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Mrc1 and Tof1 Promote Replication Fork Progression and Recovery Independently of Rad53

Short Article

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Summary

The yeast checkpoint factors Mrc1p and Tof1p travel with the replication fork and mediate the activation of the Rad53p kinase in response to a replication stress. We show here that both proteins are required for normal fork progression but play different roles at stalled forks. Tof1p is critical for the activity of the rDNA replication fork barrier (RFB) but plays a minor role in the replication checkpoint. In contrast, Mrc1p is not necessary for RFB activity but is essential to mediate the replication stress response. Interestingly, stalled forks did not collapse in *mrc1Δ* cells exposed to hydroxyurea (HU) as they do in *rad53* mutants. However, forks failed to restart when *mrc1Δ* cells were released from the block. The critical role of Mrc1p in HU is therefore to promote fork recovery in a Rad53p-independent manner, presumably through the formation of a stable fork-pausing complex.

Introduction

The S phase of the cell cycle is a period of high vulnerability for the genome of eukaryotic cells. Indeed, replication forks frequently stall when they encounter obstacles such as DNA lesions or tightly bound protein complexes and generate toxic recombination intermediates if they are not readily stabilized and restarted. To suppress this genomic instability, cells have developed complex surveillance mechanisms involving sensor proteins, checkpoint kinases, and specialized DNA helicases (Cobb et al., 2004; Nyberg et al., 2002; Rothstein et al., 2000). In *S. cerevisiae*, two protein kinases play a central role in the replication stress response. The sensor kinase Mec1p (ATR in human) detects single-stranded DNA at stalled forks together with Ddc2p and phosphorylates the effector kinase Rad53p (Chk2). Mec1p also activates specific adaptor proteins whose role is to mediate the autoactivation of Rad53p and therefore to amplify the checkpoint response (Nyberg et al., 2002).

One of the best-characterized adaptor proteins is Rad9p, which acts as a scaffold to promote the autoactivation of Rad53p in the DNA damage checkpoint (Gilbert et al., 2001). In response to a replication stress, Rad9p is replaced by an S phase-specific mediator called Mrc1p in yeast (Alcasabas et al., 2001; Tanaka and Russell, 2001) and CLASPIN in vertebrates (Kumagai and Dunphy, 2000). Additional candidates include Tof1p and Csm3p in *S. cerevisiae* (Foss, 2001; Tong et al., 2004) and Swi1p and Swi3p in *S. pombe* (Dalgaard and Klar, 2001; Noguchi et al., 2004).

In a normal S phase, Mrc1p and Tof1p are loaded on DNA shortly after initiation and travel with the replication fork (Katou et al., 2003). They are therefore in a unique position to detect replication problems. Interestingly, *mrc1Δ* cells progress slowly through S phase and accumulate spontaneous DNA damage (Alcasabas et al., 2001). These defects are independent of the checkpoint function of Mrc1p, as the checkpoint-deficient mutant *mrc1^{AQ}* progresses normally through S phase (Osborn and Elledge, 2003). Interestingly, the viability of *mrc1Δ* cells depends on Rrm3p, a DNA helicase promoting fork progression through natural pause sites (Torres et al., 2004). In fission yeast, the Tof1p homolog Swi1p also acts as sensor of natural pause sites (Codlin and Dalgaard, 2003; Krings and Bastia, 2004; Lambert et al., 2005; Noguchi et al., 2004). These data suggest that Mrc1p and Tof1p are required for the replication of specific regions of the genome. However, the role of Tof1p in S phase progression has not been addressed yet.

In the presence of a replication stress, Mrc1p and Tof1p mediate the amplification of the replication checkpoint cascade and regulate several downstream events, including the firing of late origins (Nyberg et al., 2002). Recent evidence indicates that the only crucial function of the replication checkpoint is the stabilization of arrested forks (Tercero et al., 2003). However, the exact role of the Mrc1p and Tof1p in this process has remained unclear. Indeed, although Rad53p is essential to prevent fork collapse (Lopes et al., 2001), its activation is dispensable to prevent the disassembly of DNA polymerases (Cobb et al., 2003; Katou et al., 2003). Moreover, Mrc1p and Tof1p contribute to the formation of a stable pausing complex independently of Rad53p (Katou et al., 2003). Finally, the fact that *mrc1Δ* and *tof1Δ* cells are not nearly as sensitive as *rad53* mutants to genotoxic drugs suggests that Rad53p plays a critical role at stalled forks that is independent of Mrc1p and Tof1p.

Here, we have used a single-molecule approach and two-dimensional (2D) gel electrophoresis to investigate the role of Mrc1p and Tof1p in fork progression, pausing, and restart. We show that both proteins are required for normal fork progression but are differentially required for recovery after a replication stress.

