The EMBO Journal (2008) 27, 1378–1387 | © 2008 European Molecular Biology Organization | All Rights Reserved 0261-4189/08
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THE EMBO JOURNAL

Mus81 is essential for sister chromatid recombination at broken replication forks

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Recombination is essential for the recovery of stalled/collapsed replication forks and therefore for the maintenance of genomic stability. The situation becomes critical when the replication fork collides with an unrepaired single-strand break and converts it into a one-ended double-strand break. We show in fission yeast that a unique broken replication fork requires the homologous recombination (HR) enzymes for cell viability. Two structure-specific heterodimeric endonucleases participate in two different resolution pathways. Mus81/Eme1 is essential when the sister chromatid is used for repair; conversely, Swi9/Swi10 is essential when an ectopic sequence is used for repair. Consequently, the utilization of these two HR modes of resolution mainly relies on the ratio of unique and repeated sequences present in various eukaryotic genomes. We also provide molecular evidence for sister recombination intermediates. These findings demonstrate that Mus81/Eme1 is the dedicated endonuclease that resolves sister chromatid recombination intermediates during the repair of broken replication forks.

The EMBO Journal (2008) **27,** 1378–1387. doi:10.1038/emboj.2008.65; Published online 3 April 2008
Subject Categories: genome stability & dynamics
Keywords: recombination; replication collapse; sister chromatid recombination

Introduction

Increasing evidence from bacteria to mammalian cells indicates that the DNA replication period is the most active phase of the cell cycle for homologous recombination (HR) repair in non-pathological conditions. When the replication fork collides with an unrepaired single-strand break (SSB), a polar one-ended double-strand break (DSB) is formed. Such a polar one-ended DSB is *a priori* not a substrate for non-homologous end-joining (NHEJ), for single-strand annealing or for the classical double-strand break repair pathways, as in these processes two free double-stranded ends are required. It is generally accepted that a one-ended DSB uses the intact sister chromatid for repair, as it is the closer homologous sequence and may simultaneously repair the broken end and restore the fork structure, allowing replisome reassembly and replication to restart. The current understanding of how disintegrated replication forks are repaired by HR comes from genetic, biochemical and cytological studies in diverse organisms (reviewed by McGlynn and Lloyd, 2002; Vilenchik and Knudson, 2003; Lisby and Rothstein, 2004). The product of HR between sister chromatids is genetically invisible, because DNA replication by definition produces two identical chromosomes. Cytogenetic studies have strongly suggested that sister chromatid repair occurs through HR, although the evidence is indirect. Duplicated or repetitive intact homologous sequences, when they are available, can also be used for repair. However, utilization of an ectopic homologous sequence is a venturesome issue in regard to genetic stability and can occasionally result in genome rearrangements or loss of heterozygosity in diploid cells. The synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR) pathways are thought to follow similar HR initiation events and involve DNA synthesis in the repair process. For BIR, DNA synthesis can proceed several hundred kilobases to the telomere end (Lydeard *et al*, 2007; Smith *et al*, 2007) and, for SDSA, the newly synthesized strands must be able to anneal to a complementary sequence in a way that precludes crossing over (see, for review, Pâques and Haber, 1999).

The HR proteins in the *S. cerevisiae* Rad52 epistasis group (Rad50, Mre11$^{S.p.Rad32}$, Xrs2$^{S.p.Nbs1}$, Rad52$^{S.p.Rad22A}$, Rad51$^{S.p.Rhp51}$, Rad54$^{S.p.Rhp54}$, Rad57$^{S.p.Rhp57}$, Rad55$^{S.p.Rhp55}$, S.p. means *Schizosaccharomyces pombe*, which is used in this work) are recruited to sites of DSBs during the S and G2 phases of the cell cycle to form a nucleoprotein filament (see, for review, Krogh and Symington, 2004). Once assembled, the nucleoprotein filament is competent to search, pair and eventually exchange DNA with an intact homologous double-stranded DNA molecule. The joint molecule or D-loop, when stabilized, can promote DNA repair synthesis. In humans, the Rad51C- and XRCC3-containing complex, a probable counterpart of Rhp55/Rhp57 (Tsutsui *et al*, 2000), has been shown to be involved in Holliday junction (HJ) processing in mammalian cell-free extracts (Liu *et al*, 2004). Recently, in fission yeast, other Rhp51 mediator complexes have been identified. Swi5$^{S.c.SAE3}$/Sfr1$^{S.c.MEI5}$ (S.c. means *S. cerevisiae*) is involved in global HR repair and Swi5/Swi2 is dedicated to mating-type (MT) switching in fission yeast (Egel *et al*, 1984; Akamatsu *et al*, 2007).

The last activity for DNA repair is provided by the structure-specific endonucleases from the XPF family, the heterodimer Swi9$^{S.c.RAD1}$/Swi10$^{S.c.RAD10}$ (homologous to XPF/ERCC1 in mammals) (Rodel *et al*, 1997) and the heterodimer Mus81/Eme1$^{S.c.MMS4}$ (Boddy *et al*, 2000; Interthal and Heyer, 2000). *In vitro*, Rad1/Rad10 has a major role in numerous

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Received: 13 November 2007; accepted: 6 March 2008; published online: 3 April 2008

DNA repair pathways and has been implicated in the SDSA pathway in *S. cerevisiae* and *S. pombe* by cleaving heterologous 3′ tails from branch intermediates (Pâques and Haber, 1999). *In vitro*, the Mus81/Eme1 complex (called Mus81) cleaves a variety of branched molecules, including D-loops, nicked HJs and also intact HJs, although less efficiently (Boddy *et al*, 2001; Gaillard *et al*, 2003; Osman *et al*, 2003; Gaskell *et al*, 2007).

We have used the fission yeast *S. pombe* to study the fate of a unique broken fork of replication at a specific locus. In *S. pombe*, a stable, site- and strand-specific DNA lesion has been found at the MT locus, *mat1* (Arcangioli, 1998). The lesion was described as an SSB, with 3′OH and 5′OH termini, or with one or two ribonucleotides (Kaykov and Arcangioli, 2004; Vengrova and Dalgaard, 2004). In the following report, we will refer to the lesion as an SSB for simplicity.

The polarity of *mat1* DNA replication is controlled by a strong replication block (*RTS1*) on the proximal side of the *mat1* locus constraining *mat1* to be replicated in a unique direction. In this configuration, the *mat1* pause site (*MPS1*) localized distal to *mat1* will be proficient for SSB formation (Dalgaard and Klar, 2001). By using an inducible MT switching system, it was shown that the SSB appears on the neosynthesized lagging strand during *mat1* DNA replication and remains stable. During the following round of DNA replication, the leading-strand DNA polymerase converts the SSB into a polar blunt-ended DSB (Kaykov *et al*, 2004; Holmes *et al*, 2005; Figure 1A and Supplementary Figure S3). The fate of the polar DSB will depend on the presence or absence of homologous sequences for repair. In the wild-type strain, the silent donor alleles (*mat2*P and *mat3*M), embedded in the heterochromatin, provide intact DNA templates for recombinational repair, allowing MT switching (Figure 1B; reviewed by Egel, 2005; Arcangioli *et al*, 2007). In the absence of donor loci, the steady-state SSB level is similar to the level observed in wild-type cells and the donorless strain is perfectly viable, indicating an alternative repair process (Klar and Miglio, 1986).

