after release (8 μm) and was shorter than wild-type but longer than *cds1Δ* cells.

Both fiber and FACS analysis confirmed that *cds1Δ* and *mrc1Δ* cells synthesized substantial DNA during HU treatment and after release. One explanation for this synthesis is that a fraction of replication forks remain functional and collapse during release. An alternative is that DNA synthesis we observed after HU release represents late origin firing. To distinguish these possibilities, we used a temperature-sensitive *orp1-4^{ORC1}* mutant to inactivate unfired origins, so that only forks that initiated during HU treatment could continue DNA synthesis after release. We labeled cells sequentially with EdU during HU treatment and then with BrdU after HU release at either restrictive (36°C) or permissive (25°C) temperature (Fig. 3C). A fork that synthesizes DNA during HU treatment (EdU) and continues extension following release (BrdU) will show adjacent tracts of label, a pattern we observed experimentally.

We found that DNA synthesis occurred in *orp1ts cds1Δ* and in *orp1ts mrc1Δ* cells, although it was somewhat reduced compared to the *orp1ts* single mutant (see Fig. S3D and E in the supplemental material). The median length of BrdU tracts after release (0.5 h post-HU synthesis) was shortest in *orp1ts cds1Δ* cells, longer in *orp1ts mrc1Δ* cells, and longest in *orp1ts* single mutants (Fig. 3D), consistent with less efficient synthesis in the checkpoint mutants. Thus, late origin firing contributed to some, but not all, EdU incorporation (Fig. 3D; statistics are given in Fig. S3C in the supplemental material). When we released cells at permissive temperature, we saw evidence for origin firing, synthesis, and extension in all genotypes (Fig. 3F; see Fig. S3F in the supplemental material). We conclude that *orp1ts cds1Δ* and *orp1ts mrc1Δ* synthesis during release is due to extension of existing forks and not origin firing.

Interestingly, while the *orp1ts* single mutant displayed discrete tracts of EdU adjacent to BrdU (consistent with replication fork extension), we found the two labels frequently overlapped in *orp1ts cds1Δ* and *orp1ts mrc1Δ* cells (Fig. 3E). This signal overlap was often extensive and covered most of the tract (Fig. 3D). In contrast, small signal overlaps bridging the block and release synthesis tips were similar in all genotypes. Evidence for overlapping synthesis might represent differences in incorporation in leading versus lagging strands, single-strand gaps, or resection. Given the dynamic nature of this synthesis, we next examined helicase activity and DNA damage markers in checkpoint mutants during and after HU treatment.

Accumulation of ssDNA and DNA damage markers during block and release. The replication checkpoint limits ssDNA accumulation (12, 23, 29) caused by helicase and polymerase uncoupling (9). The accumulation of phospho-H2A during HU block and release suggested that ssDNA and DNA damage are dynamic markers of replication instability during HU block and release. To examine these factors, we performed time courses on live cells in a microfluidics chamber, allowing medium changes in a block-and-

FIG 3 Replication proceeds in checkpoint-deficient cells during HU block and release. (A) Frequency histograms of spread-DNA fiber EdU tract lengths after 3.5 h HU block (red bars) and then after 0.75 h post-HU release (blue bars). EdU labeling is continuous throughout block and release, according to the scheme in Fig. 2A. Histogram bins are 2 μm, and median tract lengths are given. Refer to Fig. S3 in the supplemental material for additional details. Strains FY3858, FY3859, and FY3866 were used in panels A and B. (B) Representative EdU tracts (green) during block and release (REL), as shown in panel A. (C) Experimental design to test late origin-dependent synthesis in *orp1ts*^{ORC1} mutant cells. Asynchronous cells were incubated with HU for 0.5 h and then shifted to 36°C. EdU was added at 1 h of HU treatment for 0.5 h at 36°C to label block synthesis. After a total of 3 h in HU (2.5 h at 36°C), HU was washed out, and the cells were resuspended in fresh medium containing BrdU. Recovery from HU treatment proceeded for 0.5 h before fibers were harvested. (D) Histograms of BrdU tract lengths (release synthesis) on *orp1ts*, *cds1Δ orp1ts*, and *mrc1Δ orp1ts* fibers. Post-HU treatment synthesis was performed at 36°C to inhibit additional origin firing. Examples of fibers are shown for each genotype, with EdU (red) and BrdU (green) shown separated and merged. BrdU tracts in *cds1Δ orp1ts* and *mrc1Δ orp1ts* cells frequently overlapped with EdU signal. The median length is indicated for each genotype. Scale bars, 5 μm. Refer to Fig. S3B in the supplemental material for additional data. (E) Proportions (±95% CI) of extensively overlapping BrdU/EdU tracts for each genotype. *, $P < 0.05$. (F) Recovery synthesis was allowed to proceed at 25°C, demonstrating that late origin firing is possible at permissive temperature. Shown is an example of an *orp1ts cds1Δ* fiber, labeled during HU block (EdU; red) and release (BrdU; green). The indicated green-only region is an origin that fired after release from HU treatment.

