Replication fork blockage by *RTS1* at an ectopic site promotes recombination in fission yeast

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Homologous recombination is believed to play important roles in processing stalled/blocked replication forks in eukaryotes. In accordance with this, recombination is induced by replication fork barriers (RFBs) within the rDNA locus. However, the rDNA locus is a specialised region of the genome, and therefore the action of recombinases at its RFBs may be atypical. We show here for the first time that direct repeat recombination, dependent on Rad22 and Rqh1, is induced by replication fork blockage at a site-specific RFB (*RTS1*) within a 'typical' genomic locus in fission yeast. Importantly, when the RFB is positioned between the direct repeat, conservative gene conversion events predominate over deletion events. This is consistent with recombination occurring without breakage of the blocked fork. In the absence of the RecQ family DNA helicase Rqh1, deletion events increase dramatically, which correlates with the detection of one-sided DNA double-strand breaks at or near *RTS1*. These data indicate that Rqh1 acts to prevent blocked replication forks from collapsing and thereby inducing deletion events.

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Introduction

The path of a replication fork is often littered with obstacles that prevent its easy passage. Such obstacles can be accidental, for example lesions in the DNA template or proteins bound to the DNA template, or can be programmed (Rothstein *et al.*, 2000). The appropriate response to these obstacles is important for both efficient DNA synthesis and the maintenance of genome stability.

Proteins that promote homologous recombination play important roles in processing stalled and blocked replication forks (McGlynn and Lloyd, 2002; Michel *et al.*, 2004). For example, they can promote fork reversal where the nascent strands are unwound from their templates and annealed to each other (McGlynn and Lloyd, 2002). Fork reversal may enable polymerases to switch templates and bypass DNA lesions (Higgins *et al.*, 1976), as well as provide room

for DNA repair enzymes to remove blocking lesions in the DNA template (Courcelle *et al.*, 2003). Recombination proteins can also protect the replication fork from excessive nucleolytic attack (Courcelle *et al.*, 2003), and help the restart of replication by generating DNA structures at which replication proteins can reload (Liu *et al.*, 1999). However, the cell has to be circumspect about its use of recombination. Inappropriate recombination can result in genome instability, for example recombination between repetitive DNA elements can result in the expansion and contraction of tandem arrays, or even translocations between different chromosomes. Such events are hallmarks of a number of diseases including cancer.

Studies of replication fork stalling in *Escherichia coli* have established that the way in which a blocked replication fork is processed depends on the type of block (Michel *et al.*, 2004). For example, an ultra violet light (UV)-induced lesion in the leading strand template may be side-stepped by a recombination-mediated transient switch of the polymerase onto the nascent lagging strand (Higgins *et al.*, 1976), whereas a UV-induced lesion in the lagging strand template may be left unreplicated, the resultant daughter strand gap being repaired by recombination postreplication (Rupp and Howard-Flanders, 1968). At other types of stalled or blocked replication fork, it may be sufficient to simply stabilise the fork until the block is removed and DNA synthesis can be resumed. This appears to be true in yeast for forks that are stalled by dNTP depletion mediated by the drug hydroxyurea (HU), which inhibits ribonucleotide reductase. The maintenance of fork stability here depends on the intra-S checkpoint kinase Rad53 (Lopes *et al.*, 2001; Sogo *et al.*, 2002).

Members of the RecQ family of DNA helicases, which includes Sgs1 and Rqh1 in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively, and BLM and WRN in humans, also play crucial roles in maintaining fork stability (Hickson, 2003). The importance of this is underlined by the fact that defects in BLM and WRN give rise to the cancer-prone diseases Blooms syndrome and Werners syndrome, respectively. In *S. cerevisiae*, Sgs1 prevents polymerase dissociation from replication forks stalled by HU treatment (Cobb *et al.*, 2003). RecQ helicases may also reset reversed replication forks by branch migrating Holliday junctions (HJs) that are formed when nascent strands anneal to each other (Doe *et al.*, 2000; Karow *et al.*, 2000). It has been suggested that this activity might prevent replication fork breakage by the XPF-related endonuclease Mus81-Eme1 (Kaliraman *et al.*, 2001; Doe *et al.*, 2002). The ability to branch migrate HJs may also be used by RecQ helicases to abort inappropriate recombination at stalled forks, and, together with Topoisomerase III, to process double HJ intermediates as noncrossover recombinants (Ira *et al.*, 2003; Wu and Hickson, 2003). These activities would reduce the risk of genome rearrangements caused by crossing over between repeated DNA sequences.