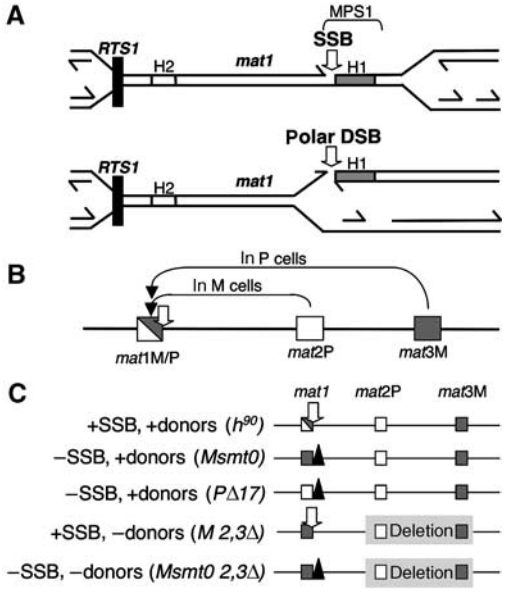

Figure 1 Replication of the *mat1* locus and MT loci of strains used. (**A**) A site- and strand-specific break, SSB (white arrow), at *mat1* is shown. *RTS1* at the proximal side of *mat1* constrains replication in a unique direction. During replication, the leading strand converts the SSB into a polar DSB. (**B**) In the wild-type strain, the DSB allows MT switching: *mat2P* (white square) provides the intact DNA template in M cells and *mat3M* (grey square) in P cells. (**C**) We used five tester strains with (+SSB) or without (−SSB) the SSB, shown as a white arrow and black triangle, respectively, and with (+donors) or without (−donors) silent donor loci.

In this report, we provide genetic evidence that, in the absence or presence of homologous DNA sequences, the HR machinery is essential for viability when the replication fork collides with the unique *mat1*-SSB. Furthermore, we show that Mus81 is dispensable for MT switching, but essential when the sister chromatid is used for repair; conversely, Swi10 is dispensable in the absence of the silent donors loci, but essential for MT switching. The mechanism of choice/exclusion of one or the other nuclease is unknown. Consistent with these results, we found that Mus81 accumulates *in vivo* at *mat1* in an SSB-dependent manner in the absence of donors. Furthermore, an inducible SSB formation system allowed us to observe the accumulation of sister recombination intermediates in the absence of Mus81.

Results

The Rad22A epistasis group is essential for MT switching

To determine the contribution of the HR gene products when a replication fork encounters the SSB at *mat1*, we analysed the phenotypes of several HR mutants in four related strains, with or without (+/−) the SSB and with or without (+/−) donors (*mat2*P and *mat3*M), which serve as template donor for repair. The strains used in this study are as follows: the wild-type or h^{90} strain (+SSB, +donors); the *mat1-Msmt-0* strain (−SSB, +donors), in which *mat1*-M contains a deletion of the *cis*-acting elements essential for SSB formation (Styrkarsdottir *et al*, 1993); the *mat1-M(2,3Δ)* strain (+SSB, −donors), in which *mat1*-M contains a deletion of the *mat2*-P and *mat3*-M silent donors (Klar and Miglio, 1986); and the *mat1-Msmt-0(2,3Δ)* strain (−SSB, −donors), which combines the modifications of the two previous variants (Dalgaard and Klar, 1999) (Figure 1C). These four tester strains are isogenic, fully viable and have similar growth rates.

First the *rad22A*, *rhp51*, *rhp54*, *rhp50*, *exo1*, *rhp57* and *swi5* null mutations have been introduced into the stable *mat1-PΔ17* strains, containing a similar deletion as the *mat1-Msmt-0* strain inhibiting break formation, except that *mat1* expresses the P allele (Arcangioli and Klar, 1991). Then, we crossed the *mat1-PΔ17* strains, containing the null mutations, with the wild-type h^{90} strain. Following mating, several diploids were selected, incubated on sporulation plates and four spores from several diploids were dissected and analysed. MT switching efficiency was monitored by the iodine vapour staining method. Iodine stains black the starch produced before sporulation and therefore indirectly indicates the MT switching efficiency in individual colonies. The results obtained are listed in Table I and representative tetrads from the *rhp51*Δ cross are shown in Figure 2A, left panel).

*rhp22A*Δ, *rhp51*Δ and *rhp54*Δ mutants do not form colonies in the wild-type h^{90} strain, as already shown for *rhp22A*Δ (Ostermann *et al*, 1993), although they are viable in *mat1-*

Table I Phenotypes of the mutants studied in this work

S. pombe	Orthologues		Mutants		Comment
	S. cerevisiae	Human	+SSB, +donors	+SSB, −donors	
Rhp51	RAD51	RAD51	Lethal	Lethal	Search for homology
Rad22A	RAD52	RAD52	Lethal	Lethal	
Rhp54	RAD54	RAD54	Lethal	Lethal	
Rhp57	RAD57	XRCC3	sd	ok	
Swi5	SEA3	AAH21748.1	sd	ok	
Rhp57–Swi5			Co-lethal	Co-lethal	
Rad50	RAD50	RAD50	sg sd	sg	DSB processing
Exo1	EXO1	EXO1	ok	ok	
Rad50–Exo1			Microcolony	Co-lethal	
Swi8	MSH2	MSH2	sd	ok	MMR
Swi10	RAD10	ERCC1	sd	ok	Endonuclease
Mus81	MUS81	Mus81	ok	Lethal	
Mus81–Swi10			sg sd	Lethal	
Rad2	RAD27	FEN1	ok	ok	
Slx1(Slx4)	SLX1(4)	GIYD1(nf)	ok	ok	
Ku70	YKU70	KU70	ok	ok	NHEJ
Rqh1	SGS1	BLM, WRN	ok	ok	Helicases
Srs2	SRS2	nf	ok	ok	
Fbh1	nf	FBH1	ok	ok	
Pfh1	PIF1, RRM3	PIF1	sd	ok	
Rqh1–Top3	SGS1-TOP3	BLM/WRN-TOP3A	ok	ND	
Rad3	MEC1	ATR	ok	ok	Checkpoint
Tel1	TEL1	ATM	ok	ok	
Cds1	RAD53	CHK2	ok	ok	
Chk1	CHK1	CHK1	ok	ok	
Crb2	RAD9	53BP1	ok	ok	
Dcc1	DCC1	DCC1	ok	ok	RFC-like
Elg1	ELG1	ELG1	ok	ok	

sg: slow growth, sd: switching defect, ok: switching and growth similar ±SSB, ND: not determined, nf: not found.