release experiment. We monitored RPA-cyan fluorescent protein (CFP) and Rad52-yellow fluorescent protein (YFP) recruitment relative to HU treatment and mitosis using the spindle pole body (SPB) marker Sad1-DsRed. This single-cell approach allowed us

to monitor cells in sufficient numbers for a robust view of population dynamics (Fig. 4; see Fig. S4 in the supplemental material).

Few wild-type cells had RPA or Rad52 foci prior to HU block (Fig. 4C and D, time zero). RPA and Rad52 foci only appeared in

FIG 4 RPA and Rad52 accumulate in checkpoint-deficient HU block. (A) Movie outline. Cells were photographed every 0.25 h for RPA (Rad11-CFP), Rad52 (Rad52-YFP), SPB marker (Sad1-DsRed), and transmitted light (strains FY4744, FY4919, FY4929, and FY4752). Refer to Fig. S4 in the supplemental material for conditions, scoring, and colocalization. (B) Percent mitotic cells—the proportion of M to S phase cells—is the sum of duplicated SPB, dividing nuclei, and binucleate and septated cells at each time point. (C) Accumulation of nuclear RPA foci (none, 1, or ≥ 2) over time. (D) Live-cell Rad52 nuclear foci, plotted over time, are consistent with previous work using static time points. (B to D) HU treatment is represented by the red boxes. (E) Images from selected time points: asynchronous (AS), during HU treatment (2 h and 4 h), and after release (1 h and 6 h). Early wild-type release had transient accumulation of RPA and Rad52. Shown is a heat map of RPA signal intensity, from low (purple) to high (white), where foci are orange to white. Rad52 foci are shown merged with transmitted light.

untreated binucleate cells coincident with S phase, and the signals partly overlapped (see Fig. S4 in the supplemental material). During HU treatment, we observed a modest increase in single RPA foci, but not in Rad52, consistent with evidence showing ssDNA is required to activate the replication checkpoint (66). RPA foci increased at 0.5 to 1.5 h postrelease, which was followed by a burst of Rad52 at 1 h postrelease (0.5 h later than the RPA peak). This indicates that RPA recruitment is temporally distinct from Rad52. Both signals decreased by 1.5 h post-HU treatment, followed by SPB duplication and mitosis by 2 h post-HU treatment, consistent with population synchrony from HU treatment (Fig. 4B). Most wild-type cells (78%) divided at least once during the 6 h after HU treatment.

RPA foci were present even in asynchronous *cds1Δ* and *mrc1Δ* cells (Fig. 4C) and increased further after 2 h of HU treatment. After 4 h of HU treatment, RPA foci were present in >80% of cells. RPA accumulation continued after release, observed in >90% of *cds1Δ* and *mrc1Δ* cells. Strikingly, RPA foci merged into masses covering the nucleus and appeared dramatically different from the small, discrete foci in the wild type (Fig. 4E; see Fig. S4 in the supplemental material).

Rad52 foci were also more common in untreated *cds1Δ* and *mrc1Δ* cells, consistent with low levels of constitutive damage (Fig. 4D). Rad52 foci increased in *cds1Δ* cells by 2 h of HU treatment. In *mrc1Δ* cells, Rad52 accumulated more slowly, starting after 3 h of HU treatment. In contrast to the brief burst observed in the wild type after HU release, Rad52 foci steadily accumulated in both checkpoint mutants during release. Rad52/RPA colocalization also increased dramatically during and after HU treatment in *cds1Δ* cells (see Fig. S4 in the supplemental material). The increased RPA and Rad52 signal in *cds1Δ* and *mrc1Δ* cells correlated with loss of viability after 4 h of HU treatment (see Fig. S1 in the supplemental material). A small subpopulation of *cds1Δ* and *mrc1Δ* cells divided during early HU exposure (Fig. 4B), but only infrequent division was observed later, consistent with cell cycle arrest (see Movies S2 and S3 in the supplemental material).