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A growing number of programmed replication fork barriers (RFBs) or pause sites have been identified (Rothstein *et al*, 2000). Important roles for RFBs include ensuring the successful merging of opposing replication forks, and promoting the avoidance of collisions between replication forks and transcription complexes (Brewer and Fangman, 1988; Krabbe *et al*, 1997; Rothstein *et al*, 2000). Unlike most accidental blockades, the majority of programmed RFBs are unidirectional. Replication can therefore be completed when the blocked fork merges with the opposing fork. In theory, recombination at such blocked forks would be an unnecessary risk, and therefore should be suppressed. However, like accidental blockades, programmed RFBs can elicit different responses. In *E. coli*, replication forks blocked at *Ter* sites bound by the Tus protein remain stable, and only provoke recombination if a subsequent round of replication runs into the back of them to generate free double-strand ends (Bidnenko *et al*, 2002). In contrast, forks blocked at RFBs in the rDNA locus of *S. cerevisiae* appear to more directly stimulate recombination resulting in the expansion and contraction of the array, as well as the production of extrachromosomal rDNA circles (ERCs) (Kobayashi *et al*, 1998; Weitao *et al*, 2003b; Burkhalter and Sogo, 2004).

Here, we have analysed whether a third RFB, *RTS1*, provokes recombination in *S. pombe*. *RTS1* ensures that replication of the *mat1* locus in *S. pombe* occurs in the centromere-proximal direction (Dalgaard and Klar, 2001; Vengrova *et al*, 2002). This is necessary for laying down an imprint, which stimulates mating-type switching in the next generation (Dalgaard and Klar, 1999). Essential to *RTS1* function are four repeated ~55 bp motifs, whose function is enhanced by an ~60 bp purine-rich region (Codlin and Dalgaard, 2003). The barrier function of these *cis*-acting elements depends on the Swi1–Swi3 complex and the *trans*-acting replication termination factors Rtf1 and Rtf2 (Dalgaard and Klar, 2000).

We show that replication fork blockage by *RTS1* massively stimulates recombination between repeated DNA sequences with a bias toward conservative gene conversion events. This recombination, like that induced by stochastic events, depends on the *S. pombe* homologues of the recombinases Rad51 (called Rhp51) and Rad52 (called Rad22). These data provide the first demonstration that a site-specific replication fork blockage outside of the rDNA locus directly stimulates recombination. We also show that *RTS1*-induced recombination is constrained by Rqh1. In particular, *rqh1*[−] mutants exhibit a dramatic increase in deletion recombinants, which correlates with the detection of one-sided double-strand breaks (DSBs) in the vicinity of the RFB. This provides further evidence that RecQ DNA helicases protect stalled replication forks from collapse.

Results

Agents that stimulate direct repeat recombination

To measure inter/intra-chromosomal recombination in *S. pombe*, we have used a direct repeat of *ade6*[−] heteroalleles with an intervening *his3*⁺ gene positioned at the *ade6* locus on chromosome 3. With this system, *ade6*⁺ recombinants can be divided into those that retain the intervening marker (conversion-types) and those that lose it (deletion-types) (Figure 1A). Under our normal assay conditions, approxi-

mately 3.0–4.5 *Ade*⁺ recombinants arise spontaneously in every 10⁴ viable cells, and typically between 70 and 80% of these are deletion-types (Figure 1B and Table I). Various agents, which are known to impede replication forks, stimulate direct repeat recombination (Galli and Schiestl, 1999). However, a comparison of their affect on the ratio of deletion and conversion-types has not been made. UV, which generates lesions such as pyrimidine dimers that block the normal replicative polymerases, stimulates the formation of conversion-types more than it does deletion-types, altering the ratio of these recombinants from approximately 1:3 to 1:1 (Figure 1B) (Osman *et al*, 2000). In contrast camptothecin (CPT), which inhibits the religation step during the reaction cycle of topoisomerase I and consequently generates single-strand breaks in DNA at which replication forks collapse (Pommier *et al*, 2003), stimulates mostly deletion-type recombinants (Figure 1C). Finally, fork stalling induced by HU only slightly stimulates deletion-type recombinants (Figure 1C). These data confirm that the impedance of a replication fork can induce recombination, and that the nature of the impedance influences the type of recombinant that is generated. Intriguingly, the fact that recombinant formation is only weakly stimulated by acute HU exposure, suggests that replication fork stalling *per se* is insufficient to promote recombination.