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Results and Discussion

Mrc1p and Tof1p Are Required for Normal S Phase Progression

Previous work has shown that Mrc1p is required for efficient DNA replication in unchallenged growth conditions (Alcasabas et al., 2001). However, the role of Tof1p in this process has not been addressed. Here, we have used pulsed-field gel electrophoresis (PFGE) to compare the kinetics of S phase progression in wild-type (wt), *mrc1Δ*, and *tof1Δ* cells (Versini et al., 2003). Cells were released into S phase from a G_1 arrest, and BrdU incorporation in fully replicated chromosomes was quantitated after electrophoretic separation on gel. Wt cells completed S phase 20 and 15 min earlier than *mrc1Δ* and *tof1Δ* cells, respectively (Figures 1A and 1B). Interestingly, we also observed an accumulation of Ddc1- and Rad52-GFP foci in *mrc1Δ* and *tof1Δ* cells (C.A., unpublished data), which indicates that DNA damage accumulates spontaneously in *tof1Δ* cells, as it does in *mrc1Δ* mutants (Alcasabas et al., 2001). We therefore conclude that Mrc1p and Tof1p are both required for normal DNA replication in unchallenged growth conditions.

A longer S phase could either reflect a lower frequency of initiation or a slower rate of fork progression. To discriminate between these two possibilities, we first measured the rate of initiation at the level of individual chromosomes by DNA combing (Michalet et al., 1997).

The Prolonged S Phase of *mrc1Δ* and *tof1Δ* Cells Is Not Due to Inefficient Initiation

Wt, *mrc1Δ*, and *tof1Δ* cells were released synchronously into S phase in the presence of BrdU to label active origins, and elongation was blocked with HU. DNA fibers were purified and stretched on silanized coverslips as described (Pasero et al., 2002). Initiation sites were detected with an anti-BrdU antibody, and DNA fibers were counterstained with an anti-guanosine antibody (Figure 1C). Statistical analysis revealed that the mean distance between active origins is significantly shorter in *mrc1Δ* cells and to a lesser extent in *tof1Δ* mutants than in wt cells (Figure 1D). We assume that the higher rate of initiation in these mutants reflects the derepression of late origins (Katou et al., 2003), as it was not observed in the absence of HU (Figure S1A available in the Supplemental Data with this article online). The slow S phase in *mrc1Δ* and *tof1Δ* cells is therefore not due to a general initiation defect.

Fork Progression Is Altered in the Absence of Mrc1p and Tof1p

We next asked whether the longer S phase in *mrc1Δ* and *tof1Δ* cells reflects a slower progression of replication forks. Early log-phase cultures were pulse labeled with BrdU, and the length of newly replicated tracks was measured by DNA combing (Versini et al., 2003). We found that BrdU tracks were ~40% longer in wt (29.3 kb ± 13.9) than in *mrc1Δ* (21.0 kb ± 7.9) and *tof1Δ* (20.2 kb ± 8.0) cells (Figure 1E). These data suggest that elongation is impaired in *mrc1Δ* and *tof1Δ* cells. Alternatively, this slow replication could reflect an extended pausing at natural pause sites (Ivessa et al., 2003). To discriminate between these two possibilities, short BrdU pulses were used in order to label BrdU tracks that would be shorter than the average distance between natural pause sites (~25 kb; [Ivessa et al., 2003]). Yet, newly replicated segments remained 30% longer in wt cells (7.8 kb ± 3.1) than in *mrc1Δ* cells (5.9 kb ± 2.8; Figure S1B) in these conditions. These data, together with unpublished results from the Shirahige (personal communication) and Aparicio (Szyjka et al., 2005) labs, suggest that forks do not accumulate at programmed pause sites in *mrc1Δ* cells but are intrinsically slower. This slow replication is independent of the checkpoint function of Mrc1p, as forks move at a normal rate in *rad53* cells (Versini et al., 2003), and S phase is normal in the checkpoint-deficient mutant $mrc1^{AQ}$ (Osborn and Elledge, 2003).