Finally, we compared the dynamics of RPA and Rad52 accumulation in *rad51Δ* (repair-deficient) cells. The *rad51Δ* cells did not divide during HU treatment (Fig. 4B), indicating robust checkpoint arrest. However, *rad51Δ* cells formed many RPA and Rad52 foci, even during asynchronous growth, which increased both during and after HU treatment (Fig. 4C and D; see Movie S4 in the supplemental material). Significantly, RPA remained in discrete foci (Fig. 4E) and did not develop into the large RPA masses seen in checkpoint mutants. Thus, ssDNA and DNA repair signals that accumulated in *cds1Δ* and *mrc1Δ* cells during and after HU treatment are specific to loss of the replication checkpoint and distinct from defects in DNA repair.

MCM helicase drives ssDNA accumulation. Accumulation of ssDNA during HU treatment could reflect two replisome activities: (i) MCM helicase-dependent unwinding at existing forks, which can be blocked by a temperature-sensitive degron allele of the Mcm4 subunit (*mcm4ts-dg*), or (ii) late origin firing and assembly of new replication forks, which can be blocked using the *hsk1-1312* temperature-sensitive allele (*hsk1ts*). We compared RPA accumulation in *cds1Δ* cells to that in *cds1Δ mcm4ts-dg* or *cds1Δ hsk1ts* cells (Fig. 5A and B) and observed a significant reduction of RPA in the *cds1Δ mcm4ts-dg* strain at restrictive temperature (36°C). In comparison, *cds1Δ* or *cds1Δ hsk1-ts* cells accumulated multiple RPA foci by all metrics at 36°C in HU (Fig. 5C;

see Fig. S5A in the supplemental material). Thus, high levels of RPA and ssDNA in *cds1Δ* cells were MCM dependent and Hsk1 kinase independent, reflecting extension of active forks and not firing of inactive origins.

The relationship of helicase and origin firing to Rad52 focus formation was less clear (see Fig. S5B in the supplemental material). Asynchronous *cds1Δ mcm4ts-dg* cells had many Rad52 foci at 25°C, and their numbers increased only slightly during HU block. *cds1Δ hsk1ts* cells had more Rad52 foci than other genotypes and accumulated additional Rad52 foci only at 36°C. This suggests that Hsk1 might restrain Rad52 accumulation, or the damage it recognizes, independently of Cds1. Despite the increased Rad52 accumulation, *hsk1ts* cells retained viability in HU, and *hsk1ts* partly rescued the viability of *cds1Δ* cells in HU (see Fig. S5C in the supplemental material).

We next asked whether MCM activity contributed to DNA synthesis by monitoring EdU incorporation (Fig. 5D and E). First, we observed that synthesis kinetics differed slightly at 36°C from what we observed at lower temperatures. Wild-type DNA synthesis began to leak through the HU block by 4 h at 36°C, while *cds1Δ* EdU incorporation was reduced in HU treatment at higher temperatures (compare with EdU incorporation at 32°C) (Fig. 2C). There may be differences in checkpoint regulation at high temperatures (as proposed in reference 16). However, leak-through synthesis was largely blocked in *mcm4ts-dg* and *cds1Δ mcm4ts-dg* cells (Fig. 5E), indicating that DNA synthesis remains MCM helicase dependent.

DISCUSSION

Our results map DNA replication and damage dynamics during HU arrest in the wild type and the intra-S phase checkpoint mutants. The replication checkpoint stabilizes forks during stalling or stopping to prevent lethality due to fork collapse. This is loosely defined as accumulation of DNA damage, disconnected helicase and polymerase, dissociation of replisome components at the fork, and an end of DNA synthesis (10, 11, 13, 14, 40). Current models suggest that forks collapse during HU treatment and are unable to recover and restart replication after HU removal, leading to a failure of recovery and cell death. However, our study shows that fork collapse is a delayed process, accompanied by significant DNA synthesis after HU treatment.

This leads to a revised model for replication fork behavior in HU and for the roles of Mrc1 and Cds1 (Fig. 6). Our results suggest that HU treatment in the wild type slows polymerase activity before the replisome starves for nucleotides, which results in little EdU incorporation during HU block, consistent with previous reports (25, 38). However, in the absence of Cds1 or Mrc1, this monitoring is lost, and we observe ongoing synthesis during HU treatment and after release. Both mutants display increased levels of DNA damage markers (phospho-H2A and Rad52 foci), increased RPA foci, and activation of the DNA damage checkpoint, which indicates that this ongoing synthesis is abnormal and accompanied by DNA damage. Moreover, the phenotypes of *cds1Δ* and *mrc1Δ* are sufficiently different that we can distinguish their individual effects.

