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A Central Role for DNA Replication Forks in Checkpoint Activation and Response

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Summary

The checkpoint proteins Rad53 and Mec1-Ddc2 regulate many aspects of cell metabolism in response to DNA damage. We have examined the relative importance of downstream checkpoint effectors on cell viability. Checkpoint regulation of mitosis, gene expression, and late origin firing make only modest contributions to viability. By contrast, the checkpoint is essential for preventing irreversible breakdown of stalled replication forks. Moreover, recruitment of Ddc2 to nuclear foci and subsequent activation of the Rad53 kinase only occur during S phase and require the assembly of replication forks. Thus, DNA replication forks are both activators and primary effectors of the checkpoint pathway in S phase.

Introduction

Eukaryotic cells have developed sophisticated surveillance mechanisms called checkpoints to regulate responses to DNA damage and perturbations of DNA replication (Elledge, 1996; Nyberg et al., 2002; Weinert, 1998; Zhou and Elledge, 2000). Checkpoint failure can be potentially catastrophic, leading to an elevated mutation rate, chromosome instability, and the development of cancer (Hartwell and Kastan, 1994).

Checkpoints were initially described as pathways required to arrest the cell cycle in response to DNA damage, thereby coordinating cell cycle progression and repair (Hartwell and Weinert, 1989; Weinert and Hartwell, 1988). More recently, a wider vision of checkpoints has emerged. Checkpoint pathways control not only cell cycle progression but also processes such as the transcription of DNA damage response genes, the activation of DNA repair pathways, the regulation of telomere length and chromatin structure, and the recruitment of proteins to sites of damage (Foiani et al., 2000; Lowndes and Murguia, 2000; Nyberg et al., 2002; Rhind and Russell, 2000; Zhou and Elledge, 2000).

How checkpoints are activated during S phase is an area of intense interest. Drugs that perturb replication fork progression such as the ribonucleotide reductase inhibitor, hydroxyurea (HU), cause checkpoint activation. In addition, many replication mutants show aberrant checkpoint signaling. It has not been possible to unify these genetic data in a coherent model because some replication mutants cause checkpoint induction even in the absence of exogenous agents while others are defective in checkpoint activation even in the presence of agents such as HU (Araki et al., 1995; D'Urso et al., 1995; Griffiths et al., 2001; Longhese et al., 1996; McFarlane et al., 1997; Navas et al., 1995, 1996; Noskov et al., 1998; Reynolds et al., 1999; Shimada et al., 1999; Sugimoto et al., 1997; Wang and Elledge, 1999; Weinert et al., 1994).

Somewhat confusingly, in addition to its role in checkpoint signaling, DNA replication is itself regulated by checkpoint mechanisms. The central checkpoint kinases Mec1 and Rad53 in *S. cerevisiae* inhibit the activation of late-firing replication origins in HU and MMS (Santocanale and Diffley, 1998; Santocanale et al., 1999; Shirahige et al., 1998; Tercero and Diffley, 2001; Weinberger et al., 1999). In addition, Mec1 and Rad53 have an essential role in maintaining DNA replication fork stability in the face of DNA damage and replication fork blocks (Lopes et al., 2001; Tercero and Diffley, 2001). These different roles of replication forks in the genome integrity checkpoint have not been considered together and require clarification. In this work we have examined the requirements for replication forks in checkpoint activation and have determined the relative importance of known downstream effectors of the checkpoint in maintaining cell viability.

Results

Protein Synthesis Is Not Required for Checkpoint Activation, Recovery, or Viability

We have previously shown that hypersensitivity to MMS requires *mec1* and *rad53* mutant cells to enter S phase (Tercero and Diffley, 2001). Previous work has shown that preventing entry into mitosis with nocodazole is completely ineffective in rescuing *rad53* and *mec1* mutants from the lethal effects of MMS and HU, indicating that prevention of mitosis cannot account for the importance of Mec1 and Rad53 in maintaining cell viability (Desany et al., 1998; Tercero and Diffley, 2001).

The transcriptional induction of genes is thought to be another important checkpoint target (Aboussekha et al., 1996; Allen et al., 1994). However, protein synthesis is not required for the G2/M checkpoint (Weinert and Hartwell, 1990). To examine the importance of gene regulation in responding to DNA replication fork stalling, we exploited the fact that new protein synthesis is not required for passage of cells through S phase after the *cdc7* arrest point (Hereford and Hartwell, 1973, 1974). The top panel of Figure 1A confirms that cycloheximide (CHX) prevents S phase in cells released from α factor arrest but does not prevent S phase in cells released from *cdc7* arrest.

Previous work indicated that CHX does not prevent checkpoint recovery after HU arrest in wild-type cells

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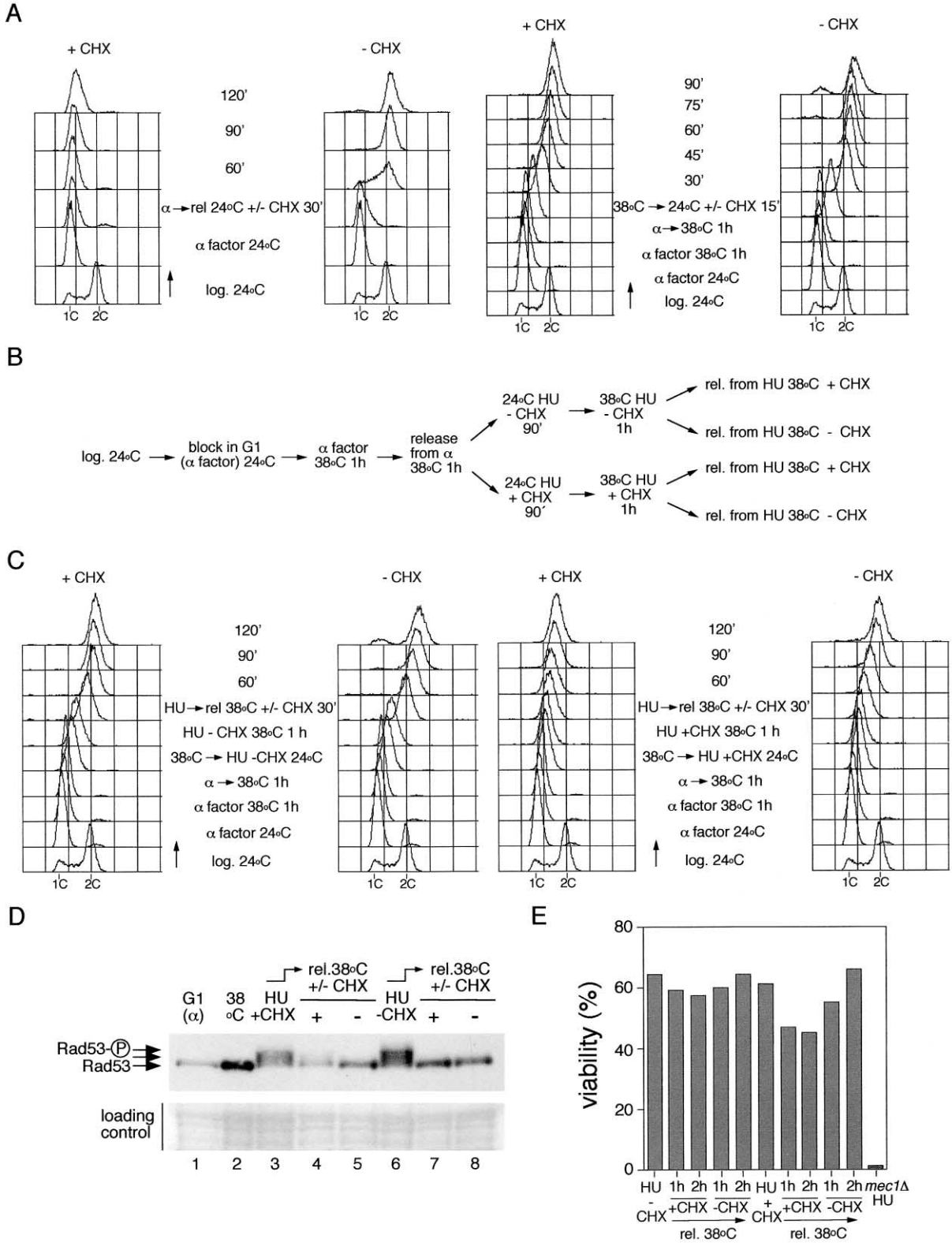

Figure 1. Protein Synthesis Requirements for Checkpoint Functions during S Phase

(A) New protein synthesis is not required to complete S phase after the *cdc7*-arrest point. *cdc7-4* cells were arrested in G1 with α factor at 24°C. Cultures were then divided in two. One half was maintained at 24°C, divided in two, and released from α factor arrest at 24°C either in the presence or absence of cycloheximide (CHX; 100 μg/ml). The other half was held in α factor for 1 hr at 38°C, released for 1 hr at 38°C, and then the culture was divided in two and shifted to 24°C either in the presence or absence of CHX. Samples were taken at the time points indicated and analyzed by flow cytometry.

