Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint

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The Rad52 pathway has a central function in the recombinational repair of chromosome breaks and in the recovery from replication stress. Tolerance to replication stress also depends on the Mec1 kinase, which activates the DNA replication checkpoint in an Mrc1-dependent manner in response to fork arrest. Although the Mec1 and Rad52 pathways are initiated by the same single-strand DNA (ssDNA) intermediate, their interplay at stalled forks remains largely unexplored. Here, we show that the replication checkpoint suppresses the formation of Rad52 foci in an Mrc1-dependent manner and prevents homologous recombination (HR) at chromosome breaks induced by the HO endonuclease. This repression operates at least in part by impeding resection of DNA ends, which is essential to generate 3' ssDNA tails, the primary substrate of HR. Interestingly, we also observed that the Mec1 pathway does not prevent recombination at stalled forks, presumably because they already contain ssDNA. Taken together, these data indicate that the DNA replication checkpoint suppresses genomic instability in S phase by blocking recombination at chromosome breaks and permitting helpful recombination at stalled forks.

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Introduction

The genome of eukaryotic cells is particularly vulnerable during the S phase of the cell cycle, when replication forks encounter DNA lesions or natural obstacles such as tightly bound protein complexes and highly expressed genes. Stalled forks are unstable structures that can induce chromosomal rearrangements if they are not properly processed and restarted (Labib and Hodgson, 2007; Tourriere and Pasero, 2007). Arrested forks are detected by a surveillance pathway called the intra-S checkpoint, which coordinate fork repair mechanisms and arrest cell cycle progression (Branzei and

Foiani, 2008). In budding yeast, this checkpoint response is initiated with the recruitment of the checkpoint kinase Mec1 onto RPA-coated single-strand DNA (ssDNA) in a Ddc2-dependent manner (Rouse and Jackson, 2002). Phosphorylation of the checkpoint mediator Mrc1 by Mec1 promotes the hyperactivation of the effector kinase Rad53 (Alcasabas *et al.*, 2001; Osborn and Elledge, 2003). Mec1 and Rad53 execute multiple functions, the most important being the maintenance of arrested forks (Lopes *et al.*, 2001; Tercero *et al.*, 2003). To this end, checkpoint kinases regulate the activity and/or the association of several fork components, including RPA (Brush *et al.*, 1996), DNA polymerases (Pelliccioli *et al.*, 1999; Cobb *et al.*, 2003), MCMs (Cobb *et al.*, 2005) and Exo1 (Cotta-Ramusino *et al.*, 2005; Segurado and Diffley, 2008). These modifications prevent the collapse of arrested forks and the formation of abnormal replication intermediates, including double-strand breaks (DSBs).

DSBs can be repaired by different pathways, the most important being homologous recombination (HR) and non-homologous end joining (NHEJ). Although NHEJ operates throughout the cell cycle, HR is restricted to S and G₂/M phases. Recombination is initiated at DSBs with the nucleolytic degradation of DNA ends to generate 3'-ended ssDNA. This resection process depends on the coordinated action of Mre11, Exo1, Sgs1 and Sae2 (Paques and Haber, 1999; Krogh and Symington, 2004; Llorente and Symington, 2004; Clerici *et al.*, 2005; Mimitou and Symington, 2008; Zhu *et al.*, 2008) and requires high cyclin-dependent kinase (CDK) activity (Aylon *et al.*, 2004; Ira *et al.*, 2004; Huertas *et al.*, 2008; Zierhut and Diffley, 2008). Homology search and DNA-strand exchange is catalysed by the assembly of Rad51-ssDNA nucleoprotein filaments on RPA-coated ssDNA, a process that is mediated by Rad52 and by the Rad55–57 heterodimer. Strand invasion, promoted by the dsDNA translocase Rad54, leads to the synthesis of the DNA sequence disrupted at the DSB (San Filippo *et al.*, 2008).

Recombination-related processes have a central function in the recovery of stalled or collapsed replication forks. Broken forks can be repaired by a single-ended invasion recombination mechanism called break-induced replication (Wang *et al.*, 2004; Llorente *et al.*, 2008). Arrested forks can also be restarted by recombinational repair, a process that has been extensively studied in *Escherichia coli* (Michel *et al.*, 2007). However, the importance of recombination-mediated fork restart mechanisms remains to be established in eukaryotic cells. Indeed, unlike bacteria, eukaryotic genomes contain a large excess of replication origins, which can be used to compensate for stalled forks (Ge *et al.*, 2007; Ibarra *et al.*, 2008). Moreover, although cells mutated for members of the RAD52 epistasis group are hypersensitive to replication inhibitors (Chang *et al.*, 2002; Lundin *et al.*, 2005), it has been difficult to determine whether this pathway is necessary for

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the recovery of stalled forks in S phase or for the repair of residual replication-dependent DSBs in G₂.