Previous studies showed that Cds1 limits ssDNA accumulation in part by restraining MCM activity while also blocking Mus81-dependent damage and preventing late origin firing (5, 14, 24, 32). Our data are consistent with this: in *cds1Δ* cells, we observe increased RPA accumulation that is MCM dependent, together with

FIG 5 RPA foci and DNA synthesis in *cds1Δ* cells is dependent on MCM helicase activity. (A) Experimental outline of RPA/Rad52 response to HU treatment with *hsk1ts* or *mcm4ts-dg* (FY4743, FY5286, FY5279, FY5277, FY5296, and FY5299). Cells were photographed asynchronously (AS) at 1 h of HU treatment (25°C) and then 3 h of HU treatment/2 h at 36°C. (Right) Examples of RPA and Rad52 focus categories scored at each time point. Multiple RPA or Rad52 foci were used as a metric for ssDNA and DNA damage induction. (B) Examples of RPA foci (cyan with transmitted light; RPA/BF) or Rad52 foci (right; yellow). Scale bar, 10 μm. (C) Induction of multiple RPA foci in *cds1Δ* backgrounds. Two independent experiments were combined for categorical analysis (proportion ± 95% CI; $n > 400$). RPA induction is the difference between foci from 1 h of HU treatment to 3 h of HU treatment/2 h at 36°C (± 95% CI). RPA accumulation was greater in *cds1Δ* and *cds1Δ hsk1ts* than *cds1Δ mcm4ts-dg* cells, which had more RPA foci at the start. The asterisk indicates a 1.29-fold increase in *cds1Δ mcm4ts-dg* RPA foci, which was significantly lower ($P < 0.05$) than the 1.8-fold increase in either *cds1Δ* or *cds1Δ hsk1ts* cells. (D) As in panel A, but EdU was added at 1 h of HU treatment at the time of temperature shift to 36°C. Samples were removed hourly for EdU-FACS during the time course. EdU signal is presented as a log scale, and a line in each sample marks the maximal incorporation in the wild type at 5 h. Shown is one representative time course. (E) Median EdU peak values from panel D were calculated and normalized to AS values and then plotted over time in HU at 36°C. An EdU-incorporated sample without Click reagent (no Click) is a negative control (obscured by the *mcm4dg cds1+* line). Both wild-type and *cds1Δ* cells incorporated EdU, while the *mcm4-dg* and *cds1Δ mcm4-dg* cells incorporated little EdU above the negative control.

Mus81-dependent phospho-H2A. However, we also see Mus81-independent DNA damage in *cds1Δ mus81Δ* cells that is sufficient to activate the Chk1 damage checkpoint. Thus, the breaks caused by Mus81 are not the only source of DNA damage in *cds1Δ* cells.

Strikingly, we found that *cds1Δ* cells continue DNA synthesis during and after HU block. This synthesis was not observed in previous studies that used bulk DNA FACS, possibly because concurrent ssDNA accumulation reduces the SytoxGreen signal. We found that replicated tracts of DNA elongate in the absence of late origin firing. Both ssDNA (measured by RPA) and DNA synthesis require MCM helicase activity, and this is accompanied by increased phospho-H2A after release. The coincident timing of DNA synthesis and RPA accumulation could indicate uncoupling between leading-strand (Polε) and lagging-strand (Polα/Polδ) synthesis. Uncoupling could allow Polα to reprime at the ssDNA gaps behind the helicase and promote DNA synthesis in ssDNA behind the replication fork. This model is consistent with longer tracts of new synthesis that we observed in *cds1Δ* cells in HU.

However, Polα-dependent synthesis is insufficient to fully replicate DNA in *cds1Δ* + HU, since Polα overexpression does not rescue viability (33).

After *cds1Δ* cells are released from HU treatment, we detect a brief burst of DNA synthesis. This increased rate of synthesis is not observed in *mrc1Δ* cells. We suggest that Mrc1 protein in *cds1Δ* cells still links the helicase to polymerase during HU treatment, which attenuates but does not block polymerase activity during HU exposure. This polymerase attenuation presumably operates through Polε-Mrc1 interactions on the leading strand, as in budding yeast (28). This repression of Polε-Mrc1 ends after HU is removed, which causes a burst of accelerated synthesis during the initial release period in *cds1Δ* cells and in the wild type.