(Pellicioli et al., 1999). However, the completion of S phase in wild-type cells occurs both by resumption of DNA synthesis from forks which had stalled in HU and by establishing new replication forks from origins which had not fired in HU. *cdc7* cells released from HU at the restrictive temperature can complete S phase; thus, new initiation is not required after HU arrest. The completion of S phase is slower in the absence of Cdc7 function, indicating that late origin firing contributes to timely completion of S phase (Bousset and Diffley, 1998; Diffley et al., 2000).

To specifically examine the role of protein synthesis in resumption of replication from stalled forks, we examined S phase after release from HU at the *cdc7* restrictive temperature (37°C). Cells completed S phase with similar kinetics after release from HU arrest in either the absence or presence of CHX (Figure 1C). Rad53, which became hyperphosphorylated in the HU arrest (Figure 1D, lane 6), was dephosphorylated after release from HU in either the presence or absence of CHX (lanes 7 and 8). HU arrest in the absence of CHX resulted in approximately 60% viability. This level of survival was not reduced after release from HU arrest either with or without CHX and is much higher than that of the *mec1*Δ mutant (Figure 1E). Together, these experiments show that new protein synthesis is not required for stalled DNA replication forks to resume synthesis after release from HU arrest.

In the preceding experiment, Rad53 had been activated by HU before protein synthesis was inhibited. Thus, checkpoint-dependent transcriptional induction could have already directed the synthesis of some protein(s) required to maintain viability in the HU arrest. To test this, we released cells from *cdc7* arrest into medium that contained both HU and CHX (HU/CHX). Rad53 phosphorylation occurred normally under these conditions (Figure 1D), indicating that new protein synthesis is not required for checkpoint activation. Furthermore, the presence of CHX during the release from *cdc7* into HU did not lead to any reduction in viability. These experiments demonstrate that new protein synthesis is not required for checkpoint activation or for maintenance of viability during fork stalling in HU.

When cells were released from the HU/CHX arrest in either the presence of absence of CHX, Rad53 became dephosphorylated normally (Figure 1D) and viability was unaffected (Figure 1E). In both cases, DNA synthesis resumes after release from HU although it is considerably slower, especially in cells released from HU/CHX into CHX. This indicates that synthesis of some protein(s) contributes to the resumption of normal rates of DNA synthesis. However, because Rad53 is dephosphorylated after release from HU, and given that viability remains high, we conclude that the replication forks, though slow-moving, are functional and can support replication of the entire genome.

Therefore, new protein synthesis is not required to activate or recover from the checkpoint or to maintain the stability of stalled replication forks, although there appears to be a role for protein synthesis in reestablishing rapid DNA synthesis from these forks.

Late Origin Firing and Replication Fork Stabilization Are Genetically Separable Functions

In addition to regulating DNA damage-induced gene expression, the Mec1/Rad53 checkpoint controls at least two aspects of DNA replication: late origin firing and replication fork progression (Lopes et al., 2001; Santocanale and Diffley, 1998; Shirahige et al., 1998; Tercero and Diffley, 2001). The *mec1-100* allele shows characteristics of a separation of function mutant (Paciotti et al., 2001). As in the *mec1*Δ mutant, MMS does not significantly slow down S phase in *mec1-100* cells, suggesting that *mec1-100* is defective in the intra-S phase checkpoint. Since rapid S phase in MMS is due to inappropriate late origin firing, this suggested that *mec1-100* was defective in restraining late origin firing. However, unlike the *mec1*Δ mutant, *mec1-100* cells are not hypersensitive to MMS (Paciotti et al., 2001), suggesting that the *mec1-100* mutant maintains stable replication forks. To test this, we examined activation of an early origin (ARS305) and a late origin (ARS501) in HU-arrested cells. Using the ARS305 probe, replication intermediates (RIs) are detected in all the strains after 30 min in HU (Figure 2A), indicating that the early origin fires efficiently. When the same samples were probed with ARS501 sequences (Figure 2B), RIs were not detected in checkpoint-proficient strains (wild-type or *sml1*Δ) but were detected at similar levels in both *mec1*Δ and *mec1-100* cells. Therefore, like the *mec1*Δ, the *mec1-100* mutant is unable to prevent the firing of late origins when replication forks from early-firing origins are perturbed by HU.

We next examined the progression and stability of DNA replication forks though alkylated DNA using dense isotope substitution (Reynolds et al., 1989; Tercero et al., 2000). After release from α factor arrest, *mec1-100* cells proceed rapidly through S phase in the presence of MMS, as shown previously (Figure 2C; Paciotti et al., 2001). Six restriction fragments along the chromosome VI replicon from cells pregrown in heavy isotopes and arrested in G1 phase begin entirely in the heavy-heavy (HH) peak in α factor (Figure 2D, top row) and are converted rapidly to the heavy-light (HL) position after release from α factor arrest into medium containing light isotopes in the absence of MMS (bottom row). In the presence of MMS, only fragment 1, containing ARS607, was shifted to the HL peak 30 min (row 2) after release from α factor arrest in medium with light isotopes. By

(B) New protein synthesis is not essential for checkpoint activation or recovery. *cdc7-4* cells were grown at 24°C, blocked in G1 with α factor at 24°C, held in α factor for 1 hr at 38°C, and released at 38°C for 1 hr. The culture was then divided in two and released into HU at 24°C, either in the presence or absence of CHX. In each case, cultures were shifted to 38°C and maintained in HU for 1 hr, with or without CHX, divided then in two, and released from the HU arrest at 38°C either in the presence or absence of CHX.
(C) DNA content was determined by flow cytometry at the indicated stages of the experiment described in (B).
(D) Immunoblot analysis of Rad53 during the experiment. Loading control is a Ponceau-S-stained membrane coincident with Rad53 migration.
(E) Cell viability during the course of the experiment. The viability of *mec1*Δ cells in HU is also included.