Msmt-0 and *mat1-PΔ17* backgrounds. Further observation of the germinating spores showed that the mutated h^{90} cells elongated and eventually divided, but never formed visible colonies (data not shown). Altogether, these results indicate that the presence of the SSB at *mat1* leads to cell death after few cell divisions in the absence of HR gene products.

In contrast to the three mutants described above, the h^{90} *rad50*Δ or h^{90} *mre11*Δ (data not shown) mutant forms small colonies. However, these colonies contain many dead cells and few spores. The h^{90} *exo1*Δ mutant is viable and does not exhibit MT switching defects, whereas the double h^{90} *rad50*Δ *exo1*Δ mutant is not viable, but can eventually form micro-colonies (Figure 2B, left panel). We next analysed the Rhp51 mediators, Rhp57- and Swi5-containing complex (Akamatsu *et al*, 2003, 2007; Hope *et al*, 2007). The h^{90} *rhp57*Δ strain produces colonies with a mild defect in MT switching and the h^{90} *swi5*Δ mutant produces healthy colonies but MT switching is drastically reduced (Egel *et al*, 1984; Jia *et al*, 2004). However, the h^{90}*rhp57*Δ *swi5*Δ double mutant is not viable (Figure 2C). Interestingly, upon re-streaking, the h^{90} *rhp57*Δ cells progressively produced colonies defective in MT switching, indicating that Rhp57 participates in efficient MT switching. The *mat1* sequences of several independent streaky and white *rhp57*Δ colonies were sequenced and found to contain the same mutation, in which 8 bp of the H1 distal sequence from *mat2P* was transferred to *mat1* (Figure 2D). As expected, *mat1-Msmt-0* and *mat1-PΔ17* strains, which do not exhibit SSBs, are fully viable regardless of the mutant status. Altogether, these results clearly show that in the wild-type h^{90} strain, the major players of the HR pathway of DSB repair are also essential for MT switching.

The Rad22A epistasis group is essential for sister chromatid recombination

In the *mat1-M(2,3Δ)* donorless strain (+SSB, −donors), the SSB is observed at wild-type levels and cells are perfectly viable. This observation clearly indicates that MT switching is not the only possible outcome for repair (Klar and Miglio, 1986). We genetically investigated the role of the HR gene products in the absence of donor loci. All of the single mutants and combinations of mutants studied above exhibit similar viabilities as observed for the wild-type h^{90} strain (Table I; Figure 2A–C, right panels). Altogether, these results provide evidence that only one unrepaired SSB, subsequently transformed into a DSB during the replication period, requires the HR enzymes for repair. In the presence of the donors, HR uses the opposite donor allele for repair, and in the absence of donors, HR uses the sister chromatid, which is the only homologous template available for repair. Because the converging replication fork coming from the centromere-proximal side of *mat1* is arrested by the *RTS1* element, independently of the presence of the donors (Dalgaard and Klar, 2000), the HR repair process must re-establish a replication structure suitable to restart and complete *mat1* DNA replication.

Mus81 is not required for MT switching but is essential for sister chromatid recombination

Genetic epistasis studies with the *rad22* group have suggested that Mus81 also participates in HR pathway to properly replicate broken DNA (Boddy *et al*, 2000). Consequently, we introduced the *mus81*Δ mutation into the two strains containing the SSB with or without donors to analyse viability and/or MT switching of the segregants. The

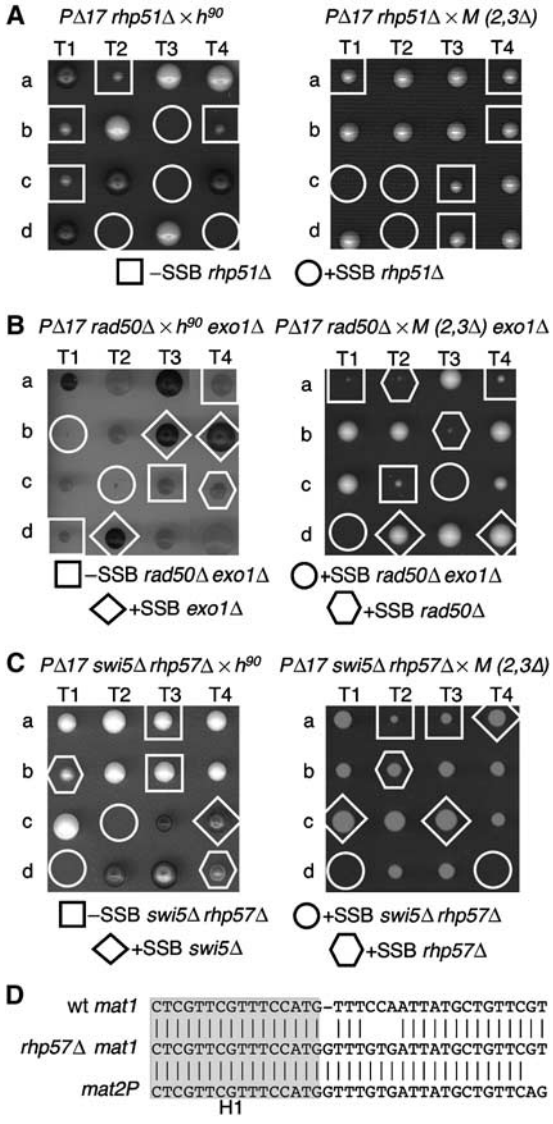

Figure 2 The Rad22A epistasis group is essential for MT switching and sister chromatid recombination. (**A**) Tetrad dissections of *PΔ17 rhp51Δ* crossed with h^{90} (left) or with *M(2,3Δ)* (right). (**B**) Tetrad dissections of *PΔ17 rad50Δ* crossed with h^{90} and *M(2,3Δ) exo1Δ* mutants. (**C**) Tetrad dissections of *PΔ17 swi5Δ* crossed with h^{90} and *M(2,3Δ) rhp57Δ* mutants. A circle, rhombus or hexagon surrounds mutants of interest with the SSB (h^{90} or *M(2,3Δ)*), whereas squares surround mutants without the SSB (*PΔ17*). (**D**) Alignment of *mat1* distal and *mat2P* distal sequences from wild type and *rhp57Δ* variants with a stable MT locus.