Homologous recombination must be tightly regulated to prevent genomic instability in S phase. Central to this regulation are specialized DNA helicases such as Srs2, Mph1 and Sgs1, which modulate different steps of the HR process (Gangloff *et al*, 2000; Krejci *et al*, 2003; Veaute *et al*, 2003; Branzei and Foiani, 2007; Prakash *et al*, 2009). Some of these mechanisms depend on the ubiquitinylation and/or sumoylation of PCNA (Pfander *et al*, 2005; Branzei *et al*, 2006; Davies *et al*, 2008). The DNA replication checkpoint has also been implicated in the regulation of HR. However, it is not clear whether the Mec1 pathway has a positive or a negative role in this process. Thus, recent reports indicate that Mec1 promotes HR in budding yeast by phosphorylating recombination proteins such as Rad55 and Slx4 in response to MMS (Bashkurov *et al*, 2000; Herzberg *et al*, 2006; Flott *et al*, 2007). Similarly, ATR and Chk1 activate HR in mammalian cells by phosphorylating Rad51, Nbs1 and FANCD2 (Sorensen *et al*, 2005; Branzei and Foiani, 2008). In contrast, the checkpoint kinase Cds1 prevents the activity of Mus81 and Rad60 at stalled forks in fission yeast (Boddy *et al*, 2003; Kai *et al*, 2005; Raffa *et al*, 2006). Moreover, live cell imaging studies in budding and fission yeast indicate that Rad52–GFP recombination foci are absent from wild-type cells exposed to hydroxyurea (HU), but accumulate in checkpoint-deficient cells (Lisby *et al*, 2004; Meister *et al*, 2005). These data suggest that HR foci are actively suppressed by the replication checkpoint. Alternatively, recombination foci could be absent from wild-type cells because checkpoint-proficient cells do not accumulate recombinogenic substrates in HU.

To help clarify the effect of the replication checkpoint on recombinational repair during S phase, we have monitored how *Saccharomyces cerevisiae* cells respond to genotoxic agents that generate different types of replication stress. HU is an inhibitor of ribonucleotide reductase that slows down fork progression by reducing dNTP pools (Koc *et al*, 2004; Alvino *et al*, 2007). Methyl methanesulphonate (MMS) induces a stable fork arrest by methylating DNA on N⁷-deoxyguanine and N³-deoxyadenine (Vázquez *et al*, 2008). Importantly, both HU and MMS elicit a robust replication checkpoint response but do not generate DSBs in checkpoint-proficient cells (Lundin *et al*, 2005; Tourriere and Pasero, 2007). In contrast, the topoisomerase I inhibitor camptothecin (CPT) induces checkpoint-blind lesions that are not detected by the replication checkpoint but generate DSBs upon passage of the fork (Redon *et al*, 2003). Finally, the radiomimetic agent Zeocin induces chromosome breaks independently of DNA replication. Using different combinations of these drugs, we show here that both the formation of recombination foci and the recombinational repair of DSBs are actively repressed by drugs that activate the DNA replication checkpoint—such as HU and MMS—but not by CPT. Moreover, we provide direct evidence that recombination occurs during S phase to promote the recovery of stalled forks, regardless of checkpoint activity. Finally, we show that HU and MMS impede the resection of DNA ends in an Mrc1-dependent manner. As ssDNA is already present at stalled forks, we propose that inhibition of resection under replication stress prevents toxic recombination events in S phase without interfering with helpful recombination at stalled replication forks.

Results and discussion

HR foci assemble in cells exposed to Zeocin or CPT, but not to HU or MMS

To monitor the cellular response to different types of genotoxic insults, we first examined the ability of *S. cerevisiae* cells to assemble Rad52 subnuclear foci at Zeocin-induced DSBs. Haploid cells carrying a GFP-tagged version of Rad52 (Lisby *et al*, 2001) were arrested in G₁ with α -factor and were exposed or not to Zeocin. Cells were then either released into S phase or maintained in G₁ for the indicated period of time (Figure 1A). The percentage of cells presenting HR foci was determined microscopically. As reported earlier (Lisby *et al*, 2001, 2004), we measured a 5- to 10-fold increase of spontaneous Rad52 foci upon entry into S phase (Figure 1B), which form at chromosome breaks occurring during normal DNA replication. After Zeocin treatment, we observed a further five-fold increase of Rad52 foci in S-phase cells (Figure 1B). However, Zeocin did not induce Rad52 foci in G₁-arrested cells, which is consistent with the fact that high CDK activity is required for the resection of DNA ends and the formation of RPA-coated ssDNA, the substrate recognized by Rad52 (Lisby *et al*, 2001; Aylon *et al*, 2004; Ira *et al*, 2004).