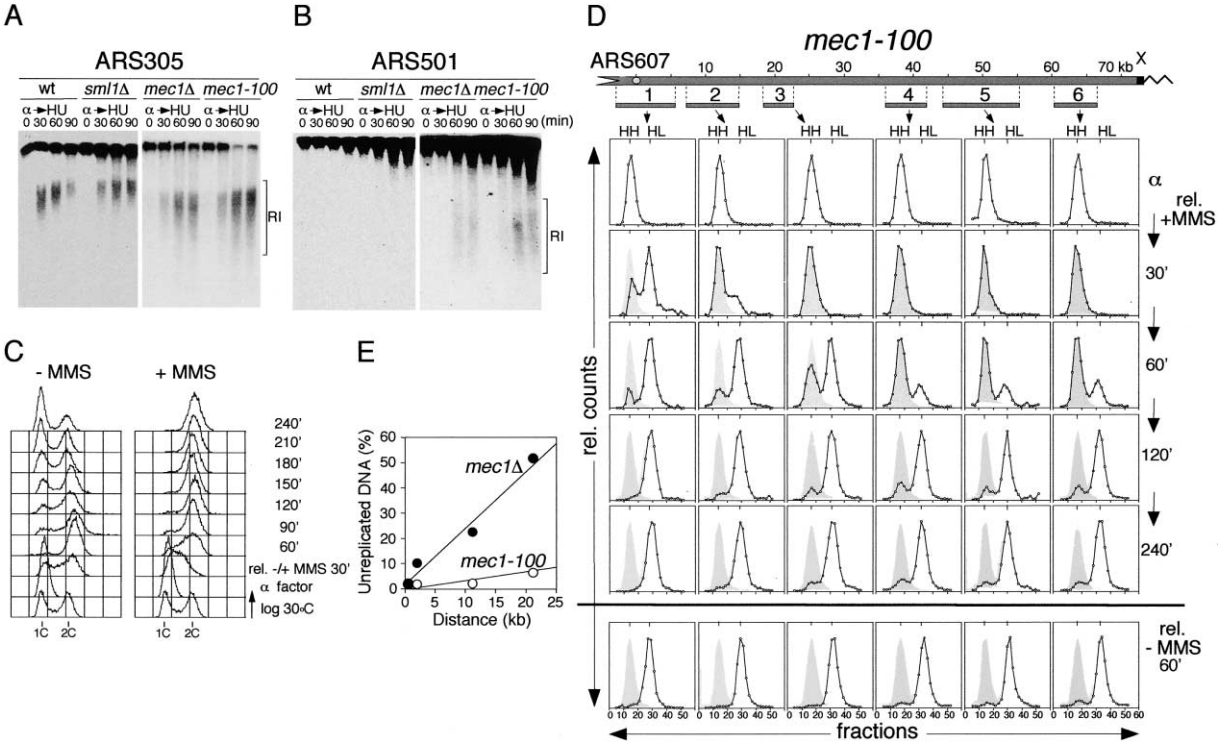

Figure 2. Analysis of Replication Intermediates and Replication Fork Progression in *mec1-100* Cells

(A) Detection of replication intermediates from stalled replication blocks from the early active origin ARS305 in *MEC1*$^+$, *mec1*Δ, and *mec1-100* strains. Cells arrested in G1 with α factor were released into medium containing 0.2 M HU at 24°C for 0–90 min. Replication intermediates were analyzed using the alkaline agarose method (Santocanale and Diffley, 1998) with ARS305 DNA as a probe.

(B) Detection of RIs from the late origin ARS501. The same samples used in (A) were analyzed with ARS501 DNA as a probe.

(C) Replication fork progression in *mec1-100* cells. *mec1-100* cells were arrested in G1 with α factor in the presence of heavy isotopes and released from the block into medium containing light isotopes either in the presence or in the absence of MMS (0.033%). DNA content was determined by flow cytometry at the indicated time points.

(D) The time course of DNA replication in a replicon of chomosome VI was analyzed by density transfer, using specific probes recognizing the ClaI/SalI fragments 1–6 as indicated. The positions of replication origins are shown at the top. The time points indicated correspond to those analyzed by flow cytometry in (C). The relative amounts of radioactivity in the hybridized DNA are plotted against the fraction number. The position of unreplicated heavy-heavy (HH) and fully replicated heavy-light (HL) DNA peaks is indicated. At later time points the position of the initial HH peak is shown by comparison (gray area).

(E) Replication fork breakdown rates. The percentage of unreplicated DNA at the end of the experiment in (D) for fragments 1–3 was plotted against distance from the ARS consensus sequence.

60 min (row 3), replication can be seen progressing from left to right in fragments 1–3. The slow fork rate is virtually the same as that previously described for wild-type and checkpoint null mutants (Tercero and Diffley, 2001). As with *mec1*Δ, forks also appear to originate from the right end of the replicon by 60 min, suggesting that the X element-associated ARS becomes activated (Tercero and Diffley, 2001). At 120 (row 4) and 240 (row 5) min, the six restriction fragments are primarily in the HL position (i.e., they are almost fully replicated); very little terminally unreplicated (HH) DNA remains. This is in sharp contrast to the *mec1*Δ mutant, in which large amounts of DNA remain terminally unreplicated (Tercero and Diffley, 2001). We estimate that the rate of DNA fork breakdown in the *mec1-100* strain (0.2% per kb) is considerably reduced (~10-fold) compared to that in the *mec1*Δ mutant (Figure 2E). Thus, in *mec1-100* cells, DNA replication forks move slowly through alkylated DNA but, like wild-type cells, remain functional and ultimately replicate the entire replicon.

Thus, *mec1-100* is unable to prevent the firing of late origins but can still prevent damage-induced DNA replication fork catastrophe. Because the *mec1-100* mutant is not nearly as MMS sensitive as the *mec1*Δ mutant (Paciotti et al., 2001), the main cause of cell death in checkpoint null mutants in the presence of MMS appears to be replication fork breakdown rather than inappropriate replication origin firing.

Irreversibility of Rad53 Arrest

Mec1/Rad53 may be required during fork stalling to prevent some irreversible, catastrophic event at forks; alternatively, they may play no role during fork arrest but may be specifically required to resume DNA synthesis after stalling. To distinguish between these alternatives, we constructed a strain in which the sole copy of *RAD53* was replaced by a copy of *RAD53* under the regulation of the *GAL1,10* promoter. This allows us to induce Rad53 at specific times before or after fork stalling. Cells re-

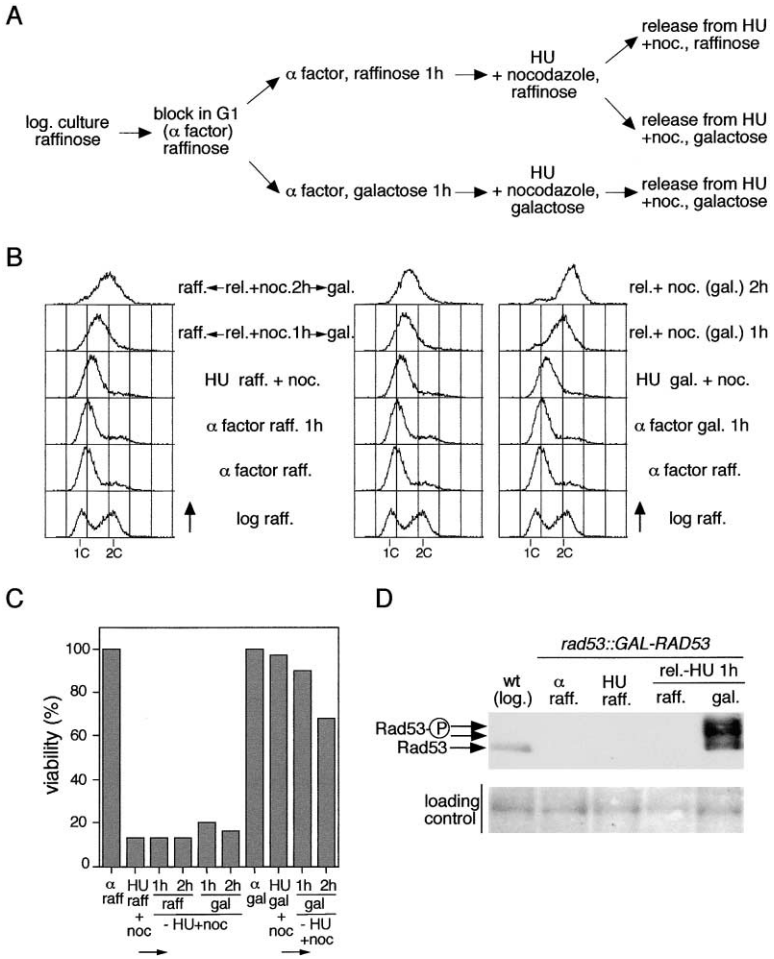

Figure 3. DNA Replication Fork Arrest in the Absence of Rad53 Is Irreversible

(A) Cells carrying a *Gal-RAD53* fusion as the only source of Rad53 protein were grown in YP-raffinose at 30°C and arrested in G1 with α factor. The culture was divided in two and incubated in either YP-raffinose or YP-galactose for 1 hr. In both cases, cells were released from the G1 block into HU plus nocodazole, in medium with raffinose or galactose, respectively. The culture arrested in HU in raffinose-containing medium was divided in two, and each half was released in fresh medium with nocodazole, either in raffinose- or in galactose-containing medium. The culture arrested in HU in galactose-containing medium was released in fresh galactose-containing medium plus nocodazole.

(B) DNA content was determined by flow cytometry at the indicated stages of the experiment described in (A).

(C) Cell viability during the course of the experiment.