The *RTS1* RFB stimulates recombination when placed at the *ade6* locus

To study the effect of a site-specific RFB on direct repeat recombination, we have engineered strains in which an 889 bp fragment containing the *RTS1* element has been placed at two different positions within the *ade6*[−] heteroallelic repeat described above (Figure 2A). The two different positions for *RTS1* are between the *his3*⁺ and *ade6-M375* genes (site A), and within the *ade6-L469* coding region (site B). As *RTS1* is a polar RFB, strains have been constructed that contain *RTS1* in both possible orientations at sites A and B. *RTS1* in orientation 1 is active for blocking forks coming from the direction of the centromere (*cen3*), whereas orientation 2 blocks forks moving toward the centromere (Figure 2A). Although the direction of replication across the *ade6* locus is unknown, the positions of putative origins of replication are such that there is a predicted bias in replication proceeding across the *ade6* locus towards *cen3* (Figure 2A) (Segurado *et al*, 2003). If true then only *RTS1* in orientation 2 would result in replication forks being blocked within the *ade6*[−] direct repeat. To investigate this, we used a native two-dimensional (2-D) agarose gel electrophoresis technique for studying replication intermediates (Brewer and Fangman, 1987). Genomic DNA from logarithmically growing cells was digested with *Eco*NI, enriched for replication intermediates by fractionation on BND-cellulose, and separated in a 2-D gel. The gel was then analysed by Southern blotting using radiolabelled probe A, which is specific to the *his3*⁺ gene (Figure 2A). An uninterrupted arc of Y-shaped replication forks was detected in a strain without the *RTS1* element and in one with *RTS1* at site A in orientation 1 (Figure 2B). However, in the strain with *RTS1* at site A in orientation 2 the replication forks accumulate as a comet-shaped spot at the top of the ascending part of the Y-arc (Figure 2B). Closer inspection of this comet-shaped signal reveals that it consists of at least two discrete spots (Figure 2B, inset). This is

Figure 1 Induction of direct repeat recombination by different genotoxins. (A) Schematic of intrachromosomal recombination substrate and recombinant products. Solid and open circles represent the *ade6-L469* and *ade6-M375* mutations, respectively. (B) The effect of increasing doses of UV on the frequency and type of *Ade⁺* recombinants in a wild-type strain (MCW39). (C) The effect of a timed exposure to CPT or HU on the frequency and type of *Ade⁺* recombinants in a wild-type strain (MCW39). Note that the ~ 2 -fold increase in spontaneous *Ade⁺* recombinants in these assays compared to that in 'B' is due to the slightly different protocols that were used.