Next, we investigated whether genotoxic drugs that affect replication fork progression such as HU, MMS and CPT are also able to induce Rad52 foci in S phase. CPT blocks the Top1–DNA cleavable complex and generates nicks that are converted into DSBs upon passage of the replication fork (Pommier, 2006). As expected, we found that CPT induces the formation of Rad52 foci to a level comparable to Zeocin (Figure 1C). In contrast, the fraction of cells presenting Rad52 foci after HU or MMS exposure was even lower than in untreated cells (Figure 1C). The lack of Rad52 foci could reflect the fact that HU- and MMS-treated cells are arrested too early in S phase to be competent for HR. To address this possibility, we monitored the ability of cells arrested at the *CDC7* execution point to assemble HR foci at DSBs induced by Zeocin. The Cdc7 kinase is essential for the activation of replication origins and *cdc7-4* mutants accumulate at the G₁/S transition at 37°C with an unreplicated genome and elevated CDK activity (Bousset and Diffley, 1998). Interestingly, we found that *cdc7-4* cells are equally able to form Rad52 foci when arrested at the G₁/S transition or released into S phase in the presence of Zeocin (Figure 1D and E). Taken together, these data indicate that exit from G₁, but neither the initiation of DNA replication nor the presence of homologous sequences, is required to form recombination foci at Zeocin-induced DSBs.

HU and MMS prevent the formation of HR foci at DSBs induced by Zeocin

Our data indicate that CPT induces Rad52 foci in S phase, but HU or MMS do not (Figure 1C). This difference could be due to the fact that unlike CPT, HU and MMS do not generate DSBs in checkpoint-proficient cells (Sogo *et al*, 2002; Lundin *et al*, 2005). To test this hypothesis, we induced DSBs with Zeocin prior to the addition of HU, MMS or CPT and we monitored cells' ability to assemble Rad52 foci. As shown in Figure 2A, addition of CPT did not prevent HR foci assembly in cells previously exposed to Zeocin. In contrast, formation of Rad52 foci at Zeocin-induced breaks was strongly suppressed by HU or MMS. As HU and MMS, but not CPT, elicit a

Figure 1 Genotoxic agents differentially affect the formation of Rad52 foci in S phase. **(A)** Overview of the assay. Wild-type cells (PP534) expressing Rad52-GFP were arrested in G₁ with α -factor for 120 min. Half of the culture was exposed to the radiomimetic agent Zeocin (ZEO) for 30 min to generate DSBs. Cells treated with or without Zeocin were either maintained in G₁ or released into S phase for the indicated time. **(B)** The percentage of G₁- and S-phase cells forming spontaneous and Zeocin-induced Rad52 foci was scored as described in Materials and Methods. Representative images of cells showing Rad52-GFP foci are shown. **(C)** Percentage of wild-type cells forming Rad52 foci in response to genotoxic drugs. Rad52-GFP cells were arrested in G₁ and were released into S phase in the absence of drugs (untreated) or in the presence of 200 mM hydroxyurea (HU), 0.033% methyl methanesulphonate (MMS), 20 μ M camptothecin (CPT) or 100 μ g/ml Zeocin (ZEO). The fraction of cells showing Rad52-GFP foci was determined for the indicated time after release from α -factor. **(D, E)** Cells arrested at the G₁/S transition are competent to form Rad52 foci. Thermosensitive *cdc7-4* cells (PP544) expressing Rad52-GFP were arrested in G₁ with α -factor for 120 min and exposed to 100 μ g/ml Zeocin for 30 min in the presence of α -factor. Cells were then released into S phase at the permissive (24°C) or restrictive (37°C) temperature for the *cdc7-4* mutation and the percentage of cells forming Rad52 foci was scored 90 min after release from G₁. Flow cytometry analysis indicates that although *cdc7-4* cells are blocked at the G₁/S transition (D), they are able to assemble Rad52 foci (E).

robust activation of the replication checkpoint (Redon *et al*, 2003), these data suggest that the DNA replication checkpoint prevents the formation of Rad52 foci at DSBs.