Loss of Mrc1 destabilizes the replication fork (37, 58), slows fork movement (53), inhibits Cds1 activation (63, 64), promotes ssDNA accumulation (1, 59), and disrupts origin timing (17). *mrc1Δ* cells synthesize many short, asymmetric tracts of DNA during HU block, which is consistent with slower, unstable forks

FIG 6 Model of fork stabilization by Cds1 and Mrc1 preventing replication fork collapse. HU exposure activates Cds1 and Mrc1, leading to Pol ϵ inhibition. Active Cds1 restrains the MCM helicase, preventing excessive unwinding. Activated Cds1 additionally inhibits late origin firing and regulates Mus81 activity. A small amount of RPA accumulates at the wild-type stabilized forks, and induction of phospho-H2A and DNA damage is largely prevented. (Right, top) In *cds1* Δ cells, Mrc1 is present and restrains Pol ϵ while ssDNA accumulates. A leading-strand ssDNA loop is presumed to occur in *cds1* Δ cells but is omitted here for clarity. Mus81 nuclease is active, causing DSBs, yet additional damage signals accumulate in the absence of Mus81, suggesting that other nucleases contribute to DNA damage. Late origins fire during HU treatment, and active replisomes retain some synthesis ability for the first 0.5 h after HU release. (Right, bottom) In *mrc1* Δ cells, helicase and Pol ϵ are uncoupled, causing inefficient leading-strand synthesis. The rate of this synthesis is not changed with HU release, reflecting overall instability at *mrc1* Δ forks. The interaction between Mcl1, lagging-strand Pol α , and the helicase stabilizes the fork, but not sufficiently to avoid damage during HU treatment. Cds1 is not activated, and extensive ssDNA accumulates at the fork, in addition to DNA damage.

and progressive loss of replisome proteins at active forks during HU treatment (23, 28). There is little change in the rate of EdU incorporation following HU release, suggesting that in the absence of Mrc1, there is no mechanism to slow replication forks in HU. We propose that the short asymmetric tracts observed in *mrc1* Δ cells reflect Pol α inefficiencies in the absence of robust coupling to the leading strand. Pol α is attached to the helicase through Mcl1 (60), which may preserve replisome structure in *mrc1* Δ cells. Consistent with this, we have seen that *mrc1* Δ *mcl1* Δ double mutants are synthetically lethal (J.-P. Yuan and S. L. Forsburg, unpublished result).

Our data suggest that the DNA synthesis and ssDNA accumulation that we observe in *cds1* Δ and *mrc1* Δ cells both during HU block and after release reflect ongoing replication fork activity. An alternative model is that synthesis results from the inappropriate firing of late replication origins during HU treatment in *cds1* Δ cells (12, 18, 19, 24) and/or the firing of cryptic origins after release. However, our data suggest that unfired origins are not required to generate ssDNA or DNA synthesis in *cds1* Δ or *mrc1* Δ cells. First, RPA accumulates in the *cds1* Δ *hsk1ts* double mutant, which inactivates the origin-activating kinase Hsk1^{Cdc7}. Interestingly, *cds1* Δ *hsk1ts* cells have increased viability compared to *cds1* Δ cells in HU (see Fig. S5C in the supplemental material); perhaps, in this case, origin suppression in *cds1* Δ *hsk1ts* cells at 36°C preserves enough unfired origins to allow them to fire and complete S phase after release from HU and temperature.

We also examined DNA synthesis in strains with temperature-sensitive *orp1*^{ORC} to inactivate origin firing. By labeling with two different nucleoside analogues, we determined that tracts are extended after HU release in the absence of ORC activity. Thus, origin firing is not solely responsible for DNA synthesis or replication fork collapse in any genotype. However, the slight reduction of labeling in *mrc1* Δ *orp1ts* and *cds1* Δ *orp1ts* strains indicates that late origin firing contributes modestly to the extent of replication, allowing more forks to start and later fail. Interestingly, *cds1* Δ and *mrc1* Δ release tracts frequently show coincident signals

of block and release label. This may represent leading- and lagging-strand uncoupling. We also found that wild-type cells recover from HU treatment more quickly at 36°C with increased RPA foci. This is consistent with previous studies in fission yeast that indicate checkpoints are temperature sensitive (16, 21).

Distinguishing arrest from collapse: defining the fork collapse point. If DNA synthesis is ongoing during HU treatment and after release, when do replication forks collapse? We observe continued accumulation of RPA and Rad52 in *mrc1* Δ and *cds1* Δ cells released from HU treatment, suggesting that unwinding and DNA damage continue into the attempted recovery phase. During the initial 0.5 h after HU release, both *cds1* Δ and *mrc1* Δ cells incorporate amounts of nuclear-specific label similar to those of the wild type, only to slow synthesis by 0.75 h postrelease. Live-cell analysis showed transient accumulation of RPA, followed by Rad52, in wild-type cells at 0.5 h post-HU treatment. We suggest this represents a period of fork repair and recovery that may also depend on Mus81 (7, 14, 22), Exo1 (34, 50) or helicases, such as Rqh1 (31, 61). Exonucleases are controlled by activated Cds1 (7, 22, 61); thus, inappropriate resection and cleavage may contribute to HU-dependent DNA synthesis and damage. We propose that loss of synthesis, disrupted fork structure, ssDNA accumulation, and induction of DNA damage define a fork “collapse point,” at which time DNA damage and instability at individual forks impede further replication. Based on our observations, this execution point occurs during release in *cds1* Δ and *mrc1* Δ cells.