(D) Immunoblot analysis of Rad53 during the experiment. The first line is a control of the level of Rad53 in exponentially growing wild-type cells. Loading control is a Ponceau-S-stained membrane coincident with Rad53 migration.

leased into HU plus galactose and subsequently released from HU arrest into fresh medium containing galactose serve as a control for cells that express Rad53 throughout the experiment (see Figure 3A). These control cells complete S phase efficiently (Figure 3B) and retain high viability (Figure 3C). By contrast, cells released into HU in raffinose-containing medium and subsequently released from HU into fresh raffinose-containing medium were unable to efficiently resume DNA synthesis after release from HU (Figure 3B), consistent with earlier work (Desany et al., 1998), and exhibited a severe loss in viability (Figure 3C). To examine reversibility, cells were released into HU in raffinose-containing medium, then transferred to galactose-containing medium and released from HU arrest. These cells were unable to resume DNA synthesis (Figure 3B) and lost viability (Figure 3C). Rad53 was efficiently expressed in this experiment (Figure 3D), and the newly synthesized Rad53 remains hyperphosphorylated even up to an hour after HU withdrawal. This strongly suggests that the irreversible fork arrest seen in the absence of Rad53 generates a structure that is recognized by the checkpoint machinery as DNA damage. Despite efficient checkpoint activation, however, cells are unable to resume DNA synthesis from these damaged forks or to regain viability.

Checkpoint Activation by MMS Is Limited to S Phase

Given that the checkpoint cannot act to restore forks after they have stalled, it might act to prevent/delay entry into S phase by arresting cells at some point in the cell cycle outside of S phase or it might act more directly during S phase to regulate DNA replication. If the former contributes to cell viability, then MMS treatment should lead to checkpoint activation outside of S phase. To examine this, we utilized the fact that Mec1-dependent hyperphosphorylation of Rad53 is required for its activation as a kinase (Allen et al., 1994; Sanchez et al., 1996; Sun et al., 1996). G1 (α factor-arrested), S (α factor-arrested and released), or G2/M (nocodazole-arrested) phase cells were treated with different concentrations of MMS. Significant phosphorylation of Rad53 occurred only in cells allowed to proceed through S phase in the presence of MMS (Figure 4A). Rad53 appears as a doublet in nocodazole-arrested cells, even in the absence of MMS (lane 15), presumably as a consequence of cell cycle arrest, not DNA damage. Rad53 activation in S phase cells is seen at MMS concentrations as low as 0.005%, reaching a maximum at 0.010% MMS. Rad53 kinase activity (Pellicioli et al., 1999) could not be detected in either G1- or G2/M-arrested cells in the presence of MMS (Figure 4A, bottom); kinase activity could only be detected when cells were allowed to proceed

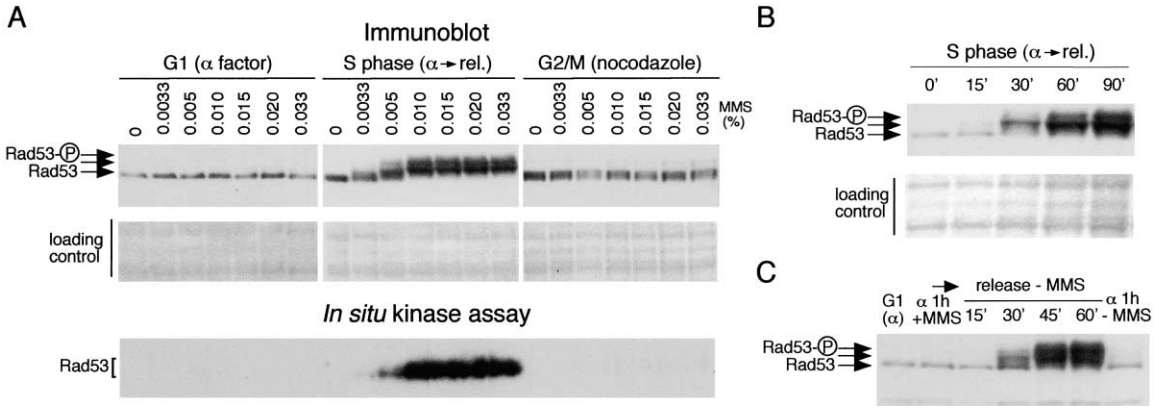

Figure 4. Efficient Activation of Rad53 in Response to MMS Occurs in S Phase

(A) Immunoblot analysis of Rad53. W303-1a cells blocked in G1, S, or G2/M were treated with different concentrations of MMS (as indicated) for 1 hr. Phosphorylated and unphosphorylated forms of Rad53 were identified. The loading control was a region of Ponceau-S-stained membrane coincident with Rad53 migration. In situ autophosphorylation assay for Rad53 protein kinase, as described in the Experimental Procedures. Samples are the same as in (A).

(B) Time course analysis of Rad53 phosphorylation in S phase by immunoblot. W303-1a cells were blocked in G1 with α factor and released into fresh medium containing 0.033% MMS. Samples were taken at the time points indicated.

(C) MMS treatment in G1 activates Rad53 in the subsequent S phase. W303-1a cells were blocked in G1 with α factor and held in G1 for an extra hour in the presence of 0.033% MMS. The culture was split in two; half was released into S phase in the absence of MMS and the other held in G1 with α factor for 1 hr without MMS. Samples were taken at the time points indicated.

through S phase in the presence of MMS. Rad53 first became phosphorylated 30 min after release from α factor (Figure 4B), by which time 85% of cells had budded. Phosphorylation increased at 60–90 min after release as cells proceeded into S phase.

The above results show that Rad53 activation only occurs during S phase in the presence of MMS. There are two possible explanations for this. First, alkylated DNA may only be sensed when replication forks encounter it. In such a case, DNA damaged outside of S phase would cause Rad53 activation only when cells subsequently enter S phase. Second, some aspect of DNA replication forks (e.g., ssDNA) may be required for MMS to generate specific DNA lesions that are sensed by the checkpoint pathway. In this case, MMS would have to be present during S phase to cause checkpoint activation. To test these alternatives, cells blocked in G1 with α factor were treated with MMS for 1 hr. The culture was then divided in two; one half was released into S phase in the absence of MMS and the other half was held in G1 with α factor also in the absence of MMS. As shown in Figure 4C and in agreement with Figure 4A, no Rad53 activation was detected in cells held in G1. However, when these cells were allowed to enter S phase in the absence of MMS, Rad53 was phosphorylated after 30 min. In addition, consistent with Figure 4B, Rad53 phosphorylation increases with S phase progression. We conclude that DNA damage incurred outside of S phase can activate Rad53 during a subsequent S phase.

To examine this correlation between S phase and MMS-dependent activation of Rad53 DNA synthesis, we sought to determine whether conditions that delay or extend S phase similarly affect the kinetics of Rad53 activation. Deletion of both S phase cyclins (Clb5, Clb6) delays entry into S phase, presumably until later Clbs (e.g., Clb3, Clb4) are synthesized (Kuhne and Linder, 1993; Schwob and Nasmyth, 1993). Wild-type and *clb5*Δ *clb6*Δ cells were blocked in G1 with α factor and released from the arrest into S phase in either the absence or presence of MMS. Wild-type cells entered S phase 15 min after release from α factor and by 30 min were well into S phase, while *clb5*Δ *clb6*Δ cells entered S phase at around 45 min (Figure 5A). In both cases, the time of S phase entry was unaffected by MMS (Figure 5A). In the absence of MMS, phosphorylation of Rad53 was not detected in either wild-type or *clb5*Δ *clb6*Δ cells. In wild-type cells, after release from α factor arrest in the presence of MMS, Rad53 phosphorylation was first observed after 30 min and increased as cells progressed further into S phase. In *clb5*Δ *clb6*Δ cells, phosphorylated Rad53 was first detected after 45 min, coincident with the start of DNA synthesis. The extent of phosphorylation increased with time, reaching a maximum after 75 min, when all cells were clearly in S phase. Therefore, Rad53 activation is delayed in the *clb5*Δ *clb6*Δ strain, and the onset of DNA synthesis and Rad53 activation in response to DNA alkylation are correlated in both strains.