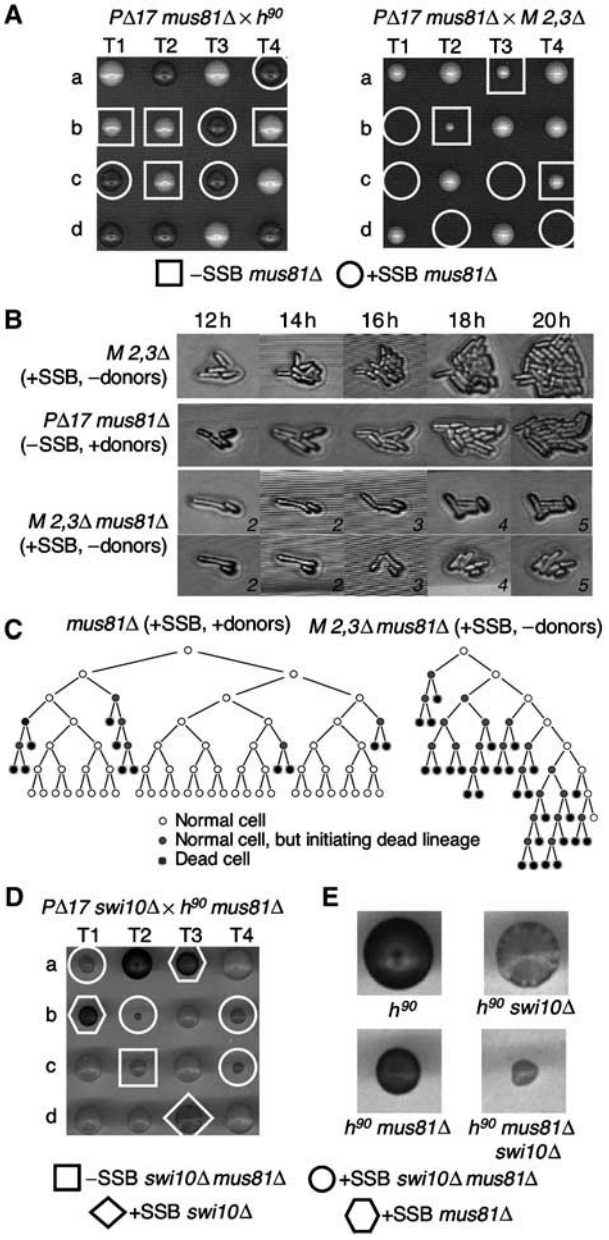

Figure 3 Mus81 is essential for sister chromatid recombination. (**A**) Tetrad dissections of *PΔ17 mus81Δ* crossed with h^{90} (left) or *M(2,3Δ)* (right). The circles and the squares surround *mus81Δ* mutants with (h^{90} or *M(2,3Δ)*) or without (*PΔ17*) SSB, respectively. (**B**) Time course of germinating wild type and *mus81Δ* mutants with (*M(2,3Δ)*) or without (*PΔ17*) the SSB. Numbers of cells are indicated for *mus81Δ M(2,3Δ)* mutants. (**C**) Pedigree of the *mus81Δ* mutant with or without donors. The empty circles indicate dividing cells, the grey circles indicate elongated cells and the black circles indicate undividing cells. (**D**) Tetrad dissections of h^{90} *mus81Δ* crossed with *PΔ17 swi10Δ*. A circle, rhombus or hexagon surrounds mutants of interest with the SSB (h^{90} or *M(2,3Δ)*), whereas squares surround mutants without the SSB (*PΔ17*). (**E**) Iodine staining of wild-type, *swi10Δ*, *mus81Δ* and *swi10Δ mus81Δ* h^{90} colonies, after 4 days at 33°C and 4 days at 25°C.

h^{90}*mus81Δ* mutant strain produces homogeneous iodine-black colonies (Figure 3A, left panel), whereas the germinating donorless *mat1-M(2,3Δ) mus81Δ* mutant barely produces visible colonies (Figure 3A, right panel). Similar growth defects were observed with the nuclease-dead *mus81-DD* mutant (Boddy *et al*, 2001), demonstrating that Mus81 endonuclease activity is essential for viability in the *mat1-M(2,3Δ)* (+SSB, −donors) background (data not shown). We microscopically observed the germinating spores of *mat1-M(2,3Δ) mus81Δ* from the tetrad dissection plates and showed that the germinating cells elongate, indicating an active cell cycle checkpoint arrest and they eventually divide with a quasi-linear division mode (Figure 3B). An example of the lineage of *mus81Δ* strains, with or without donors, is presented (Figure 3C, right panel) and shows that only one of the two daughter cells inherits the potential to grow. Knowing that the SSB is formed on only one of the two *mat1*-containing chromatids during DNA replication, we conclude that the dividing daughter cell follows the segregation of the unbroken *mat1* chromatid, whereas its daughter, inheriting

the broken *mat1* chromatid, rapidly dies in the absence of Mus81. These findings showed that the Mus81 endonuclease complex is necessary at the collapsed replication fork for sister chromatid recombination repair to complete replisome reassembly, as proposed previously (reviewed by Whitby, 2004). Notably, Rad2$^{S.c.RAD27}$ (5′–3′ flap exo/endonuclease), required for Okazaki fragment processing, and the Slx1/Slx4 structure-specific endonuclease required for maintaining ribosomal DNA (Mullen *et al*, 2001; Coulon *et al*, 2004) are not necessary for viability and MT switching (Table I).

The h^{90} wild-type strain lacking either Swi9$^{S.c.Rad10/ERCC1}$ (also named Rad16) or Swi10$^{S.c.Rad1/XPF}$ endonuclease subunits rapidly produces MT region rearrangements containing duplications and extrachromosomal circles of the MT region, probably resulting from HR resolution errors (Egel *et al*, 1984). Similar results have been observed in strains lacking Swi4$^{S.c.Msh3}$ or Swi8$^{S.c.Msh2}$, two proteins related to the mismatch repair proteins (Egel *et al*, 1984; Fleck *et al*, 1992, 1994). When the *swi10*Δ or *swi8*Δ mutations were genetically introduced into the other three tester strains, no loss of viability or significant slow growth phenotypes were observed (Table I). These results indicated that in the donorless *mat1-M(2,3*Δ*)* (+SSB, −donors) strain, the Swi9/10 nuclease and Swi4/8 complexes are not required, or can be replaced by other activities, when the sister chromatid is used for HR repair.

Next, we analysed MT switching and viability of the wild-type h^{90} strain in the absence of both Swi10 and Mus81 by crossing the *mat1-P*Δ*17 swi10*Δ strain with the h^{90} *mus81*Δ mutant strains (Figure 3D). Among the segregants, the h^{90} *mus81*Δ *swi10*Δ double mutant gives rise to small irregular colonies, containing many dead cells, and exhibits a white/pale iodine staining, indicative of an MT switching inhibition associated with deadly recombination events (Figure 3D). Therefore, we analysed by Southern blot the MT region in the single and double mutants and showed that indeed the double mutant exhibits a higher level of DNA rearrangements (Supplementary Figure S1, h^{+N*}). These phenotypes (Figure 3E) are different for both single mutants and indicate that the Mus81 endonuclease might act to prevent further aberrant chromosomal rearrangements, when Swi10 is absent.