Our findings are in agreement with newly published work on budding yeast, which shows that HU alters origin firing in *rad53* Δ mutants and promotes additional synthesis despite low deoxynucleoside triphosphate (dNTP) levels (45). The concept that replication proceeds in HU at a glacial pace was previously demonstrated in *Saccharomyces cerevisiae* (3) and is consistent with an inability of checkpoint mutants to sufficiently slow or arrest fork activity under depleted-dNTP conditions. Replisome proteins migrate farther away from origins in *rad53* and *mec1* mutant cells, demonstrating that helicase and polymerase are retained in bud-

ding yeast replication checkpoint mutants and are able to migrate away from origins during HU stress (11). Consistent with the results of De Piccoli et al. (11), we saw tract elongation in fission yeast checkpoint mutants, suggesting polymerase components are retained.

Our data suggest that replication fork arrest and collapse are separated both temporally and spatially and that DNA unwinding and damage accompany both events. In checkpoint mutants, the final moments of replication fork collapse are extended into the time of HU release. We propose that replication fork collapse in *cds1Δ* and *mrc1Δ* cells occurs progressively, with a final loss of replication capacity during attempted recovery. A key question is whether cell death is caused by unwinding and DNA synthesis occurring during HU treatment or during an abortive attempt to rescue damaged replication forks. Future studies will further define the symptoms and timing of replication fork collapse.

ACKNOWLEDGMENTS

We thank JiPing Yuan for technical assistance, Forsburg laboratory members for beneficial discussion, and anonymous reviewers who made helpful suggestions during the review process. We thank Beth Sullivan for assistance with DNA fiber methods, Toshio Tsukiyama for helpful discussion, and Johanne Murray, Oscar Aparicio, and Matthew Michael for critical reading of the manuscript.

This work was supported by grant NIH R01 GM059321 to S.L.F.