Mrc1p and Tof1p Are Dispensable to Maintain the Integrity of Paused Forks at the rDNA

Although programmed pause sites do not affect the rate of elongation in *mrc1Δ* cells, they could induce fork collapse and chromosome breaks, especially when Rrm3p is absent (Alcasabas et al., 2001; Katou et al., 2003; Torres et al., 2004). Hence, forks arrested at the RFB of the ribosomal DNA frequently break and generate extrachromosomal rDNA circles (ERCs) by homologous recombination (Brewer and Fangman, 1988; Rothstein et al., 2000). ERC species accumulate in mutants with increased fork instability, such as *sgs1Δ*, *srs2Δ*, *dna2Δ*, or *rrm3Δ* (Ivessa et al., 2000; Versini et al., 2003; Weitao et al., 2003). To test whether this is also the case for *mrc1Δ* and *tof1Δ* cells, we monitored in parallel ERC levels and RFB activity. To this aim, cells were synchronized in S phase (Figure S2C), and genomic DNA was prepared in agarose plugs to avoid shearing. 2D gel analyses revealed a 2-fold increase of RFB signal in *mrc1Δ* cells (Figure 2C). This increased pausing, which was also observed in asynchronous cultures (Figure S2D), is independent of the checkpoint function of Mrc1p (Figure S2E). Interestingly, this increased RFB activity was accompanied with a significant reduction of ERCs (Figures 2D and 2E). In *tof1Δ* cells, we measured a 4-fold reduction of the RFB signal (Figure 2C), which is reminiscent of the phenotype of *S. pombe swi1Δ* mutants (Krings and Bastia, 2004). We also detected a sharp reduction of ERC levels (Figures 2D and 2E). Taken together, these data indicate that (1) fork stalling at the rDNA RFB is an active process that depends on Tof1p, but not on Mrc1p, and (2) neither Mrc1p nor Tof1p are required to promote fork integrity at the rDNA. Because *mrc1Δ* and *tof1Δ* mutants have been reported to be sensitive to replication stress induced by genotoxic drugs (Alcasabas et al., 2001; Foss, 2001), we next asked whether Mrc1p and Tof1p are required to maintain or restart arrested forks in the presence of HU or methylmethane sulfonate (MMS).

Mrc1p and Tof1p Are Dispensable to Prevent Fork Collapse in HU

We first used 2D gel electrophoresis to monitor the integrity of stalled forks in cells exposed to HU. As indi-

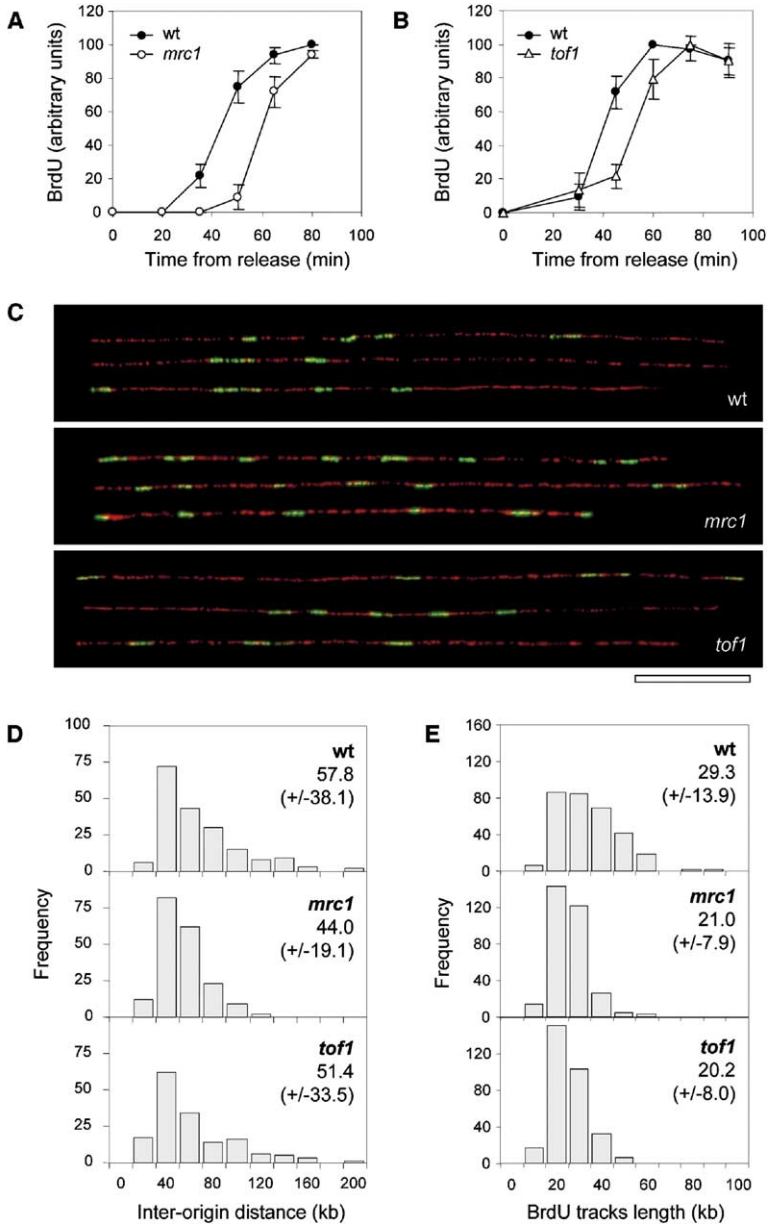

Figure 1. The Prolonged S Phase of *mrc1Δ* and *tof1Δ* Cells Is Due to Slower Forks