Next, we investigated whether HU treatment is sufficient to induce a disassembly of preformed Rad52 foci. G₁-arrested cells were exposed to Zeocin and were released into S phase as described above. HU was either added to the culture immediately after release from G₁ or was added 50 or 90 min later (Figure 2B). Cells were harvested 130 min after release and the fraction of cells presenting Rad52-GFP foci was determined. As shown above, addition of HU immediately after release from G₁ suppressed the formation of HR foci and induced a two-fold reduction of cell viability (Supplementary Figure 1A). However, Rad52 foci were not affected when HU was added 50 min later (Figure 2C). To verify that HU is able to block S-phase progression when added 50 min after release from G₁, we monitored the electrophoretic mobility of chromosomes by pulsed-field gel electrophoresis (PFGE) (Figure 2D). The fact that this mobility was still altered reflects the persistence of unreplicated chromosomes. We therefore conclude that replication fork arrest prevents the formation of HR foci but does not affect their maintenance once formed.

HU inhibits DNA replication by depleting dNTP pools. As dNTPs are also required for HR, we checked whether HU suppresses Rad52 foci by blocking fork progression or by altering dNTP pools. To this end, we generated DSBs with Zeocin in cells arrested at the G₁/S transition with the *cdc7-4* mutation and we measured the capacity of HU treatment to prevent Rad52 foci formation in the absence of DNA replication. Under these conditions, Rad52 foci formed even in the presence of HU, indicating that HU-arrested forks—and not HU itself—inhibit recombination foci (Figure 2E).

HU- and MMS-arrested forks prevent the repair of HO-induced DSBs

As MMS and HU prevent the formation of Rad52 foci, we next investigated whether these drugs also inhibit HR at DSBs. A unique DSB was generated at the *MAT* locus with the HO endonuclease and the ability of HU- or MMS-treated cells to repair this break was monitored by Southern blot hybridization after *StyI* digestion (Sugawara and Haber, 2006). Cleavage of *MATa* with a galactose-inducible HO generates a small HO-cut fragment (C) that is further converted into a larger repair product (R) by gene conversion using the *HMLa* locus as a template (Figure 3A). In untreated or CPT-treated cells, this gene conversion product was detected 2–3 h after HO induction (Figure 3B and C). In contrast, gene conversion was strongly delayed in HU-exposed cells and was completely suppressed after MMS treatment (Figure 3B and C). This repression was further confirmed by semiquantitative PCR analysis of the gene conversion product (Supplementary Figure 2). These data indicate that MMS and HU prevent both the formation of Rad52 foci and the recombinational repair of chromosome breaks.

HU and MMS impede resection at HO-induced DSBs

Homologous recombination is initiated with the resection of DNA ends to generate a 3' ssDNA overhang. To check whether this process is affected by genotoxic drugs, a non-reparable HO cut was induced at the *MATa* locus of donorless cells (Figure 4A) and the 3'–5' resection of DSB ends was

Figure 2 HU and MMS prevent the assembly of Rad52 foci at DSBs. (A) Wild-type cells (PP534) expressing Rad52-GFP were arrested in G₁, exposed to Zeocin as described in Figure 1 and released into S phase for 90 min in fresh medium containing no drug (S), 200 mM HU, 0.033% MMS or 20 μM CPT. The fraction of cells showing Rad52 foci was scored. (B) Wild-type cells expressing Rad52-GFP were arrested in G₁ for 120 min and cells were maintained in G₁ for another 30 min in the presence or absence of 100 μg/ml Zeocin (grey box). Cells were then released into S phase and HU was added to the culture at the indicated time (white boxes). (C) Percentage of cells presenting Rad52-GFP foci. (D) PFGE analysis of chromosome mobility in Zeocin-treated cells exposed to HU at the indicated time points after release from G₁. Averaged values for a representative set of chromosomes are shown. (E) *cdc7-4* mutants (PP544) were arrested in G₁ for 120 min at 24°C and exposed to 100 μg/ml Zeocin for another 60 min at 24°C. Cells were then released into S phase for 90 min in the presence (ZEO + HU) or the absence (ZEO) of 200 mM hydroxyurea. The percentage of cells presenting Rad52 foci is shown.