We next asked whether the time interval in which cells can respond to MMS is lengthened when S phase is lengthened. Deletion of Clb5 alone does not delay entry into S phase but instead causes an extended S phase because late origins are not activated (Donaldson et al., 1998; Epstein and Cross, 1992; Kuhne and Linder, 1993; Schwob and Nasmyth, 1993). Wild-type and *clb5*Δ cells were arrested in G1 with α factor and then released in the absence of MMS. Samples were taken every 10 min and transferred to medium with MMS for 30 min. Both wild-type and *clb5*Δ strains enter S phase at approximately the same time (20–30 min after release). Wild-type cells finish S phase 50 min after the release from α factor while S phase continues in the *clb5*Δ strain until 60–70 min. In wild-type cells, Rad53 activation is

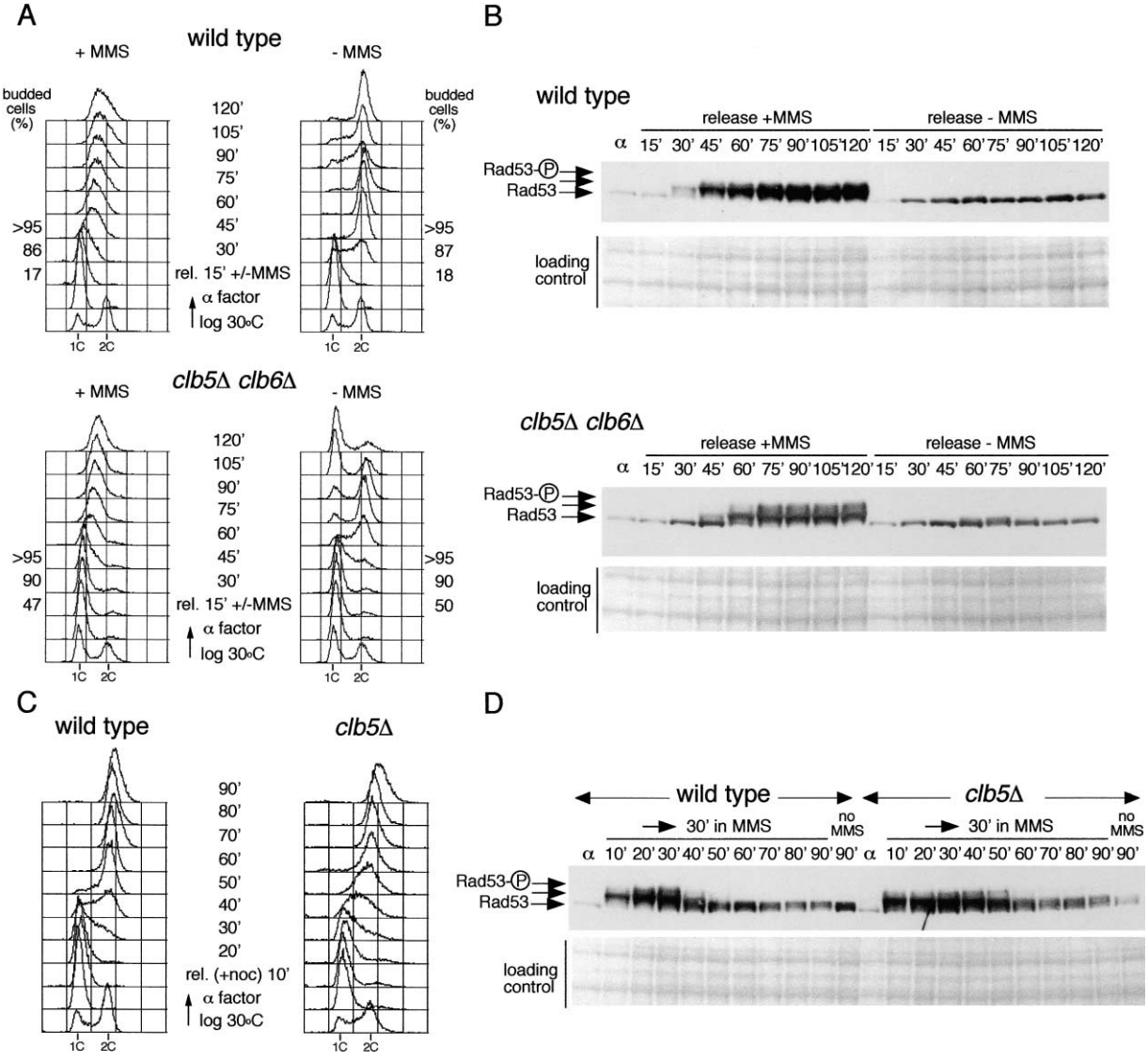

Figure 5. Rad53 Activation in Response to MMS Correlates with DNA Synthesis

(A) Logarithmic phase cultures of *clb5*Δ *clb6*Δ and wild-type cells growing in YPD at 30°C were synchronized in G1 with α factor and then released into fresh medium with or without 0.033% MMS. Samples were taken as indicated and analyzed by flow cytometry. Budding index is indicated at the relevant time points.

(B) Immunoblot analysis of Rad53 during the course of the experiment in (A). The loading control is a region of Ponceau-S-stained membrane coincident with Rad53 migration.

(C) Logarithmic phase cultures of *clb5*Δ and wild-type cells growing in YPD at 30°C were synchronized in G1 with α factor. They were then released into S phase in fresh medium lacking α factor. Nocodazole was added upon release from the α factor to prevent cells entering a new cell cycle. Aliquots were taken every 10 min and transferred to medium containing 0.033% MMS for 30 min. Flow cytometry analysis shows DNA content before MMS was added.

(D) Immunoblot analysis of Rad53 during the course of the experiment in (C), after the MMS treatment. The loading control is a region of Ponceau-S-stained membrane coincident with Rad53 migration.

observed in samples transferred to MMS between 10 and 40 min after release from α factor (Figure 5D). In some of these samples, cells were already in S phase when MMS was added while in others cells entered S phase during MMS treatment. Once cells have finished S phase (50 min and beyond), they are unable to activate Rad53 in response to MMS. In *clb5*Δ cells, which show an extended S phase, the Rad53 response is extended accordingly. Rad53 activation is detected in cells transferred to MMS from 10 min to 50–60 min after release, whereas no Rad53 response is observed after transfer at 60 min, when cells had finished S phase prior to MMS treatment. Rad53 is present as a doublet at later times due to the presence of nocodazole (see Figure 4A), used to prevent entry into the next cell cycle. These results indicate that the timing of the Rad53 response to DNA alkylation correlates with both the entry into and the duration of S phase. We note that in the *clb5*Δ strain, Rad53 is not fully hyperphosphorylated as seen in wild-type cells, suggesting that the reduced number of repli-