(A) PFGE analysis of S phase kinetics in wild-type (wt) (PP108) and *mrc1Δ* (PP374) cells. Cells were arrested in G_1 with α factor and released into S phase in the presence of 0.4 mg/ml BrdU. Samples were collected at the indicated times after release, and chromosomes were sorted by PFGE. The amount of BrdU incorporated in fully replicated chromosomes was quantitated as described (Versini et al., 2003).

(B) Kinetics of completion of DNA replication in wt (PP108) and *tof1Δ* (PP320) cells.

(C) Single-molecule analysis of the rate of initiation. Cells were released synchronously into S phase in the presence of 0.4 mg/ml BrdU and 200 mM HU. Chromosomes were purified 90 min after release from G_1 and were stretched by DNA combing. Green, anti-BrdU antibody; red, anti-guanosine antibody. Bar, 50 kb.

(D) Center-to-center distances between adjacent BrdU tracks. Average distances and standard deviations are indicated in kb.

(E) Distribution of BrdU tracks length. Exponentially growing cells were pulse labeled for 35 min with 0.8 mg/ml BrdU, and the length of BrdU tracks was measured as described above.

cated by the presence of a strong bubble arc (arrow), the early origins *ARS305* and *ARS315* fired efficiently in wt cells (Figure 3A). In contrast, initiation at the late origin *ARS1212* was repressed by the replication checkpoint (Santocanale and Diffley, 1998). In *rad53-11* cells, both early and late origins fired and forks collapsed, as indicated by the decrease of bubbles (arrow) and large Ys (asterisk) at *ARS305* and *ARS315* (Figures 3A and 3C). It has been recently reported that the replication checkpoint is also required to maintain X-shaped molecules, which form shortly after initiation and are unstable in *rad53* mutants exposed to HU (Benard et al., 2001; Lopes et al., 2003). Because these structures are sensitive to classical DNA extraction procedures, genomic DNA was prepared in agarose plugs to prevent branch migration. By using this procedure, we detected X-shaped molecules at *ARS315* in wt cells exposed to HU, but as expected, these intermediates were lost in *rad53-11* mutants (Figure 3B, arrow). In contrast to *rad53* mutants, levels of bubbles, large Ys, and joint molecules were only modestly affected in HU-arrested *mrc1Δ* and *tof1Δ* cells (Figures 3A–3D). Bubbles and large Ys were also maintained in HU in the checkpoint-deficient mutant *mrc1^{AQ}* (Figure S3A). Taken together, these data indicate that Mrc1p and Tof1p are dispensable for the maintenance of stalled forks. Moreover, we found that *tof1Δ* mutants were at least partially proficient to activate the replication checkpoint. Indeed, unlike *mrc1Δ* cells, only 20% of *tof1Δ* cells activated the late origin *ARS1212* in the presence of HU (Figure 3A)

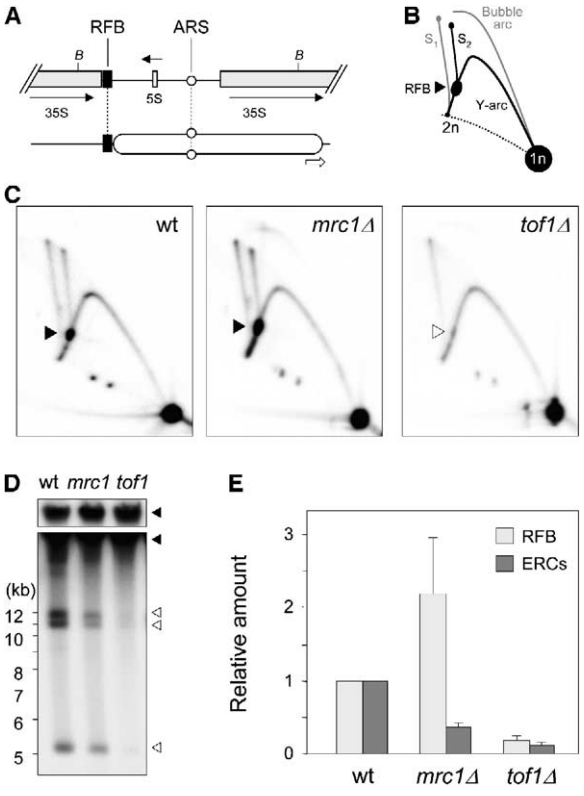

Figure 2. Mrc1p and Tof1p Have Different Roles at the rDNA Replication Fork Barrier

(A) The replication fork barrier (RFB) is a polar structure arresting forks moving in the direction opposite to transcription of 35S genes. B, BclI.