monitored on Southern blot hybridization (Ira *et al*, 2004). During the resection process, the intensity of the cut fragment (Figure 4B, fragment C) and of adjacent *StyI* fragments (Figure 4B, fragments 2 and 3) diminishes as *StyI* restriction sites are inactivated (Figure 4B; Exp.). To determine whether resection activity varies during the cell cycle and whether it is affected by replication stress, cells were arrested in G₁ with α -factor and were released in the presence of nocodazole or HU to arrest cells in G₂ or in S phase, respectively (Figure 4D). As shown earlier (Ira *et al*, 2004), we observed an efficient resection of the HO DSB in exponentially growing and G₂-arrested cells, but not in cells blocked in G₁ (Figure 4B and C). We also observed a strong inhibition of resection in cells exposed to HU (Figure 4B and C) and to MMS, but not in cells treated with CPT (Figure 4E; Supplementary Figure 3). These

data are consistent with the fact that HU and MMS exposure prevent DSB repair in S phase (Figures 2 and 3), presumably by impeding the formation of 3' ssDNA tails.

***Mrc1*-dependent inhibition of resection in response to replication stress**

Activation of Rad53, the effector kinase of the Mec1 pathway, depends on two mediator proteins called Rad9 and Mrc1 (Alcasabas *et al*, 2001; Gilbert *et al*, 2001). Although Rad9 functions as a general mediator of the DNA damage response, Mrc1 is active only at stalled replication forks (Tourriere and Pasero, 2007). To determine which branch of the intra-S-phase checkpoint is implicated in the regulation of HR, we next monitored Rad52 foci formation in *mrc1Δ* and *rad9Δ* cells. Wild-type and mutant cells were arrested in G₁ with

Figure 3 Cells exposed to HU or MMS are unable to repair HO-induced DSBs. **(A)** Overview of the assay. Early log-phase cultures of wild-type cells (PP723) bearing a GAL-HO construct were exposed to 200 mM HU for 4 h, 0.033% MMS for 2 h or 20 μ M CPT for 3 h prior to HO induction (see Supplementary Figure 1B for DNA content profiles). Expression of the HO endonuclease was induced for 60 min with 2% galactose and repressed with 2% glucose. Genomic DNA was extracted at the indicated time points and was digested with *StyI* (S). The smallest of the two *StyI* fragments (*) generates fragment C (cut) upon cleavage with HO. Homologous recombination with *HML α* generates fragment R (repair). **(B)** Southern blot analysis of DSB repair in untreated cells (ctrl) or in cells exposed to HU, MMS or CPT. **(C)** Relative intensity of the repair band in untreated control cells and in cells exposed to HU, MMS and CPT.

α -factor and treated with Zeocin, as described in Figure 2A. Cells were then released into S phase in the presence or absence of HU and their ability to form Rad52 foci was scored. In wild-type cells and in *rad9 Δ* mutants, we observed a sharp induction of Rad52 foci that was largely reduced upon addition of HU (Figure 5A). In contrast, *mrc1 Δ* cells were unable to repress the formation of HR foci in response to HU (Figure 5A) and to MMS (Figure 5B), indicating that the assembly of Rad52 foci is primarily repressed by the Mrc1-dependent branch of the Mec1 pathway.

It has recently been reported that Mrc1 has a structural function at replication forks that is distinct from its checkpoint function (Szyjka *et al*, 2005; Tourriere *et al*, 2005). To determine which function of Mrc1 is important to regulate HR, we analysed Rad52 foci formation in the separation-of-function allele *mrc1^{AQ}*, which is only defective for the checkpoint function of Mrc1 (Osborn and Elledge, 2003; Szyjka *et al*, 2005). Surprisingly, Rad52 foci were still repressed in HU-treated *mrc1^{AQ}* mutants (Figure 5A), indicating either that Mrc1 prevents HR independently of its checkpoint function or

that the replication checkpoint is still partially active in *mrc1^{AQ}* cells. We favour the second hypothesis as we observed that *mrc1^{AQ}* cells still repress a significant fraction of their late replication origins, including *ARS1212* and *ARS1413* (CA, unpublished results). As Rad9 is partially able to compensate for the checkpoint defect of *mrc1 Δ* mutants (Alcasabas *et al*, 2001), we monitored the assembly of Rad52-GFP foci in *mrc1^{AQ} rad9 Δ* double mutants. We observed a derepression of HR foci formation in HU comparable to *mrc1 Δ* mutants (Figure 5A). We therefore conclude that the checkpoint function of Mrc1 is required to prevent the formation of Rad52 foci in response to replication stress, this function being complemented by Rad9 in *mrc1^{AQ}* cells.