Table 1 *RTS1*-induced mitotic recombination frequencies

Strain	<i>RTS1</i>		Mean frequency of <i>Ade⁺</i> recombinants per 10^4 viable cells			% Conversion types ^a
	Site	Orientation	Total (<i>His⁻</i> + <i>His⁺</i>) ^a	Deletion type (<i>His⁻</i>) ^a	Conversion type (<i>His⁺</i>) ^a	
Wild type (MCW39)	No <i>RTS1</i>		4.42 (± 1.6)	3.47 (± 1.3)	0.95 (± 0.51)	21.4 (± 8.0)
Wild type (MCW1262)	A	1	3.26 (± 1.0)	2.18 (± 0.76)	1.08 (± 0.49)	33.6 (± 11.0)
Wild type (MCW1433)	A	2	189.2 (± 114)	86.2 (± 60.1)	103.0 (± 61.9)	55.9 (± 10.9)
Wild type (MCW1256)	B	1	6.57 (± 4.63)	5.75 (± 4.36)	0.82 (± 0.51)	13.4 (± 7.2)
Wild type (MCW1260)	B	2	36.1 (± 14.1)	24.2 (± 11.2)	11.9 (± 4.5)	35.1 (± 11.6)
<i>swi1</i> Δ (MCW1362)	A	1	33.7 (± 18.7)	25.1 (± 14.8)	8.6 (± 6.8)	25.2 (± 12.0)
<i>swi1</i> Δ (MCW1358)	A	2	47.9 (± 27.4)	36.2 (± 24.2)	11.7 (± 7.5)	27.0 (± 11.0)
<i>swi1</i> Δ (MCW1395)	B	1	37.6 (± 21.7)	30.8 (± 19.0)	6.77 (± 4.15)	19.1 (± 9.4)
<i>swi1</i> Δ (MCW1377)	B	2	20.9 (± 13.1)	16.8 (± 10.9)	4.1 (± 3.3)	19.3 (± 9.2)
<i>rhp51</i> Δ (MCW1691)	A	1	10.72 (± 5.6)	10.7 (± 5.6)	0.02 (± 0.04)	0.19 (± 0.45)
<i>rhp51</i> Δ (MCW1692)	A	2	34.2 (± 17.3)	32.6 (± 16.7)	1.55 (± 1.06)	4.71 (± 3.4)
<i>rad22</i> Δ (MCW1687)	A	1	1.02 (± 0.49)	1.0 (± 0.48)	0.016 (± 0.037)	1.2 (± 2.7)
<i>rad22</i> Δ (MCW1688)	A	2	3.39 (± 1.6)	3.27 (± 1.59)	0.12 (± 0.17)	3.57 (± 4.52)
<i>rhp51</i> Δ <i>rad22</i> Δ (MCW1695)	A	1	1.09 (± 0.52)	1.09 (± 0.52)	0.005 (± 0.02)	0.33 (± 1.3)
<i>rhp51</i> Δ <i>rad22</i> Δ (MCW1696)	A	2	2.52 (± 1.33)	2.49 (± 1.36)	0.028 (± 0.057)	1.75 (± 3.36)
<i>rqh1</i> Δ (MCW1443)	A	1	11.95 (± 3.9)	10.2 (± 3.4)	1.75 (± 0.77)	15.1 (± 5.7)
<i>rqh1</i> Δ (MCW1447)	A	2	2410 (± 1579)	2152 (± 1530)	258 (± 179)	13.1 (± 12.1)
<i>rqh1</i> Δ (MCW1445)	B	1	17.4 (± 8.9)	14.6 (± 6.0)	2.8 (± 4.6)	12.8 (± 10.7)
<i>rqh1</i> Δ (MCW1438)	B	2	748.7 (± 266)	678.8 (± 243)	69.9 (± 43.4)	9.2 (± 4.7)

^aThe values in parentheses are the standard deviations.

Figure 2 Effect of replication fork blockage by *RTS1* on direct repeat recombination. (A) Schematic showing the position of the intrachromosomal recombination substrate on chromosome III. Sites of *RTS1* insertion within the recombination substrate are also indicated as is probe (A) used to detect replication intermediates. (B) 2-D gel analysis of replication intermediates within the region delineated by the *Eco*NI restriction sites shown in 'A'. The far left panel is a guide for interpreting 2-D gels. The panels to the right of this show the 2-D gel analysis of DNA from asynchronously grown cultures of the wild-type strains MCW39 (no *RTS1*), MCW1262 (*RTS1* site A orientation 1), and MCW1433 (*RTS1* site A orientation 2). The inset picture shows an enlargement of the RFB signal with arrows indicating that it consists of at least two discrete spots. (C) Bar charts showing the effect of *RTS1* at different positions in the recombination substrate on the frequency and type of Ade⁺ recombinants in wild-type and *swi1Δ* mutant strains. In order of presentation the strains are MCW1262, MCW1433, MCW1362, MCW1358, MCW1256, MCW1260, MCW1395 and MCW1377. Error bars represent the standard deviations about the mean.

consistent with replication fork blockage by *RTS1*, which has previously been shown to consist of four repeated motifs that may each act as a barrier (Codlin and Dalgaard, 2003). These data are also consistent with the predicted bias in the direction of replication across the *ade6⁻* repeat.

Having established that *RTS1* acts as a strong RFB when placed within the *ade6⁻* direct repeat, we determined its effect on the frequency of recombination between the repeats (Figure 2C and Table I). In wild-type strains with *RTS1* in orientation 1, either at site A or B, the frequency of Ade⁺ recombinants is not significantly different from that in a wild-type strain without the *RTS1* element. However, strains with

RTS1 at site B do produce fewer conversion-type recombinants. This is probably due to impedance of conversion-type recombination by the presence of a large chunk of heterologous sequence (the *RTS1* element) within *ade6-L469*. In wild-type strains with *RTS1* in orientation 2, the recombinant frequency increases by ~50-fold at site A and ~5-fold at site B compared to the equivalent strains with *RTS1* in orientation 1 (Figure 2C and Table I). As replication fork blockage is specific to orientation 2 within the *ade6⁻* repeat, these data indicate that replication forks blocked by *RTS1* strongly provoke recombination. Intriguingly, the proportion of these induced recombinants that are conversion-types is signifi-

cantly greater than in the spontaneous recombinants ($P = < 8 \times 10^{-9}$). In this respect, recombination induced by the *RTS1* RFB resembles that induced by UV.