(B) Expected position of bubble arc, Y arc, X molecules (S_1), and converging forks (S_2) after 2D gel analysis of a 4.5 kb BclI fragment centered on the origin (ARS). See Figure 3B for the interpretation of the S_1 spike.

(C) 2D gel analysis of RFB activity (arrowhead) in wt (PP108), *mrc1Δ* (PP374), and *tof1Δ* (PP320) cells synchronized in early S phase.

(D) ERC species (empty arrowhead) in young, unsorted cells. Solid arrowhead, chromosomal rDNA (shorter exposure).

(E) Relative amount of arrested forks and ERC levels in wt, *mrc1Δ*, and *tof1Δ* cells. Error bars correspond to four independent experiments, with synchronous and asynchronous cultures.

or MMS (Figure S4A). This is consistent with the fact that *tof1Δ* cells activate more origins in HU than wt cells but significantly less than *mrc1Δ* mutants (Figure 1D). We therefore assume that Mrc1p is the major mediator of the replication checkpoint, with Tof1p playing only an indirect role in this process, presumably through the recruitment of Mrc1p at replication forks (Katou et al., 2003).

Mrc1p Is Required to Complete DNA Replication after Exposure to HU, but Not to MMS

Although stalled forks do not collapse in *mrc1Δ* and *tof1Δ* cells exposed to genotoxic drugs, they could lose their ability to resume replication when the block is relieved. To address this possibility, *mrc1Δ* and *tof1Δ* cells were released from HU or MMS, and completion of S phase was monitored by PFGE (Desany et al., 1998). Both *mrc1Δ* and *tof1Δ* cells recovered from MMS with normal kinetics (Figure S4B), whereas only *mrc1Δ* cells failed to recover efficiently from the HU arrest (Figure 3E). This is consistent with the fact that *mrc1Δ* cells are more sensitive to HU than *tof1Δ* cells (Figure S3B). To check whether this slow recovery reflects the inability of *mrc1Δ* cells to resume replication, fork restart was monitored at the level of individual chromosomes by DNA combing.

Forks Exposed to HU in the Absence of Mrc1p Are Unable to Resume DNA Synthesis

Wt and *mrc1Δ* cells were released synchronously in a medium containing HU and chlorodeoxyuridine (CldU) to label early origins. Cells were washed and resuspended in fresh medium containing iododeoxyuridine (IdU), and DNA fibers were analyzed after DNA combing. Specific antibodies were used to discriminate between DNA synthesis occurring before (green) or after (red) release from the block. CldU could not be completely eliminated from internal nucleotide pools and was still incorporated after release. Two adjacent IdU tracks overlapping a single CldU track correspond therefore to recovery from an origin activated before the HU arrest, and overlapping CldU and IdU tracks are new origins activated after the HU block (Figure 4A). Analysis of fork recovery 50 min after release revealed that most of the forks resumed replication synchronously in wt cells. Indeed, 85% of the replication bubbles showed less than a 25% difference between the distances covered by right-moving and left-moving forks after recovery (Figure 4B). In contrast, replicated segments appeared to be shorter and more heterogeneous in length in *mrc1Δ* cells (53.8% of outliers; Figure 4B). After 90 min postrelease, chromosomes from wt cells were fully labeled (Figure 4E), whereas *mrc1Δ* cells displayed partially replicated chromosomes and asymmetric forks (59.1% of outliers; Figures 4C–4E). The slow recovery of *mrc1Δ* cells transiently exposed to HU is therefore a reflection of their inability to reactivate a large fraction of their replication forks. This defect is specific to HU-arrested forks, as it was not detected after release from an MMS arrest (Figures S4B and S4C).

Interestingly, *tof1Δ* cells also showed defects in fork recovery, as illustrated by the persistence of unreplicated gaps 90 min after release from HU (Figure 4E). However, *tof1Δ* cells recovered more efficiently from HU than *mrc1Δ* mutants, owing to their ability to preserve most of their late-firing origins. These origins were used to complete replication after release from HU, as illustrated by the presence of large uninterrupted IdU tracks in wt and *tof1Δ* cells, but not in *mrc1Δ* mutants (Figures 4E and 4F). Tof1-deficient cells would therefore compensate their fork defect by their ability to activate late origins after release from the HU block.