RusA allows sister chromatid recombination

Previous work has shown that RusA, an *Escherichia coli* HJ resolvase (Chan *et al*, 1997), suppresses *mus81*Δ phenotypes (Boddy *et al*, 2001; Doe *et al*, 2002). In contrast, RusA does not rescue the genotoxic sensitivities of *swi10*Δ mutant (Doe *et al*, 2002). The experiments described in Supplementary data indicate that RusA does not participate in MT switching in the absence of Swi10. However, RusA suppresses, although partially, the lethality of the *mus81*Δ *mat1-M(2-3*Δ*)* mutant strain, indicating that in the absence of Mus81, HJs accumulate, which can be resolved by RusA.

The Rqh1, Srs2 and Fbh1 helicases, NHEJ and the DNA damage checkpoints

DNA helicases and Mus81 endonuclease are thought to function in different pathways for restarting stalled or collapsed replication forks. Therefore, we analysed the phenotypes of the *rqh1*Δ, *srs2*Δ, *fbh1*Δ, *pfh1*Δ and the *top3*Δ mutations (Morishita *et al*, 2005; Osman *et al*, 2005; Boulé and Zakian, 2006; Osman and Whitby, 2007) in our tester strains. None of the single helicase and essential topoisomerase 3 (in *rqh1*Δ background) mutant strains exhibit MT switching and/or viability defect, except for the *pfh1* mutant where a mild MT switching defect was observed (Table I, data not shown). The absence of any role for the helicases in replication fork repair at *mat1* is consistent with their anti-recombinational function. The *rqh1*Δ *srs2*Δ and *srs2*Δ *fbh1*Δ double mutants exhibit poor growth and *rqh1*Δ *fbh1*Δ double mutant is dead. The growth defect is independent of the presence of the SSB at *mat1* and thus prevents us from testing if these DNA helicases could substitute for each other. To definitively exclude the NHEJ process, the p*ku70*Δ mutation was also introduced into the tester strains and no defects in MT switching or viability were observed. Finally, we analysed the DNA damage checkpoints and showed that the four tester strains are equally viable in the absence of Rad3$^{S.c.MEC1}$, Cds1$^{S.c.RAD53}$, Chk1$^{S.c.CHK1}$ and Crb2$^{S.c.RAD9}$ null mutants (human *ATR*, CHK2, CHK1 homologues and 53BP1-related protein, respectively) and exhibit a wild-type level of MT switching in the h^{90} strain (Noguchi *et al*, 2003; Table I). Taken together, these results indicate that a process not requiring the DNA damage checkpoint efficiently repairs the collapsed fork at *mat1*.

Mus81 interacts with mat1 during sister chromatid recombination

The lethality of the *mus81*Δ *mat1-M(2,3*Δ*)* strain implies that Mus81 is required for resolving sister chromatid recombination intermediates that form during fork recapture. To test this, we used chromatin immunoprecipitation to assay whether Mus81 interacts *in vivo* with *mat1* during the replication period. We used the temperature-sensitive *cdc25-22* mutant in the donorless background (+SSB, −donors) and proceeded through a block and release experiment. We first arrested the cells in the late G2 phase at the nonpermissive temperature of 36°C and allowed cells to re-enter synchronously into the cell cycle at the permissive temperature of 25°C. Then, we followed by chromatin immunoprecipitation (ChIP) the binding of Mcm6, Rad22–YFP and Mus81–FLAG to *mat1* and the flanking replication origins, ars2004 and ars756 (Figure 4A). Upon release from G2 arrest, Mcm6 begins to accumulate at the origins at the 60 min time point to form pre-RC. Subsequently, Mcm6 is detected at the break site at the 90 min time point, indicating replication fork movement. Its persistence at *mat1* for 30–60 min is consistent with an active *MPS1* element. Rad22A is not present at the two origins and appears at the 90 min time point, accompanying the Mcm6 kinetics (Figure 4B). Mus81 also accumulates at *mat1* starting at the 80–100 min time point and stays until the 160 min point (Figure 4C). Importantly, neither Rad22A nor Mus81 associates at *mat1* in the absence of the SSB (*mat1-Msmt-0(2,3*Δ*)* strain), as shown in Supplementary Figure S2. Altogether, these results indicate that Rad22A and Mus81 accumulate rapidly at *mat1* concomitantly with the replication fork collapse and not with the *MPS1* pause, which is still active in the *mat1-Msmt-0(2,3*Δ*)* strain.

Sister replication/recombination intermediates

Having established genetically the essential role of the HR enzymes at a unique collapsed replication fork, we wanted to confirm by Southern blot that *mat1* is still cleaved in several

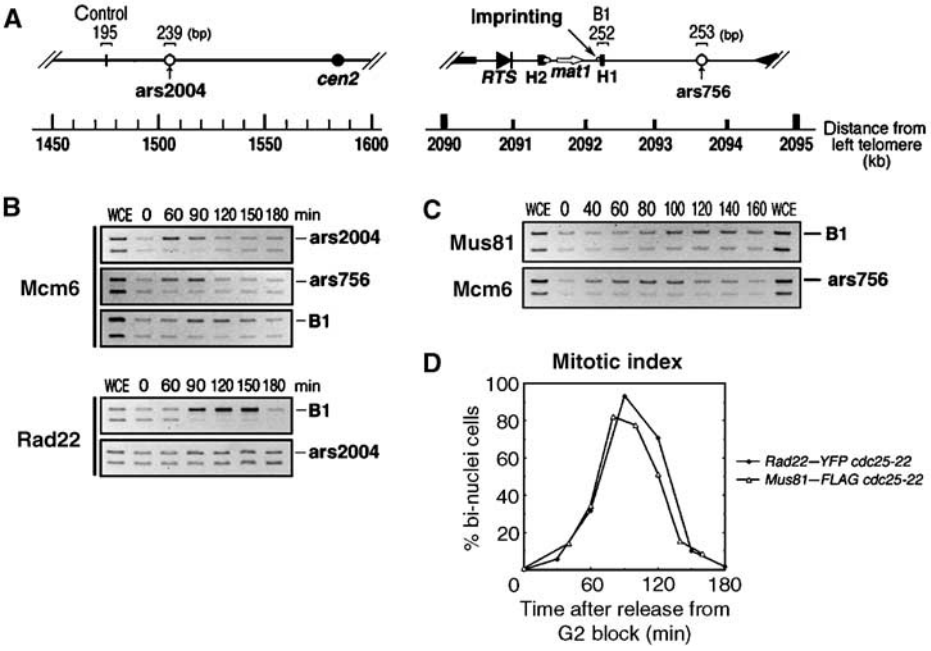

Figure 4 Mus81 accumulates at the collapsed replication fork. Cells with a *cdc25* background are first incubated at 36°C for 3 h to arrest at G2/M and then shifted to 25°C to release from G2/M block. (**A**) Positions of regions studied in this assay are shown along with the distance from the left telomere. PCR is carried out using a combination of two primer sets that amplify either replication origins or H1 (shown as B1) and control region. (**B**) Mcm6 and Rad22A ChIP. Chromatin was immunoprecipitated with anti-Mcm6 antisera and anti-GFP antibody from YYY023 *(-donor Rad22A–YFP cdc25*) as a function of time after G2/M release. DNA was analysed by PCR with the indicated primer sets. DNA amplified from total cellular DNA is shown in lane WCE. (**C**) Mus81 and Mcm6 ChIP. Chromatin was immunoprecipitated with anti-Mcm6 antisera and anti-FLAG antibody from YYY172 (*-donor Mus81-5xFLAG cdc25*). (**D**) Mitotic index was determined by analysing cells with bi-nuclei. YYY023 and YYY172 are represented by filled diamond and open triangle, respectively.