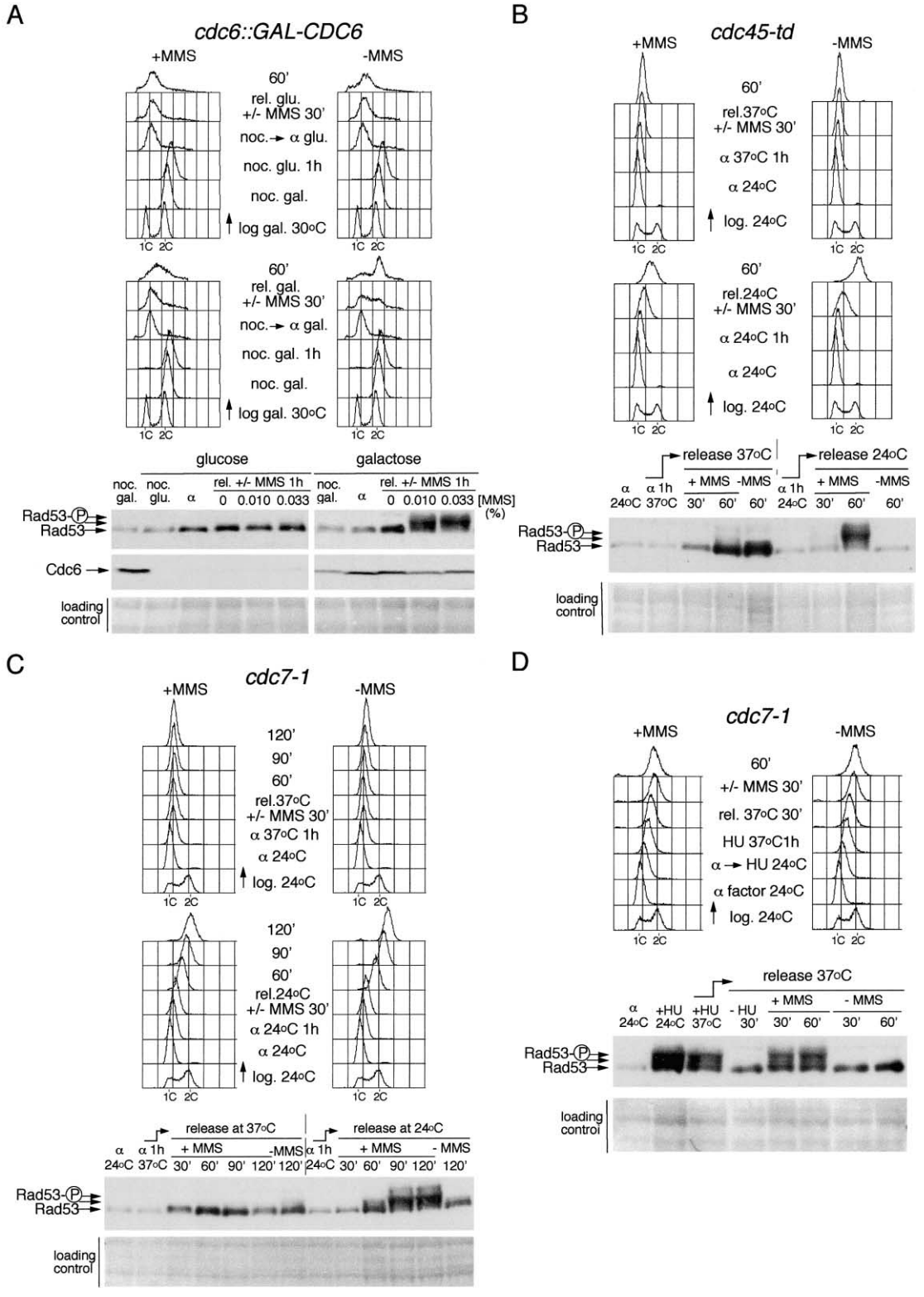

Figure 6. Establishment of Replication Forks Is Required for MMS-Induced Checkpoint Activation

(A) Cdc6 is required for Rad53 activation in response to MMS. Cells carrying a *Gal-CDC6* fusion as the only source of Cdc6 protein were grown in YP-galactose at 30°C and arrested in G2/M with nocodazole. The culture was divided in two and incubated in either YP-glucose or YP-galactose for 1 hr. In both cases, cells were released from the G2/M block into G1 (α factor block), in medium with glucose or galactose, respectively. Finally, they were released from the α factor block in the presence or absence of 0.033% MMS. Samples were taken as indicated and analyzed by flow cytometry (upper panel). The bottom panel shows an immunoblot analysis of Rad53 and Cdc6 during the course of the experiment. Also included are samples corresponding to cells released from α factor for 1 hr in 0.010% MMS. The loading control is a region

cation forks due to the reduction in origin activity in the *clb5*Δ strain quantitatively reduces checkpoint activation.

Establishment of Replication Forks Is Essential for MMS-Induced Checkpoint Activation

We next wanted to determine whether DNA replication forks per se or some other aspect of S phase was required for checkpoint activation. We exploited the fact that very tight conditional mutants which prevent the initiation of DNA replication are unable to prevent later cell cycle events such as mitosis (Labib et al., 2001; Piatti et al., 1995; Tercero et al., 2000; Toyn et al., 1995). Such mutants, therefore, can progress though the entire cell cycle in the absence of DNA replication. We examined conditional mutants in three genes, *CDC6*, *CDC45*, and *CDC7,* which act at different steps during initiation.

Cdc6 is an unstable protein which has an essential role in prereplicative complex (pre-RC) assembly (Cocker et al., 1996; Drury et al., 1997; Piatti et al., 1995). Cells containing one copy of *CDC6* expressed from the *GAL1,10* promoter were grown in galactose-containing medium and then were blocked in G2/M with nocodazole (Figure 6A). Half of this culture was held in medium containing galactose to maintain high levels of Cdc6 expression, while the other half was put in medium containing glucose to completely repress Cdc6 expression (Figure 6A). Both cultures were released from the nocodazole arrest into fresh medium containing α factor, then released from α factor arrest in the presence or absence of MMS. In the presence of galactose, cells progressed through S phase and underwent a slow S phase in MMS; in contrast, in the presence of glucose, cells are unable to initiate DNA replication (Piatti et al., 1995). When Cdc6 is present (galactose), Rad53 is phosphorylated in the presence of MMS, while in the absence of Cdc6 (glucose), Rad53 is not phosphorylated despite the presence of MMS. After 90 min these cells undergo nuclear division in the absence of DNA replication, as previously described (Piatti et al., 1995), either with or without MMS (data not shown).

Cdc45 is required for both the initiation and the elongation phases of DNA replication (Aparicio et al., 1997; Hopwood and Dalton, 1996; Owens et al., 1997; Tercero et al., 2000; Zou et al., 1997) and, unlike Cdc6, binds to origins of replication after pre-RC assembly and after cyclin-dependent kinase (Cdk) activation (Zou and Stillman, 1998, 2000). *cdc45-td* cells were synchronized in G1 with α factor at the permissive temperature (24°C). Cultures were divided in two and held in α factor for 1 hr at 24°C or 37°C (restrictive temperature), and released from the α factor arrest at 24°C or 37°C, either with or without MMS. Cells released at 24°C progress through S phase (slowly in the presence of MMS). However, at 37°C, cells cannot initiate replication and proceed through mitosis later (Tercero et al., 2000, and data not shown). At 24°C, Rad53 is fully phosphorylated by 60 min after release from α factor in the presence of MMS. In contrast, at 37°C, Rad53 remains unphosphorylated after release from α factor, indicating that Rad53 is not activated by MMS under these conditions.

cdc7-1 cells were synchronized in G1 with α factor at the permissive temperature (24°C), the culture was divided in two, and these were held in α factor for 1 hr at either the permissive or the nonpermissive temperature (37°C). Cells were then released from α factor arrest into medium either with or without MMS. After release from α factor arrest at the permissive temperature, *cdc7-1* cells initiate DNA replication and progress through S phase, while at 37°C cells remain with a 1C DNA content after release from α factor block. As previously described (Toyn et al., 1995), cells subsequently enter mitosis in the absence of detectable DNA replication (data not shown). At 24°C, once cells advance into S phase in the presence of MMS, Rad53 is activated. As is the case with the *clb5*Δ strain, the degree of Rad53 hyperphosphorylation in *cdc7-1* cells is decreased, presumably due to a lower number of active origins even at the permissive temperature. As shown above for the other initiation proteins, Rad53 is not phosphorylated at 37°C in *cdc7-1* cells even in the presence of MMS.

The experiments thus far show that Rad53 is not activated if initiation is prevented. This could mean that replication forks established during initiation are involved in sensing DNA damage. Alternatively, initiation itself could play some role in DNA damage sensing. To distinguish between these possibilities, *cdc7-1* cells were synchronized in G1 with α factor at the permissive temperature (24°C) and released into S phase at 24°C in the presence of HU. HU-arrested cultures were shifted to 37°C to inactivate Cdc7 in the presence of HU, then released from the HU arrest at 37°C. After resumption

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of Ponceau-S-stained membrane coincident with the migration of Cdc6.

(B) Cdc45 is required for Rad53 activation in response to MMS. Logarithmic phase cultures of *cdc45-td* cells were arrested in G1 with α factor at 24°C in YP plus raffinose. They were divided in two and held in α factor for 1 hr either in YP plus galactose or YP plus raffinose, at 37°C or 24°C, respectively. They were then released from the block at 37°C or 24°C, with or without 0.033% MMS. Samples were taken as indicated and analyzed by flow cytometry (upper panel). The bottom panel shows an immunoblot analysis of Rad53 during the course of the experiment. The loading control is a region of Ponceau-S-stained membrane coincident with Rad53 migration.

(C) Cdc7 is required for Rad53 activation in response to MMS (I). Logarithmic phase cultures of *cdc7-1* cells were arrested in G1 with α factor at 24°C in YP plus glucose. They were divided in two and held in α factor for 1 hr either at 37°C or 24°C. They were released from the α factor arrest at 37°C or 24°C, respectively, in the presence or absence of 0.033% MMS. Samples were taken as indicated and analyzed by flow cytometry (upper panel). The bottom panel shows an immunoblot analysis of Rad53 during the course of the experiment. The loading control is a region of Ponceau-S-stained membrane coincident with Rad53 migration.