Overview

We report here that the checkpoint mediators Mrc1p and Tof1p play multiple roles at the replication fork that are independent of Rad53p activation. We have used

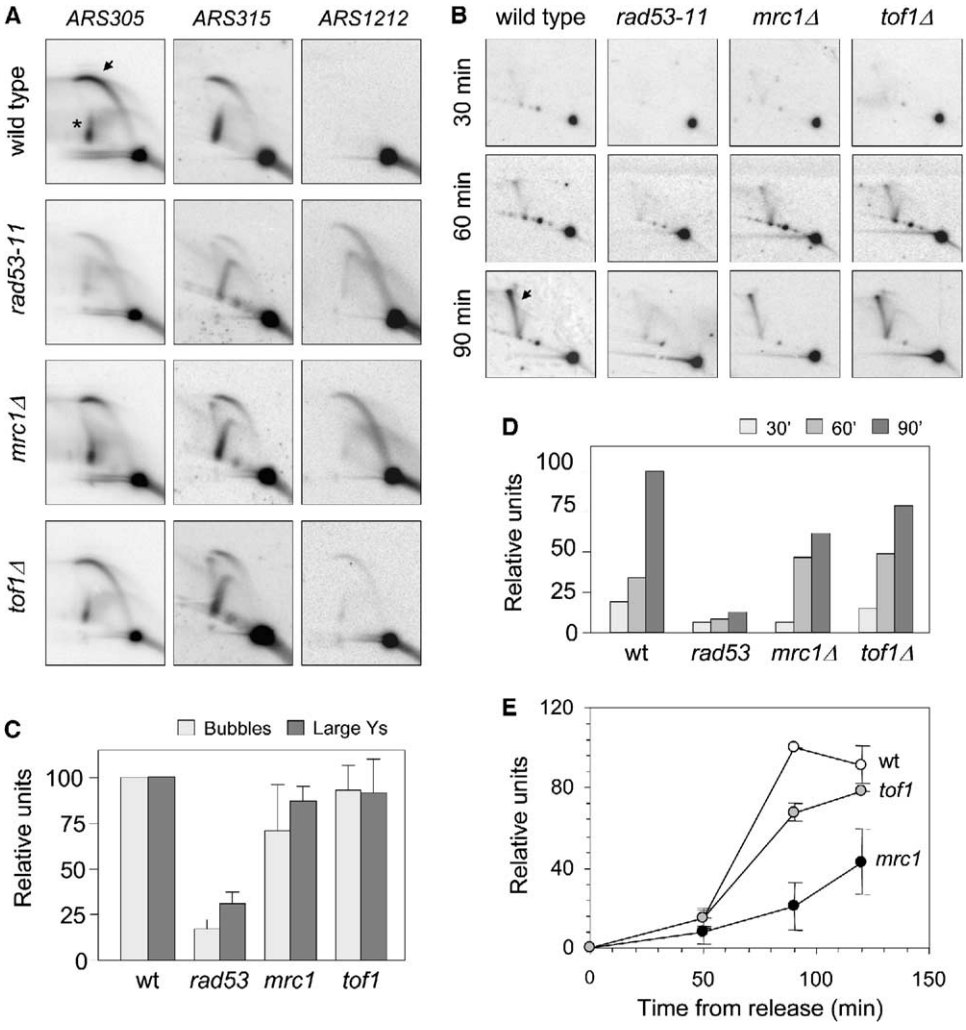

Figure 3. 2D Gel Analysis of Replication Intermediates in Cells Exposed to HU

(A) Wt (PP108), *rad53-11* (PP037), *mrc1Δ* (PP374), and *tof1Δ* (PP320) cells were arrested in G_1 with α factor and were released in a medium containing 200 mM HU for 60, 75, or 90 min. Samples were pooled and analyzed by 2D gel electrophoresis. Replication of EcoRI fragments encompassing the early origins *ARS305* and *ARS315* and the late origin *ARS1212* are shown. Arrow, bubble arc; Asterisk, large Y arc.

(B) 2D gel analysis of joint molecules (arrow) at *ARS315* in wt, *rad53-11*, *mrc1Δ*, and *tof1Δ* cells exposed to HU for 30, 60, or 90 min. DNA samples were prepared in agarose plugs to prevent branch migration.

(C) Quantitation of the relative intensity of bubbles and large Ys at *ARS305* in wt, *rad53-11*, *mrc1Δ*, and *tof1Δ* cells exposed to 200 mM HU (three experiments).

(D) Quantitation of the relative intensity of joint molecules shown in (B).