Next, we checked whether Mrc1 is also implicated in the HU-dependent repression of resection. Wild-type and *mrc1 Δ* cells were exposed to HU and resection was monitored at the *MAT α* locus after induction of HO, as described in Figure 4 (see Supplementary Figure 4A for a map of *StyI* fragments at the *MAT α* locus). Interestingly, we found that the HU- and

Figure 4 HU and MMS impede resection of HO-induced DSB. (A–C) Exponentially growing (Exp.) wild-type cells (PP1123) bearing a GAL-HO construct were arrested in G₁ with α -factor, in G₂ with 15 μ M nocodazole or in S phase with 200 mM HU. HO expression was induced as described above and the resection of HO-induced DSB at the MATa locus was monitored by Southern blot hybridization. The rate of disappearance of the HO-cut band (fragment C) and of adjacent Styl fragments (2 and 3) was expressed for each time point as a percentage of the intensity of the initial band. The intensity of resection bands was normalized to the intensity of the *RAD9* gene (*). (D) Flow cytometry analysis of DNA content in exponentially growing cells and in cells arrested in G₁, G₂ or with HU. (E) Southern blot analysis of the resection rate of HO-induced DSB in the presence of 0.033% MMS or 20 μ M CPT.

MMS-dependent inhibition of DSB resection was strongly reduced in *mrc1Δ* mutants, to a level comparable to CPT-treated cells (Figure 5C–E). Taken together, these data indicate that the DNA replication checkpoint prevents HR under replication stress by impeding the formation of 3' ssDNA overhangs at DSBs.

Rad51 is required for the recovery of stalled replication forks

Our observation that HR is suppressed under replication stress is in apparent contradiction to the fact that mutants of the Rad52 pathway are exquisitely sensitive to agents inducing fork arrest (Chang *et al*, 2002). It has been proposed that HR is repressed at stalled forks in checkpoint-proficient cells but is still required to repair residual DSBs after the completion of S phase. Alternatively, the Rad52 pathway could have a function at stalled forks that is distinct from DSB repair and that is not repressed by the replication checkpoint. The view that recombination is active at stalled forks under replication stress is supported by a recent report showing that replication forks progress at a very slow rate in MMS-treated *rad52Δ* cells (Vázquez *et al*, 2008). To test whether recombination is essential to complete S phase after MMS exposure, DNA replication was monitored in *rad51Δ* cells using a variety of assays, including PFGE, 2D

gel electrophoresis and DNA combing. Wild-type and *rad51Δ* cells were arrested in G₁ and released synchronously into S phase in the presence of BrdU and MMS. Cells were then released from MMS, and DNA samples were processed as described earlier (Luke *et al*, 2006; Zaidi *et al*, 2008). As shown in Figure 6A, chromosomes do not migrate into the PFGE gel after release from G₁ due to the presence of replication intermediates but complete replication in wild-type cells ~90 min after removal of the drug. In *rad51Δ* cells, chromosomes failed to reenter the gel even 130 min after release (Figure 6A and B), indicating that Rad51 is essential to complete DNA replication after MMS exposure.

To confirm that the altered electrophoretic mobility of chromosomes in *rad51Δ* cells reflects the persistence of stalled forks, we followed the kinetics of fork recovery at the level of individual molecules by DNA combing. DNA fibres were stretched on silanised coverslips, and BrdU-labelled sites were visualized by immunofluorescence (Figure 6C). The percentage of replication was determined for each individual DNA fibre and the number of unreplicated gaps was scored. This analysis confirmed that *rad51Δ* cells are unable to complete S phase after MMS treatment because of the persistence of unreplicated gaps (Figure 6D). After 130 min, ~40 gaps per haploid genome were detected in *rad51Δ* cells, which is sufficient to explain the

altered electrophoretic mobility of these chromosomes. Using 2D gel electrophoresis, we confirmed the presence of Rad51-dependent recombination intermediates in MMS-treated cells (Figure 6E). These intermediates generate a so-called 'X-spike', which accumulate in MMS-treated *sgs1Δ* cells used here as a positive control (Branzei and Foiani, 2007), but are not detected in *rad51Δ* cells. The presence of this X-spike correlates also with an increase of unequal sister-chromatid recombination events (Supplementary Figure 4B) in a Rad51-dependent manner (Fasullo *et al*, 2001; Duro *et al*, 2008). Taken together, these data indicate that recombination mechanisms operate at stalled forks and are essential to complete S phase after replication stress.