(D) Cdc7 is required for Rad53 activation in response to MMS (II). Logarithmic phase cultures of *cdc7-1* cells were synchronized in G1 with α factor at 24°C in YP plus glucose and then released into fresh medium containing 0.2 M HU for 90 min at 24°C. The cells arrested in S phase with HU were shifted to 37°C and held in HU for a further 60 min. They were then released from the HU block at 37°C. Thirty minutes after the release, cells were divided into two cultures and 0.033% MMS was added to one. Samples were taken at the indicated time points and analyzed by flow cytometry (upper panel). Bottom panel: immunoblot analysis of Rad53 during the course of the experiment. The loading control is a region of Ponceau-S-stained membrane coincident with Rad53 migration.

of DNA replication and recovery from checkpoint activation, MMS was added. Because Cdc7 is nonfunctional in these cells, late origins do not fire, and DNA replication can proceed only from previously stalled forks that resume DNA synthesis. When *cdc7-1* cells released from α factor are blocked in HU, Rad53 is activated because replication forks stall (Figure 6D). After the shift to 37°C, Rad53 remains phosphorylated, indicating that Cdc7 activity is not required to maintain Rad53 activation. When these cells are released from HU arrest and resume replication, replication forks resume synthesis and Rad53 becomes rapidly dephosphorylated, indicating that Cdc7 is not required for recovery. When MMS was added to the *cdc7-1* cells before completion of replication, Rad53 was reactivated, as judged by its phosphorylation (Figure 6D).

Thus, the Rad53 pathway is not activated by MMS if functional replication forks are not established, regardless of the means by which the assembly of forks is prevented. Moreover, the presence of replication forks in the absence of new initiation is sufficient to promote activation. Therefore, replication forks are required for sensing alkylated DNA.

Ddc2 Is Recruited to MMS-Induced Foci Specifically during S Phase

These results indicate that Mec1/Rad53 require replication forks for checkpoint activation and then play an essential and, perhaps, direct role at replication forks; thus, checkpoint proteins may be recruited directly to stalled replication forks where they can be activated and act directly. Mec1 is associated with a regulatory subunit called Ddc2/Lcd1, which is also essential for checkpoint responses (Paciotti et al., 2000; Rouse and Jackson, 2002). Previous experiments have shown that Ddc2-GFP is recruited to nuclear foci after DNA damage (Melo et al., 2001), so we asked whether Ddc2-GFP is also recruited to foci after MMS treatment. Ddc2-GFP foci were not observed in either G1- or G2-arrested cells treated with MMS; however, multiple sites of Ddc2-GFP foci were detected in S phase cells treated with MMS (Figure 7A). These multiple foci are reminiscent of replication foci seen during normal S phase using PCNA-GFP (M. Madine and J.F.X.D., unpublished data), suggesting that Ddc2 is recruited to replication forks that have encountered DNA damage. Recruitment also requires MMS; untreated S phase cells do not show any detectable foci. Finally, consistent with previous work using *cdc13-* and HO-induced DNA damage (Melo et al., 2001), recruitment of Ddc2 to foci is dependent upon its partner Mec1: Ddc2-GFP foci were not observed in S phase *mec1*Δ cells treated with MMS (Figure 7C). Together these experiments indicate that Ddc2 is only recruited to foci, which probably contain stalled replication forks, specifically during S phase in a Mec1- and DNA damage-dependent manner.

As described above, the *mec1-100* allele can stabilize DNA replication forks in the presence of MMS but is unable to prevent late origin firing in either MMS or HU. If our model of direct recruitment, activation, and action of Mec1 at stalled replication forks is correct, we would predict that *mec1-100*, in contrast to *mec1*Δ, should support normal recruitment of Ddc2 to foci during S phase in response to MMS treatment. To test this, *mec1-100* cells containing Ddc2-GFP were treated as in Figure 7A, but Ddc2 was not recruited to foci during either G1 or G2 phase (Figure 7D). However, just like wild-type cells and in contrast to *mec1*Δ cells, Ddc2 is recruited to foci specifically during S phase in the *mec1-100* cells after MMS treatment.

Discussion

The genome integrity checkpoint detects many types of genomic insult, coordinating a variety of responses to maximize cell survival while minimizing the loss or mutation of genetic information. Much emphasis has been placed on elaborating the complexities of this response and in determining molecular mechanisms of individual steps. This has led to a view of checkpoints as being an intranuclear signal transduction pathway where upstream sensors seek out and detect DNA damage and central transducers act in protein kinase cascades to regulate a myriad of downstream effectors. However, our results indicate that, rather than acting in a linear pathway, replication forks are required both to generate the signal which activates Mec1/Rad53 and as the primary downstream effectors of this pathway for maintaining cell viability.

Our results argue strongly that the establishment of replication forks during S phase is required for checkpoint activation. MMS can only cause Rad53 activation during S phase: conditions which delay S phase entry (*clb5,6*Δ) delay Rad53 activation, and conditions which extend S phase duration (*clb5*Δ) extend the period during which Rad53 can be activated. Mutants defective in initiation either before or after pre-RC assembly are unable to activate Rad53. The inhibition of initiation in *Xenopus* egg extracts also prevents checkpoint activation indicating that replication forks are also critical for signaling in this system (Lupardus et al., 2002; Stokes et al., 2002). Ddc2 is recruited to nuclear foci in response to DNA damage by MMS only during S phase, presumably reflecting its recruitment to sites of stalled DNA replication forks.

This requirement for replication forks in checkpoint activation by MMS has several implications. First, in contrast to prevailing views about DNA damage sensing, there is no efficient system for general genome surveillance that can detect alkylated DNA and activate Rad53 outside of S phase. Instead, it is only when a replication fork encounters alkylated DNA that Rad53 is activated. This does not mean that the repair of alkylated DNA is limited to S phase. On the contrary, our previous work has shown that simply preventing S phase entry in *mec1* and *rad53* mutants by arresting cells with α factor increases survival in a time-dependent manner, indicating that Mec1- and Rad53-independent repair must occur in G1-arrested cells (Tercero and Diffley, 2001). The second implication is that, because there are many replication forks active at any given time during S phase, and because conditions generally used to activate the checkpoint such as HU treatment affect all forks, there may be a quantitative aspect to the activation of Rad53 by MMS. Indeed, as shown in Figures 5 and 6, Rad53 phosphorylation in response to MMS treatment is re-