(E) PFGE analysis of recovery from HU. Wt, *mrc1Δ*, and *tof1Δ* cells were arrested in G_1 with α factor and were released for 90 min in S phase in the presence of 200 mM HU. Cells were washed and resuspended in fresh medium containing 0.4 mg/ml BrdU. Samples were collected at the indicated times after release, and genomic DNA was separated by PFGE. Completion of DNA replication was determined by quantitating BrdU incorporation in fully replicated chromosomes. Error bars correspond to the average of five representative chromosomes.

DNA combing to show that both proteins are required for normal fork progression. Moreover, data from our lab and others indicate that Tof1p contributes to fork arrest at the rDNA RFB while Mrc1p and Tof1p are required for pausing at tRNA genes (K. Shirahige, personal communication; Calzada et al., 2005). Whether unprotected fork pausing at these loci induces DNA damage in *mrc1Δ* and *tof1Δ* mutants is currently unknown. However, our results indicate that genomic instability does not increase at the rDNA in the absence of Mrc1p or Tof1p.

In the presence of genotoxic drugs, we show that the replication stress response is mediated primarily by Mrc1p and not by Tof1p. More surprisingly, 2D gel analyses revealed stalled forks do not collapse in *mrc1Δ* and *mrc1^{AQ}* mutants exposed to HU, as they do in *rad53-11* cells. The Mrc1p-dependent hyperphosphorylation of Rad53p is therefore dispensable for the maintenance of fork integrity, which is the most critical function of the replication checkpoint (Tercero et al., 2003). The fact that Mec1p is sufficient to activate Rad53p in the absence of Mrc1p raises the question of

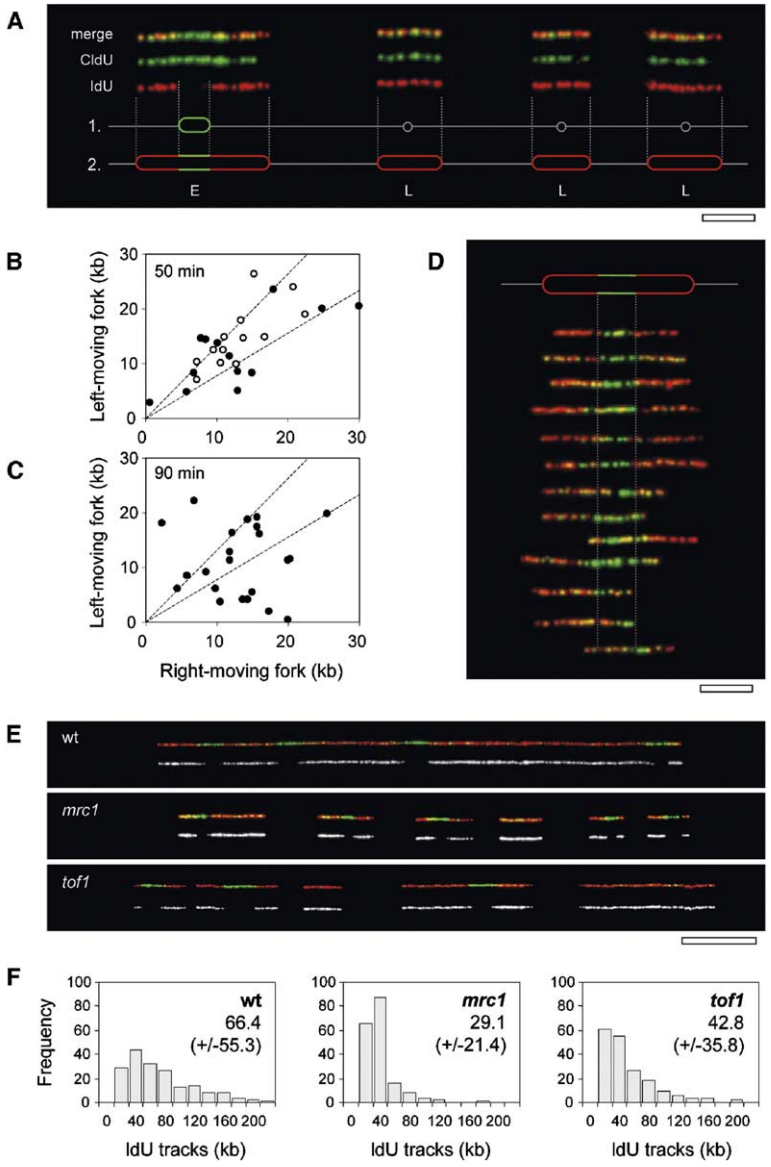

Figure 4. Mrc1p Is Required to Restart Forks after an Acute Exposure to HU

Wt (PP108), *mrc1Δ* (PP374), and *tof1Δ* (PP320) cells were released from a G_1 arrest in a medium containing 200 mM HU and 0.4 mg/ml CldU. After 90 min, cells were washed and resuspended in fresh medium containing 0.4 mg/ml IdU. DNA fibers were analyzed by DNA combing as described above.