Conclusion

Here, we show that activation of the Mrc1-dependent branch of the intra-S-phase checkpoint prevents HR at DNA DSBs, presumably to suppress HR-mediated genomic instability under replication stress. Yet, we also found that Rad51-dependent recombination events occur at stalled forks and are essential to complete DNA replication after MMS treatment. To reconcile these apparently conflicting observations, we propose that the DNA replication checkpoint differentially regulates recombination at DSBs and at stalled forks. The major difference between these two types of recombination is that DSB repair depends on the formation of 3' ssDNA overhangs, whereas ssDNA is already present at stalled forks and can be used to initiate recombination with the sister chromatid (Fabre *et al*, 2002). By impeding resection, the replication checkpoint would therefore function as a switch to prevent potentially dangerous HR at DSBs and to allow helpful SCR at stalled forks (Figure 7). It has recently been reported that SCR depends on specific factors such as Rtt109, Asf1 and Rtt101, which are fully dispensable for HR (Duro *et al*, 2008). It is therefore possible that SCR and HR take place in different subnuclear compartments and/or operate with different kinetics, which would explain why cells exposed to MMS do not show Rad52 foci. Finally, it is now important to determine how the replication checkpoint prevents the formation of ssDNA at DSBs. Recent evidence indicates that resection of DNA ends is a complex process that depends on the regulation of multiple DNA helicases and exonucleases (Raynard *et al*, 2008). One of these enzymes, Exo1, is repressed by Rad53 to protect stalled forks and uncapped telomeres (Cottar-Ramusino *et al*, 2005; Morin *et al*, 2008; Segurado and Diffley, 2008). Whether Exo1 or other resection factors are also inhibited by Rad53 to suppress HR is an important question that remains to be addressed.

Materials and methods

Strains and growth conditions

Strains used in this study are listed in Supplementary Table I. Cells were grown at 25°C in synthetic complete medium and were arrested in G₁ for 2.5 h with 2 μg/ml α-factor (GenePep, France). Cells were released from G₁ by the addition of Pronase and were resuspended in fresh medium containing 200 mM HU (Sigma), 0.033% methyl methanesulphonate (Sigma) or 20 μM CPT (Sigma).

Figure 5 Mrc1 suppresses the formation of HR foci and ssDNA overhangs in response to replication stress. (A) Wild-type (PP534), *mrc1Δ* (PP388), *rad9Δ* (PP1154), *mrc1AQ* (PP471) and *mrc1AQ rad9Δ* (PP1064) cells were arrested in G₁ and were exposed or not to Zeocin (ZEO) as indicated in Figure 2A. Cells were then released into S phase for 90 min in the presence or absence of 200 mM HU. The percentage of cells containing Rad52-GFP foci is indicated. (B) Percentage of wild-type (PP534) and *mrc1Δ* (PP388) cells forming Zeocin-induced Rad52 foci in S phase after exposure to HU, MMS or CPT. Cells were arrested in G₁, treated with Zeocin and were released into S phase for 90 min in the presence (HU, MMS and CPT) or absence (S) of drugs as described in Figure 1B. (C) Analysis of DSB resection at MATα in untreated *mrc1Δ* (PP736) cells (Exp.) or in cells exposed to HU, MMS or CPT. Experiment was performed as described in Figure 4. (D, E) Resection rate at the HO-cut band (fragment C) and at the adjacent Styl fragment (fragment 2) in untreated *mrc1Δ* cells (Exp.) and in cells exposed to drugs (HU, MMS and CPT). The ratio of intensity of fragments in the presence or absence of HU is indicated for wild-type and *mrc1Δ* cells.

Figure 6 Rad51 is required for the recovery of MMS-induced replication fork stalling. Wild-type (PP633) and *rad51Δ* (PP484) cells were arrested in G₁ with α -factor and released synchronously into S phase in the presence of 400 μ g/ml BrdU and 0.033% MMS for 60 min. Cells were then collected and resuspended in fresh medium containing BrdU for the indicated time after MMS release ($t = 0$). **(A)** PFGE analysis of chromosome mobility in wild-type and *rad51Δ* cells after release from MMS arrest. **(B)** Quantitation of chromosome mobility during release from MMS. **(C)** DNA fibres from wild-type and *rad51Δ* cells treated as indicated above were analysed by DNA combing. Representative DNA fibres from MMS-treated cells are shown. Red: DNA; green: BrdU; white: BrdU channel alone; brackets: unreplicated gaps. Scale bar is 50 kb. **(D)** Quantitation of the percentage of replication and the number of unreplicated gaps per megabyte of genomic DNA in wild-type and *rad51Δ* cells released from MMS arrest. **(E)** 2D gel analysis of replication intermediates in wild-type, *rad51Δ* and *sgs1Δ* cells exposed for 60 min to 0.1% MMS. 2D gel electrophoresis was performed as described, using a probe against the early origin *ARS305* (Tourrire *et al*, 2005). X, X-spike; B, bubble arc; Y, Y arc. Closed and open arrowheads point to the presence or the absence of X-spikes, respectively.