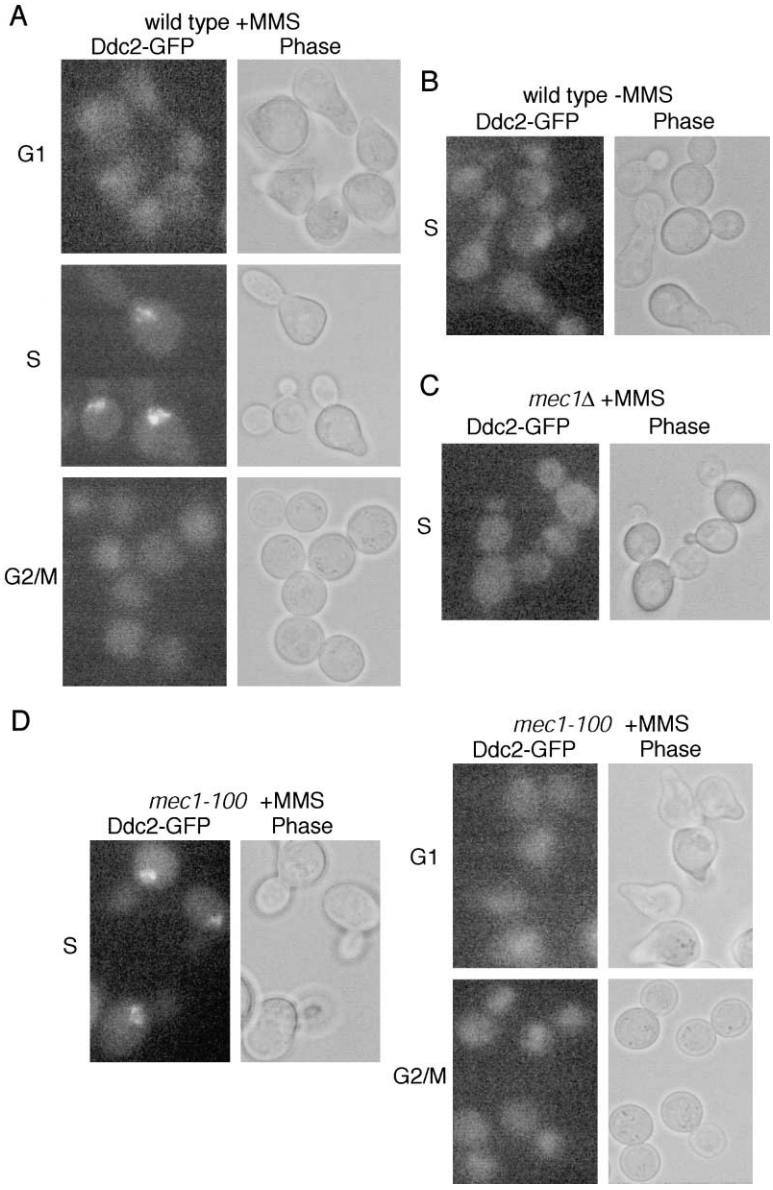

Figure 7. Ddc2-GFP Is Recruited to MMS-Induced Foci during S Phase

(A) Ddc2-GFP foci after MMS treatment are only detected during S phase. *DDC2-GFP* cells in G1 (α factor blocked), S (released from α factor arrest), or G2/M (nocodazole-arrested) phase were treated with 0.033% MMS for 60 min at 30°C and analyzed for Ddc2-GFP foci formation.

(B) Ddc2-GFP foci formed during S phase are MMS dependent. The same cultures as in (A) were analyzed for Ddc2-GFP foci formation in the absence of MMS treatment.

(C) Ddc2-GFP foci formed in response to MMS treatment are *MEC1* dependent. *mec1Δ DDC2-GFP* cells were treated as in (A) and analyzed for Ddc2-GFP foci formation.

(D) *mec1-100* cells recruit Ddc2-GFP to foci during S phase in response to MMS treatment. *mec1-100 DDC2-GFP* cells in G1 (α factor-blocked), S (released from α factor), or G2/M (nocodazole-arrested) phase were treated with 0.033% MMS for 60 min at 30°C and analyzed for Ddc2-GFP foci formation.

duced in mutants which have partial defects in initiating DNA replication and, consequently, establish fewer replication forks, such as *clb5Δ* and *cdc7^{ts}* (at 24°C). Our results, together with recent work by Shimada et al. (2002), suggest that signals from multiple stalled replication forks are integrated though Mec1/Rad53 to generate a quantitative read-out and, below some threshold, checkpoints are not activated.

The requirement for DNA replication forks in checkpoint activation is not universal; certain kinds of DNA damage can signal even in their absence. However, such DNA damage must first be processed by the DNA repair machinery before it can signal. For example, double-strand breaks can activate Rad53 outside of S phase but probably only after Mre11/Rad50/Xrs2 act to resect the break to generate ssDNA (Ivanov et al., 1994; Lee et al., 1998; Tsubouchi and Ogawa, 1998). Similarly, Rad53 can be activated by UV outside of S phase (e.g., during G1 phase); however, this activation depends entirely upon nucleotide excision repair (NER) (Neecke et al., 1999). In the absence of Rad14, an early-acting NER protein, UV can still activate Rad53 but only if cells are allowed to enter S phase with DNA damage.

Base excision repair (BER) is crucial for repairing DNA alkylated by MMS (Chen et al., 1990). BER works primarily by removing and replacing a single damaged nucleotide residue and, thus, does not generate tracts of ssDNA as an intermediate (Dianov et al., 2001; Doetsch et al., 2001; Lindahl, 2001; Memisoglu and Samson, 2000). Perhaps the absence or reduced levels of ssDNA during BER explains why MMS is very poor at activating Rad53 outside of S phase. Our results appear to disagree with previous work showing that Rad53 can be activated throughout the cell cycle in response to MMS treatment (Pellicioli et al., 1999; Sidorova and Breeden, 1997, 2002; Sun et al., 1996). However, in those studies, higher concentrations of MMS (e.g., 0.1%–0.2%), longer times in MMS, or higher temperatures were used. Addi-

tional repair pathways such as NER and homologous recombination contribute to viability in MMS (Chen et al., 1990; Xiao et al., 1996). Moreover, BER can occur by a long patch pathway (Dianov et al., 2001; Lindahl, 2001). ssDNA intermediates from one or more of these pathways may be responsible for the signaling seen at high MMS concentrations.

In addition to playing a crucial role in checkpoint signaling, our results indicate that the stabilization of stalled replication forks is also the crucial downstream effector of checkpoint activation in maintaining viability. At present we know little about this role of Mec1/Rad53 in fork stabilization except that, if Rad53 is absent during fork stalling, subsequently supplying Rad53 cannot prevent cell death and irreversible fork arrest. Whether the primary irreversible event is the generation of some unusual, irreparable DNA structure (Cha and Kleckner, 2002; Sogo et al., 2002) or the disassembly of replication factors such as Mcm2-7 that cannot be reloaded during S phase once lost (Labib et al., 2000) remains to be determined. Given the vast repertoire of cellular DNA repair mechanisms designed to deal with so many types of damaged DNA, we favor the idea that it is stabilization of the replication fork machinery that is regulated by Mec1/Rad53. Regardless, even when the checkpoint-inducing signal (HU) is withdrawn under such conditions, Rad53 becomes and remains hyperphosphorylated. This indicates that the arrested forks are seen as damaged DNA, suggesting that the replication machinery has not reassembled.

Our results indicate that damage-dependent transcription plays little role in maintaining viability after fork perturbation. However, as we have shown in this paper, when protein synthesis is prevented both during and after HU arrest, the rate of DNA synthesis during recovery is quite slow, suggesting that protein synthesis is required for the resumption of normal replication fork rates. We note that, even under these conditions, viability remains high and Rad53 is dephosphorylated normally after withdrawal from HU indicating that some aspect of normal recovery occurs. Further characterization of this slow mode of replication should yield interesting insights into the mechanisms of fork stabilization by Rad53.

The inappropriate firing of late replication origins also does not appear to have a major role in cell lethality in MMS or HU. *mec1-100* is proficient in recruiting Ddc2 and stabilizing replication forks but shows delayed Rad53 phosphorylation and defects in preventing late origin firing and mitosis (Paciotti et al., 2001, and above). It is likely that other mutants may also separate these functions. For example, the *mrc1*Δ mutant is unable to prevent late origin firing and mitosis but has relatively high viability in HU and MMS (Alcasabas et al., 2001) and thus is probably proficient in fork stabilization.

Many anticancer treatments involve drugs or treatments that damage DNA or otherwise interfere with DNA replication. We hope a deeper appreciation of the connections between replication forks and checkpoints will contribute to the understanding of how such drugs work and, therefore, lead to their improvement.

Experimental Procedures

Strains and Media

All yeast strains used in the experiments described were based on W303-1a (*MAT*a *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*) and are described in the Supplemental Data at http://www.molecule.org/cgi/content/full/11/5/1323/DC1. Ddc2 was tagged as described (Melo et al., 2001).

Cell Cycle Synchronization, Flow Cytometry, and Viability

Cell growth and cell cycle blocks with α factor, HU, and nocodazole were as described previously (Diffley et al., 1994; Donovan et al., 1997). Samples for flow-cytometric analysis (FACS) were collected and processed as described previously (Labib et al., 1999). Cycloheximide was used at a concentration of 100 μg/ml.

Cell Extracts and Assays

Yeast protein extracts were prepared from 10^8 TCA-treated cells as described (Foiani et al., 1994). Rad53 was detected with the rabbit polyclonal antibody JDI48 at a dilution of 1/1000. Detection of Cdc6 with the monoclonal antibody 9H85 has been described (Donovan et al., 1997). Density transfer assays were performed and analyzed as described (Tercero et al., 2000). Rad53 in situ kinase assay was performed as described (Pellicioli et al., 1999). The assay to detect intermediates of DNA replication in alkaline agarose gels was performed as described (Santocanale and Diffley, 1998).

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