(A) Representative DNA fiber from a wt cell showing fork recovery at an early origin and new initiation events at late origins. Bar, 10 kb.

(B and C) Length of right-moving and left-moving forks (IdU tracks) after 50 or 90 min recovery in wt (open circles) and *mrc1Δ* cells (filled circles). Dotted lines indicate pairs of diverging forks showing less than a 25% length difference.

(D) Representative images of diverging forks from *mrc1Δ* cells after 90 min recovery. Bar, 10 kb.

(E) Representative DNA fibers from wt, *mrc1Δ*, and *tof1Δ* cells after a 90 min recovery from HU. The IdU channel is shown separately for clarity. Bar, 50 kb.

(F) Distribution of IdU tracks length after release from HU (90 min).

how Rad53p is recruited to stalled forks. Recent evidence indicates that the Sgs1p helicase binds Rad53p and is potentially sufficient to target it to Mec1p-Ddc2p complexes (Bjergbaek et al., 2005). These proteins may therefore define a core checkpoint system that acts in *cis* to prevent genomic instability at stalled forks.

Finally, the fact that *mrc1Δ* cells are more sensitive to HU than *mrc1^{AQ}* mutants indicates that Mrc1p has another role at stalled forks that is independent of Rad53p. It has been recently reported that Mrc1p prevents the uncoupling of the replication machinery from sites of DNA synthesis in HU (Katou et al., 2003). Our results suggest that these abnormal structures prevent the recovery of stalled forks and are therefore responsible for the hypersensitivity of *mrc1Δ* cells to HU. It is worth mentioning that this uncoupling also occurs in *tof1Δ* cells (Katou et al., 2003), where it interferes with fork restart. Taken together, these data indicate that the repression of late origins, which is not a critical function of the replication checkpoint, may become important for viability when fork recovery is impaired.

Experimental Procedures

Strains and Media

All strains used in this study are derived from W303: PP108 (*MATa*, *RAD5*, *TK$^+$*), PP374 (*MATa*, *mrc1::HIS3*, *RAD5*, *TK$^+$*), PP320 (*MATa*, *tof1::TRP1*, *RAD5*, *TK$^+$*), PP037 (*MATa*, *rad53-11*, *TK$^+$*), and PP397 (MATa, *mrc1::HIS5::mrc1^{AQ}* [*LEU2*], *RAD5*). Cell growth, synchronization, and BrdU labeling were performed as described (Versini et al., 2003).

PFGE, 2D Gels, and ERCs Assays

Yeast cells were embedded in agarose plugs (5×10^7 cells/plug), and genomic DNA was extracted as described (Lengronne et al., 2001). Agarose plugs were used for PFGE, DNA combing, and ERCs analysis, as well as for 2D gels shown in Figures 2C and 3B. Yeast chromosomes were separated by PFGE (Gene Navigator, Amersham) and were stained with SybrGold (Molecular Probes). BrdU was detected as described (Versini et al., 2003). Fluorescent

signals were quantitated with a Typhoon (GE Healthcare). Neutral/neutral 2D agarose gels were performed as described (Brewer and Fangman, 1988; Wu and Gilbert, 1995), unless otherwise stated. Amounts of replication intermediates (bubbles, large Ys, stalled forks, and spikes) were normalized to the amount of 1 n linear fragments and are expressed relative to wt levels. ERC levels were quantitated in young, unsorted cells as described (Versini et al., 2003).

Dynamic Molecular Combing

DNA combing was performed as described (Michalet et al., 1997; Versini et al., 2003). BrdU was detected with a rat monoclonal antibody (AbCys, clone BU1/75) and a secondary antibody-coupled Alexa 488 (Molecular Probes). DNA molecules were counterstained with an anti-guanosine antibody (Argene) and an anti-mouse IgG coupled to Alexa 546 (Molecular Probes). In fork recovery experiments, CldU and IdU were detected with BU1/75 and BD44 anti-BrdU antibodies, respectively. In some recovery experiments, the human nucleotide transporter hENT1 was used on a pRS415 to facilitate CldU and IdU incorporation. Images were recorded on a Leica DMRA microscope and were processed as described (Pasero et al., 2002).

Supplemental Data

Supplemental Data include four figures and are available with this article online at http://www.molecule.org/cgi/content/full/19/5/699/DC1/.

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