mutant backgrounds (*rhp51*Δ, *mus81*Δ and *rad50*Δ). By introducing the thiamine repressible promoter (*nmt1*) in a neutral region upstream of the *mat1* locus, we can force transcription through the broken strand, repairing the SSB. This inducible system allows us to maintain good viability in the three mutant backgrounds, as long as the *nmt1* promoter is transcribing. Subsequently, by simply adding thiamine to the medium, we can turn off the transcription and follow SSB formation on one of the sister strands during the first *mat1* DNA replication and MT switching during the following DNA replication (Holmes *et al*, 2005). In this experiment, we extracted the DNA following the traditional DNA purification procedure, which breaks the DNA containing SSBs (Arcangioli, 1998). The break is observed in the three mutants, although with kinetics slightly slower than that for the wild type, consistent with their slower generation time (Figure 5A and B).

Next, to identify the replication/recombination intermediates that might accumulate in the absence of Mus81, we used thiamine repressible promoter in the donorless strain system coupled with the 2D gel electrophoresis method (Brewer and Fangman, 1987), which allows the detection of replicating and recombining molecules. In this experiment, low-melting agarose plugs have been used to preserve the replication forks and branched molecules from shearing. The replicating DNA, containing single-stranded regions, was enriched on BND cellulose, making cell cycle synchronization unnecessary. Previous work showed that *mat1* is replicated by a replication fork coming from its distal side and pauses at *MPS1* identified as a spot on the ascending side of the Y-arc (Dalgaard and Klar, 1999, 2000) a few minutes after thiamine addition in this inducible system (Holmes *et al*, 2005; Figure 6A). The position of *MPS1* is consistent with a pause inside H1, located at 1.2 and 0.9 kb from the proximal and distal *Nde*I restriction sites, respectively. Interestingly, we also observed a secondary weaker pause structure just below *MPS1*. The position of this structure is consistent with the Sap1 binding site (SAS1) localized 120 bp distal to *MPS1* (Arcangioli and Klar, 1991), also known to participate in replication fork pausing at the rDNA loci (Krings and Bastia, 2005; Mejia-Ramirez *et al*, 2005). This weak pause

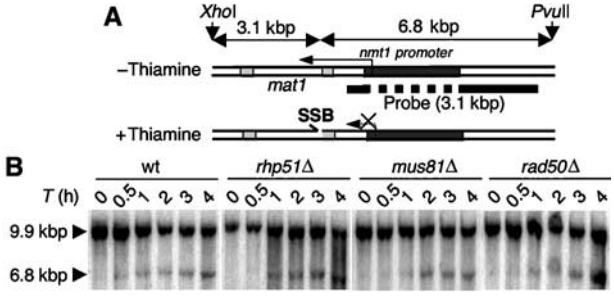

Figure 5 Break formation in *mat1P:nmt1:KAN (2,3*Δ*)* strains. (**A**) Schematic representation of the MT locus in the *mat1P:nmt1:KAN (2,3*Δ*)* strain. The strong *nmt1* thiamine repressible promoter (dark grey box) was inserted just distal to *mat1* (Holmes *et al*, 2005). The transcript is turned on (−thiamine) and the break is repaired at *mat1*. When the promoter is turned off (+thiamine), one can follow the timing of break formation. The size (in kbp) of the *Xho*I–*Pvu*II DNA fragments is indicated and the probe is shown. (**B**) Genomic DNA of wild-type (PB157), *rhp51*Δ (LR95), *mus81*Δ (LR27) and *rad50*Δ (LR164) mutants was digested with *Xho*I and *Pvu*II and analysed by Southern blot.