REFERENCES

- Alabert C, Bianco JN, Pasero P. 2009. Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint. *EMBO J.* 28:1131–1141.
- Alcasabas AA, et al. 2001. Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat. Cell Biol.* 3:958–965.
- Alvino GM, et al. 2007. Replication in hydroxyurea: it's a matter of time. *Mol. Cell. Biol.* 27:6396–6406.
- Aparicio OM, Weinstein DM, Bell SP. 1997. Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. *Cell* 91:59–69.
- Bailis JM, Luche DD, Hunter T, Forsburg SL. 2008. Minichromosome maintenance proteins interact with checkpoint and recombination proteins to promote S-phase genome stability. *Mol. Cell. Biol.* 28:1724–1738.
- Boddy MN, Furnari B, Mondesert O, Russell P. 1998. Replication checkpoint enforced by kinases Cds1 and Chk1. *Science* 280:909–912.
- Boddy MN, et al. 2000. Damage tolerance protein Mus81 associates with the FHA1 domain of checkpoint kinase Cds1. *Mol. Cell. Biol.* 20:8758–8766.
- Brondello JM, Boddy MN, Furnari B, Russell P. 1999. Basis for the checkpoint signal specificity that regulates Chk1 and Cds1 protein kinases. *Mol. Cell. Biol.* 19:4262–4269.
- Byun TS, Pacek M, Yee MC, Walter JC, Cimprich KA. 2005. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev.* 19:1040–1052.
- Cobb JA, et al. 2005. Replisome instability, fork collapse, and gross chromosomal rearrangements arise synergistically from Mec1 kinase and RecQ helicase mutations. *Genes Dev.* 19:3055–3069.
- De Piccoli G, et al. 2012. Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases. *Mol. Cell* 45:696–704.
- Feng W, et al. 2006. Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. *Nat. Cell Biol.* 8:148–155.
- Feng W, Di Rienzi SC, Raghuraman MK, Brewer BJ. 2011. Replication stress-induced chromosome breakage is correlated with replication fork progression and is preceded by single-stranded DNA formation. *G3 (Bethesda)* 1:327–335.
- Froget B, Blaisonneau J, Lambert S, Baldacci G. 2008. Cleavage of stalled forks by fission yeast Mus81/Eme1 in absence of DNA replication checkpoint. *Mol. Biol. Cell* 19:445–456.
- Green MD, Sabatinos SA, Forsburg SL. 2009. Microscopy techniques to examine DNA replication in fission yeast. *Methods Mol. Biol.* 521:463–482.
- Grenon M, Tillit J, Piard K, Baldacci G, Francesconi S. 1999. The S/M checkpoint at 37 degrees C and the recovery of viability of the mutant *poldeltats3* require the *crb2+/rhp9+* gene in fission yeast. *Mol. Genet.* 260:522–534.
- Hayano M, Kanoh Y, Matsumoto S, Masai H. 2011. Mrc1 marks early-firing origins and coordinates timing and efficiency of initiation in fission yeast. *Mol. Cell. Biol.* 31:2380–2391.
- Hayashi M, et al. 2007. Genome-wide localization of pre-RC sites and identification of replication origins in fission yeast. *EMBO J.* 26:1327–1339.
- Heichinger C, Penkett CJ, Bahler J, Nurse P. 2006. Genome-wide characterization of fission yeast DNA replication origins. *EMBO J.* 25:5171–5179.
- Jackson DA, Pombo A. 1998. Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J. Cell Biol.* 140:1285–1295.
- Janes S, et al. 13 July 2012. Heat induction of a novel Rad9 variant from a cryptic translation initiation site reduces mitotic commitment. *J. Cell Sci.* [Epub ahead of print.] doi:10.1242/jcs.104075.
- Kai M, Boddy MN, Russell P, Wang TS. 2005. Replication checkpoint kinase Cds1 regulates Mus81 to preserve genome integrity during replication stress. *Genes Dev.* 19:919–932.
- Katou Y, et al. 2003. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* 424:1078–1083.
- Kim SM, Huberman JA. 2001. Regulation of replication timing in fission yeast. *EMBO J.* 20:6115–6126.
- Koc A, Wheeler LJ, Mathews CK, Merrill GF. 2004. Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. *J. Biol. Chem.* 279:223–230.
- Lindsay HD, et al. 1998. S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe*. *Genes Dev.* 12:382–395.
- Lopes M, et al. 2001. The DNA replication checkpoint response stabilizes stalled replication forks. *Nature* 412:557–561.
- Lou H, et al. 2008. Mrc1 and DNA polymerase epsilon function together in linking DNA replication and the S phase checkpoint. *Mol. Cell* 32:106–117.
- Lucca C, et al. 2004. Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing. *Oncogene* 23:1206–1213.
- Meister P, et al. 2005. Temporal separation of replication and recombination requires the intra-S checkpoint. *J. Cell Biol.* 168:537–544.
- Miyabe I, Morishita T, Hishida T, Yonei S, Shinagawa H. 2006. Rhp51-dependent recombination intermediates that do not generate checkpoint signal are accumulated in *Schizosaccharomyces pombe* rad60 and smc5/6 mutants after release from replication arrest. *Mol. Cell. Biol.* 26:343–353.
- Miyabe I, Morishita T, Shinagawa H, Carr AM. 2009. *Schizosaccharomyces pombe* Cds1Chk2 regulates homologous recombination at stalled replication forks through the phosphorylation of recombination protein Rad60. *J. Cell Sci.* 122:3638–3643.
- Murakami H, Okayama H. 1995. A kinase from fission yeast responsible for blocking mitosis in S phase. *Nature* 374:817–819.
- Nakada D, Hirano Y, Sugimoto K. 2004. Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway. *Mol. Cell. Biol.* 24:10016–10025.
- Nakamura TM, Du LL, Redon C, Russell P. 2004. Histone H2A phosphorylation controls Crb2 recruitment at DNA breaks, maintains checkpoint arrest, and influences DNA repair in fission yeast. *Mol. Cell. Biol.* 24:6215–6230.
- Namiki Y, Zou L. 2006. ATRIP associates with replication protein A-coated ssDNA through multiple interactions. *Proc. Natl. Acad. Sci. U. S. A.* 103:580–585.
- Nedelcheva MN, et al. 2005. Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. *J. Mol. Biol.* 347:509–521.
- Nestoras K, et al. 2010. Regulation of ribonucleotide reductase by Spd1 involves multiple mechanisms. *Genes Dev.* 24:1145–1159.
- Nitani N, Yadani C, Yabuuchi H, Masukata H, Nakagawa T. 2008. Mcm4 C-terminal domain of MCM helicase prevents excessive formation