DSBs were induced in G₁ with the addition of 100 μ g/ml Zeocin (InvivoGen). Thermosensitive *cdc7-4* cells were arrested at the G₁/S transition by shifting the culture to 37°C 1 h before release from α -factor. Cell cycle progression was monitored by flow cytometry (FACScan).

Microscopy

All experiments were performed at 25°C to allow the GFP chromophore to form efficiently. Cells were immediately fixed with 4% paraformaldehyde and immobilized on a glass slide with 1.2% low melting agarose (Lisby *et al*, 2004). Images were acquired by

Figure 7 Differential regulation of homologous recombination at DNA double-strand breaks and stalled forks under replication stress. (A) DSBs are efficiently repaired by homologous recombination (HR) during S phase after the formation of 3' ssDNA overhangs. (B) In the presence of HU or MMS, stalled replication forks induce the activation of the DNA replication checkpoint in an Mrc1-dependent manner through the recruitment of the Mec1-Ddc2 complex on RPA-coated ssDNA. Hyperphosphorylated Rad53 functions in *trans* to prevent HR at DSBs, presumably by blocking the formation of ssDNA tails. In contrast, sister-chromatid recombination occurs at stalled forks, which already contain ssDNA (see text for details). SCC, sister-chromatid cohesion.

using a CoolSNAP HQ CCD camera mounted on a Leica DM6000B microscope. For each field of view, eight fluorescent images were acquired at 0.5-μm intervals along the z-axis. Rad52-GFP foci were counted by inspecting all focal planes for at least 300 cells. To stain genomic DNA, 10 μg/ml DAPI was added to the culture 30 min before imaging.

Analysis of HO cleavage, repair and DNA resection

DNA was extracted from cells before and after HO induction and digested with *StyI* as described (Sugawara and Haber, 2006). The digested DNA was separated on 1% agarose gel, blotted to GeneScreen nylon membranes (NEN) and probed with radiolabelled probe as indicated in Figure 3A. Bands were quantitated using a Typhoon Trio (Amersham GE) and signals were normalized to a *StyI* fragment containing the *RAD9* gene. The same protocol was used for the analysis of ssDNA resulting from strand resection, except that *StyI* fragments were run on a 1% agarose gel in denaturing conditions (50 mM NaOH and 1 mM EDTA) as described (Sugawara and Haber, 2006).

Dynamic molecular combing

DNA combing was performed as described earlier (Michalet *et al*, 1997; Versini *et al*, 2003). BrdU was detected with a rat monoclonal antibody (BU1/75; AbCys; 1/20) and a secondary antibody-coupled Alexa 488 (Molecular Probes; 1/50). DNA molecules were counter-stained with a mouse monoclonal antibody against ssDNA (MAB3034; Chemicon; 1/500) and an anti-mouse coupled to Alexa 546 (Molecular Probes; 1/50). Images were recorded on a Leica DM6000B microscope and were processed as described (Pasero *et al*, 2002). For each experiment, the total length of unreplicated gaps (red-only stretches larger than 5 kb) was measured for

>20 Mb of randomly selected DNA fibres and was expressed as an average number of gaps per megabyte of total DNA. The percentage of replication was determined as the ratio of the total length of replicated stretches to the total length of unreplicated DNA gaps.

Unequal sister-chromatid exchange assay

Unequal sister-chromatid exchange was measured as described earlier (Fasullo *et al*, 2001). Briefly, cells were grown in rich medium with glucose and adenine (YPAD) to 5×10^6 cells per ml and then exposed for 45 min to 0.02% MMS. Here, 2×10^6 cells were plated on solid synthetic medium without histidine (SC-His) agar and 200 cells on YPAD plates. After 3 days at 30°C, colonies were counted. Relative unit represents the number of His⁺ colony per 200 YPAD colonies. Values represent the average of three independent experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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