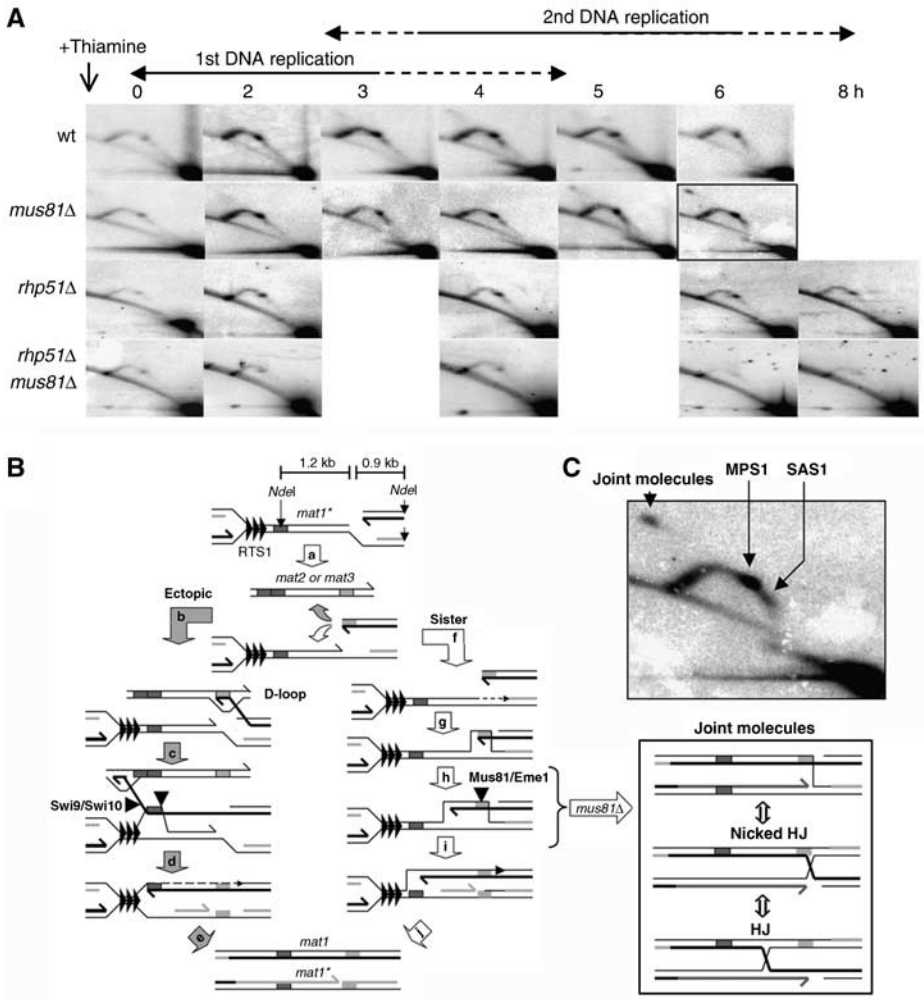

Figure 6 *mus81*Δ strain accumulates sister recombination intermediates. (**A**) DNA from mitotic time courses of PB157 (wt), LR27 (*mus81*Δ), LR95 (*rhp51*Δ) and LR294 (*rhp51*Δ *mus81*Δ) strains, in the absence of donors, was digested with *Nde*I, separated by 2D gel electrophoresis, Southern blotted and probed for *mat1-P*. Double-stranded DNA that migrates as a major spot has been used as reference to standardize the 2D gels. (**B**) Replication–recombination-coupled models at *mat1*, in the presence (left/ectopic) or absence of donors (right/sister). (a) Following formation of the polar DSB, two homologous templates can be used for repair. (b) With donors, the Swi2/Swi5 mediator complex recruits the broken end for strand invasion using the H1 sequence homology box and D-loop formation. (c) Following DNA synthesis copying the opposite silent *mat2* (or *mat3*) sequences, the Swi9/Swi10 endonuclease resolves the recombination intermediate at the non-homologous DNA junctions, allowing DNA synthesis of the second strand and resetting of the replication fork structure. (d) Lagging-strand re-initiation. (e) Generation of unbroken *mat1* switched MT allele and broken *mat1** unswitched allele. (f) Without donors, the sister chromatid is the only available template for HR repair, and DNA synthesis followed by ligation of the imprinted strand is required. (g) Invasion and D-loop formation. (h) Mus81/Eme1 resolves the D-loop and resets the replication fork structure, without crossovers. (i) Lagging-strand re-initiation. (j) Generation of unbroken *mat1* and broken *mat1**. The right panel shows the two possible representations of the *mat1* nicked HJ in *mus81*Δ strain. (**C**) Identity of the molecular intermediates observed by 2D gel analysis.

signal is not observed in the *Msmt*0 mutant, containing a deletion of SAS1 (Dalgaard and Klar, 1999). We also observed DNA accumulating in the descending side of the Y-arc in both the wild-type and *mus81*Δ strains, which might correspond to replication forks slowly restarting from *MPS1* (Vengrova and Dalgaard, 2004), as they are observed after thiamine addition during the first DNA replication, one generation before one-ended DSB formation and HR repair. Strikingly, a new structure forming a dot at the tip of the spike accumulates within 5–6 h in *mus81*Δ strain but not in the wild type (Figure 6C), giving an estimation of the kinetics of this repair process. This new structure is absent in the *rhp51*Δ single and *rhp51*Δ *mus81*Δ double mutant strains (Figure 6A), and this supports the genetic conclusion and indicates that Mus81 acts later in the recombination process. This structure, appearing during the second replication period, is indicative of unresolved sister recombination intermediates that are accumulating in the absence of Mus81. The accumulation of recombination intermediates strongly argues that DNA synthesis/replication restart can occur in the absence of cleavage by Mus81/Eme1.

Discussion

Checkpoint responses to a single polar one-ended DSB

Previous works have shown that the polar DSB is formed when the fork collides at *mat1* with the SSB (Arcangioli, 1998; Kaykov *et al*, 2004; Supplementary Figure S3). The poor viability inflicted by this DSB (in *mre11*Δ or *rad50*Δ mutants), together with the synthetic lethality observed for

the *rad50*Δ *exo1*Δ double mutant, indicates that the function of the MRN complex can be partially substituted by Exo1 (reviewed by Tran *et al*, 2004). Given the fact that recombination factors such as Rhp51 and Rad22 are required for sister chromatid recombination, RPA is also likely to be involved; however, the lack of extensive 5′ to 3′ resection may not generate a strong enough signal for Rad3/ATR checkpoint activation (Zou and Elledge, 2003).

Finally, under physiological conditions, Mus81 associates with chromatin throughout S phase and dissociates from chromatin in a Cds1-dependent manner in the presence of HU, which stalls replication forks but not in the presence of camptothecin (CPT), which breaks replication forks (Boddy *et al*, 2000; Kai *et al*, 2005). Collectively, these data are consistent with the absence of a requirement for the intra-S checkpoint and Cds1 activation during *mat1* replication runoff. This may, in turn, ensure that Mus81 is available for repair if MT switching has failed or if it is impossible, as in the *swi2*Δ, *swi5*Δ or donorless mutant strains.

MT switching process

The Swi6-dependent (heterochromatin protein 1 homologue) positioning/spreading of the Swi5/Swi2 heterodimer at one of the two donor loci, depending on the allele expressed at *mat1*, together with the physical interaction between Swi2 and Rhp51 might allow the capture of the Rhp51 nucleoprotein filament (Thon and Klar, 1993; Akamatsu *et al*, 2003; Jia *et al*, 2004). These interactions have been proposed to direct the choice of the donor to guarantee MT switches, before DNA sequence recognition. A secondary consequence of this developmental bias is also to avoid sister chromatid usage, which would reduce MT switching efficiency. This interpretation is supported by the slow growth phenotype of the *swi5*Δ *mus81*Δ double mutant (data not shown), where the absence of Swi5 mimics to a certain extent the absence of donors, leaving the sister chromatid as the main template for repair, hence requiring Mus81. During MT switching, a second Rhp51 mediator Rhp55/Rhp57 complex is also involved and progressively accumulates mutations at *mat1*. Indeed, h^{90} *swi8*Δ (Fleck *et al*, 1994), h^{90} *rhp55*Δ (Vagin *et al*, 2006) and h^{90} *rhp57*Δ (this work) mutant strains generate identical small 8 bp substitution mutations (in bold in Figure 2D) next to *mat1*, due to another small homology of 12 bp common to *mat1* and *mat2* (Figure 2D). This substitution mutation removes the *cis*-acting element SAS2 next to *mat1* required for efficient SSB formation and switching, stabilizing the mutation (Arcangioli and Klar, 1991; Kaykov *et al*, 2004). These results suggest that the mismatch repair Swi8/Swi4 and Rhp57/Rhp55 mediator complexes work together to stabilize the invading single end allowing for efficient gene conversion. The proposed D-loop structure, joining the H1 sequence of *mat1* with the H1 sequence of the appropriate donor, uses the invading 3′-end as a primer to allow DNA synthesis to proceed through the donor template. As the donor contains the opposite allele of *mat1*, DNA synthesis has to extend to the other end of the silent cassette (about 1 kb) and reach the H2 homologous sequence. The annealing between the two H2 sequences forms a structure with two non-homologous 3′ tails. The new strand can be recognized/stabilized by the Swi4/8 complex and clipped off by Swi9/10 and the old *mat1* strand can also be cleaved by Swi9/10 or degraded by the MRN complex (Figure 6B, left panel).

Recent work indicates that cohesin complexes that hold sister chromatids together are important for equal sister chromatid recombination on plasmids and rDNA loci (Sjogren and Nasmyth, 2001; Unal *et al*, 2004; Cortés-Ledesma and Aguilera, 2006). Here, we do not know if the cohesins are absent or inactive at *mat1* in order to preferentially use the donors instead of the sister chromatid to favour MT switching.