- of single-stranded DNA at stalled replication forks. *Proc. Natl. Acad. Sci. U. S. A.* 105:12973–12978.
40. Noguchi E, Noguchi C, Du LL, Russell P. 2003. Swi1 prevents replication fork collapse and controls checkpoint kinase Cds1. *Mol. Cell. Biol.* 23:7861–7874.
 41. Osborn AJ, Elledge SJ. 2003. Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. *Genes Dev.* 17:1755–1767.
 42. Pacek M, Tutter AV, Kubota Y, Takisawa H, Walter JC. 2006. Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. *Mol. Cell* 21:581–587.
 43. Pacek M, Walter JC. 2004. A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. *EMBO J.* 23:3667–3676.
 44. Patel PK, Arcangioli B, Baker SP, Bensimon A, Rhind N. 2006. DNA replication origins fire stochastically in fission yeast. *Mol. Biol. Cell* 17:308–316.
 45. Poli J, et al. 2012. dNTP pools determine fork progression and origin usage under replication stress. *EMBO J.* 31:883–894.
 46. Rasband, W. S. 2011. ImageJ. National Institutes of Health, Bethesda, MD. <http://imagej.nih.gov/ij/>.
 47. Robert T, Dervins D, Fabre F, Gangloff S. 2006. Mrc1 and Srs2 are major actors in the regulation of spontaneous crossover. *EMBO J.* 25:2837–2846.
 48. Sabatinos SA, Forsburg SL. 2010. Molecular genetics of *Schizosaccharomyces pombe*. *Methods Enzymol.* 470:759–795.
 49. Santocanale C, Diffley JF. 1998. A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature* 395:615–618.
 50. Segurado M, Diffley JF. 2008. Separate roles for the DNA damage checkpoint protein kinases in stabilizing DNA replication forks. *Genes Dev.* 22:1816–1827.
 51. Snaith HA, Brown GW, Forsburg SL. 2000. *Schizosaccharomyces pombe* Hsk1p is a potential cds1p target required for genome integrity. *Mol. Cell. Biol.* 20:7922–7932.
 52. Sogo JM, Lopes M, Foiani M. 2002. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science* 297:599–602.
 53. Szyjka SJ, Viggiani CJ, Aparicio OM. 2005. Mrc1 is required for normal progression of replication forks throughout chromatin in *S. cerevisiae*. *Mol. Cell* 19:691–697.
 54. Takeda T, et al. 2001. Regulation of initiation of S phase, replication checkpoint signaling, and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. *Mol. Biol. Cell* 12:1257–1274.
 55. Tanaka K, Boddy MN, Chen XB, McGowan CH, Russell P. 2001. Threonine-11, phosphorylated by Rad3 and atm in vitro, is required for activation of fission yeast checkpoint kinase Cds1. *Mol. Cell. Biol.* 21:3398–3404.
 56. Tanaka K, Russell P. 2004. Cds1 phosphorylation by Rad3-Rad26 kinase is mediated by forkhead-associated domain interaction with Mrc1. *J. Biol. Chem.* 279:32079–32086.
 57. Tanaka K, Russell P. 2001. Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1. *Nat. Cell Biol.* 3:966–972.
 58. Tanaka T, et al. 2010. Fission yeast Swi1-Swi3 complex facilitates DNA binding of Mrc1. *J. Biol. Chem.* 285:39609–39622.
 59. Tsolou A, Lydall D. 2007. Mrc1 protects uncapped budding yeast telomeres from exonuclease EXO1. *DNA Repair* 6:1607–1617.
 60. Williams DR, McIntosh JR. 2005. Mcl1p is a polymerase alpha replication accessory factor important for S-phase DNA damage survival. *Eukaryot. Cell* 4:166–177.
 61. Willis N, Rhind N. 2009. Mus81, Rhp51(Rad51), and Rqh1 form an epistatic pathway required for the S-phase DNA damage checkpoint. *Mol. Biol. Cell* 20:819–833.
 62. Wu PY, Nurse P. 2009. Establishing the program of origin firing during S phase in fission yeast. *Cell* 136:852–864.
 63. Xu YJ, Davenport M, Kelly TJ. 2006. Two-stage mechanism for activation of the DNA replication checkpoint kinase Cds1 in fission yeast. *Genes Dev.* 20:990–1003.
 64. Xu YJ, Kelly TJ. 2009. Autoinhibition and autoactivation of the DNA replication checkpoint kinase Cds1. *J. Biol. Chem.* 284:16016–16027.
 65. Zeng Y, et al. 1998. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. *Nature* 395:507–510.
 66. Zou L, Elledge SJ. 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 300:1542–1548.