Mus81 resolves sister chromatid recombination

This work does not address the proposed early role of Mus81 at stalled replication forks but instead examines the role of Mus81 after the fork is broken and the HR machinery has engaged. At *mat1*, in the absence of donors when the sister chromatid is used for repair, the Rhp55/57 and Swi5/Sfr1 mediators can replace each other (reviewed by Haruta *et al*, 2008) allowing initial strand invasion and D-loop formation. In *S. cerevisiae*, Mus81 was initially found by two-hybrid analysis to interact with Rad54 (Interthal and Heyer, 2000) and might be positioned very early by Rad54 following D-loop formation. Biochemical studies have shown that Mus81 exhibits a similar DNA structure specificity among different organisms and that its preferred substrate *in vitro* is nicked HJs and D-loops, although intact HJs are also cleaved, but less efficiently (reviewed by Osman and Whitby, 2007). Formally, resolution of the recombination intermediate does not require DNA synthesis from the 3′ invading end and the simplest model proposes a resolution whereby Mus81 cleaves the D-loop (Figure 6B, right panel). As shown *in vitro*, the Mus81 endonuclease subunit from *S. cerevisiae* or *S. pombe* preferentially cleaves 5′ to the junction point (Gaskell *et al*, 2007). Subsequently, the 3′ end of the cleaved molecule, annealed to the initial invading strand, can prime DNA synthesis to accommodate ligation, restoring the replication fork structure and potentially allowing replication restart (Figure 6B, step i). Another alternative, which does not require Mus81 activity at an early step, might be that the D-loop initiates only leading-strand DNA synthesis, whereby full DNA replication of *mat1* will be completed by the release of the leading-strand polymerase blocked at *RTS1* or carrying along re-initiation of the lagging strand as in the BIR process (Lydeard *et al*, 2007). These three models are not exclusive and require a single cleavage by Mus81 of a D-loop or nicked HJ to prevent sister chromatid exchanges. This view is supported by the observation that mammalian Eme1 is not required for sister chromatid exchanges (Abraham *et al*, 2003). The X-shaped structures accumulating in the *mus81*Δ mutant (Figure 6C) indicated that DNA synthesis initiates without Mus81 cleavage, supporting the last two models. A dual specificity (D-loop and nicked HJ) for Mus81 is consistent with the unstable strand invasion intermediates, followed by a hypothetical endonuclease cleavage to establish a stable replication fork during the BIR process (Smith *et al*, 2007). As only 2 kb of DNA synthesis is required before reaching *RTS1*, reconstruction of a mature replication fork might not be necessary. Finally, the nicked HJ shown in Figure 6B (right panel) could branch-migrate in either direction in a Rad54-dependent fashion (Bugreev *et al*, 2006) to form an intact HJ, consistent with the ability of RusA to replace Mus81.

Importantly, *S. cerevisiae and S. pombe mus81* mutants are hypersensitive to CPT but not to ionizing radiation. The drug

CPT works by trapping the catalytic intermediate of the topoisomerase I–DNA complexes and thus introduces SSBs (reviewed by Pommier, 2006). Prolonged incubation with CPT allows collision of the replication forks with the Top1–SSB complexes, leading to the formation of polar one-ended DSBs, revealing the role of Mus81 in DNA replication. In contrast, mouse *mus81*$^{-/-}$ cells are not hypersensitive to CPT, indicating that another endonuclease is able to maintain viability (Liu *et al*, 2004; Dendouga *et al*, 2005). Our work suggests that the ratio of unique and repeated sequences, found in various eukaryotic genomes, determines the utilization of two HR modes of resolution (Mus81 versus Swi10). This may explain, at least in part, the weak sensitivity of *mus81* mutant to CPT in mammals as compared to yeast. During meiosis in fission yeast, Mus81 is required for crossovers (Boddy *et al*, 2001; Osman *et al*, 2003; Smith *et al*, 2003; Cromie *et al*, 2006) induced by DSBs created by Rec12, the *S. pombe* orthologue of Spo11. It was recently proposed that two sequential and asymmetric single-end invasions are cleaved by Mus81 (Cromie *et al*, 2006 and references therein). The resolution by Mus81 of a single nicked HJ, as shown in Figure 6B, does not generate sister chromatid exchanges, whereas the resolution of two independent nicked HJs would lead to one crossover event as observed during meiosis in *S. pombe*.

Materials and methods

Fission yeast strains, media, techniques and plasmids

The strains used are listed in Supplementary Table S1. Media and genetic methods for studying *S. pombe* were as described by Moreno *et al* (1991).

The pREP-rus plasmids are described by Doe *et al* (2002). They express RusA (pMW413) and RusAD70N (pMW415) under the control of the inductive *nmt1* promoter. The h^{90}/*mat1-M(2,3Δ) mus81Δ*/+ diploid strain was transformed with these plasmids.

ChIP assay

ChIP assay was performed as described previously (Ogawa *et al*, 1999). Anti-FLAG antibody (Sigma), anti-GFP antibody (Abcam) and anti-Mcm6 antisera (gift from H Masukata) were used for immunoprecipitation. The nucleotide sequences of the primers used in this study are available on request.

Preparation of S. pombe genomic DNA

DNA was isolated by a classical method (Moreno *et al*, 1991), digested with *Xho*I and *Pvu*II (or with *Hin*dIII in Supplementary Figure S1) enzymes and analysed by Southern blots, using a ^{32}P-labelled *mat1*-distal (or *Hin*dIII—*Hin*dIII *mat1* fragment in Supplementary Figure S1) specific probe.

When required, the genomic DNA was prepared into low-melting agarose plugs. The agarose-embedded DNA was digested overnight with *Nde*I, as recommended by New England Biolabs.

2D gel

The *mat1P:nmt1:KAN (2,3Δ)* strains (PB157, LR27, LR95 and LR294) were grown in Edinburgh minimal medium without thiamine. Following the addition of thiamine, samples were taken at different time points, as described by Holmes *et al* (2005). DNA was prepared and digested with *Nde*I in agarose plugs, and agarose was removed by agarase treatment.

The replicating DNA was enriched on BND cellulose columns and separated by 2D gel electrophoresis (Brewer and Fangman, 1987). Gels were hybridized with a 1 kb *mat1*-P-specific fragment.

Supplementary data

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

Acknowledgements

We thank the scientific community for yeast strains, in particular H Masukata (Osaka University, Japan) for anti-Mcm6 antibody, E Hartsuiker for Rad50s and M Whitby for RusA expression plasmids; we thank A Holmes and B Llorente for critical reading of the manuscript. This work was supported by Grants from the region Martinique, the ARC to LR and ANR-06-BLAN-0271 to BA. YY is supported by a fellowship from The Uehara Memorial Foundation. Work in PR's lab was supported by NIH grant GM59447.

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