The site at which recombinases load normally acts as the recipient of genetic information during recombination. In our assay system, *ade6-L469* to *ade⁺* conversion-types can be distinguished from *ade6-M375* to *ade⁺* conversion-types by colony colour. The former appear as pale pink colonies, whereas the latter form white colonies (Osman *et al*, 1996). To see if we could get evidence that recombination was being initiated at the site of the blocked replication fork, we scored the conversion-types for the wild-type strains in Figure 2C for colony colour (data not shown). In all, >90% of conversion-types are *ade6-M375* to *ade⁺* irrespective of the position and orientation of *RTS1*. However, in the case of the strain containing *RTS1* in orientation 2 at site A, 100% of conversion-types are *ade6-M375* to *ade⁺* compared to 92% for the equivalent strain with *RTS1* in orientation 1. This difference is statistically significant ($P = 3.5 \times 10^{-5}$). Similarly, the percentage of conversion-types that are *ade6-L469* to *ade⁺* is more with *RTS1* at site B in orientation 2 (10%) than if it is in orientation 1 (4%). Again this difference is statistically significant ($P = < 0.05$). These data are consistent with recombination being initiated at the site of the blocked replication fork.

Swi1 is required for the stimulation of recombination by *RTS1*

As mentioned in the Introduction, the RFB function of *RTS1* is dependent on several *trans*-acting factors including *Swi1*. To provide further evidence that it is the RFB function of *RTS1* that is responsible for stimulating recombination, we determined the effect of *swi1* deletion on the frequency of recombination in our strains (Figure 2C and Table I). *Swi1* is known to have a general role in maintaining the stability of stalled replication forks (Noguchi *et al*, 2003). In accord with this, the frequency of *Ade⁺* recombinants in *swi1Δ* strains with *RTS1* in either orientation 1 or 2 is significantly greater than in the equivalent wild-type strains with *RTS1* in orientation 1. Importantly, however, there is little difference in the frequency of recombinants in *swi1Δ* strains containing *RTS1* in different orientations. Furthermore, the recombination induced by *RTS1* in orientation 2 at site A is abolished by *swi1Δ*. These data indicate that it is the normal RFB function of *RTS1* that is responsible for stimulating recombination.

RTS1-induced recombination depends on *Rhp51* and *Rad22*

The Rad22-dependent pathway is required for most of the direct repeat recombination in *S. pombe*, with a subpathway, dependent on *Rhp51*, generating conversion-types (Doe *et al*, 2004). Deletion-types form independently of *Rhp51*, and in fact *rhp51Δ* mutants exhibit a hyperrecombination phenotype for these recombinants (Doe *et al*, 2004). To determine whether the recombination induced by *RTS1* is similarly dependent on these known pathways of recombination, we compared the frequency of *Ade⁺* recombinants in wild-type, *rhp51Δ*, *rad22Δ* and *rhp51Δ rad22Δ* strains containing *RTS1* at site A (Figure 3A and Table I). Virtually all of the induced recombinants are dependent on Rad22, with *Rhp51* being additionally required for most of the induced conversion-

Figure 3 The dependency on *Rhp51* and *Rad22* for the recombination induced by replication fork blockage at *RTS1*. (A) Bar chart showing the frequency of *Ade⁺* recombinants in wild-type, *rhp51Δ*, *rad22Δ* and *rhp51Δ rad22Δ* strains containing *RTS1* at site A in either orientation 1 or 2 as indicated. In order of presentation, the strains are MCW1262, MCW1433, MCW1691, MCW1692, MCW1687, MCW1688, MCW1695 and MCW1696. (B) Bar chart showing the percentage of *Ade⁺* recombinants in 'A' that are conversion-types. (C) 2-D gel analysis of replication intermediates detected by probe A in the *Eco*NI DNA fragment shown in Figure 2A. The strains are MCW1692 (*rhp51Δ*), MCW1688 (*rad22Δ*) and MCW1696 (*rhp51Δ rad22Δ*).

types (Figure 3A and B). Surprisingly, *Rhp51* is also required for more than half of the induced deletion-types (Table I). 2-D gel analysis of genomic DNA from these strains confirms that the RFB function of *RTS1* is fully intact in *rhp51Δ*, *rad22Δ* and *rhp51Δ rad22Δ* strains (Figure 3C). Together, these data confirm that the recombination induced by *RTS1* is mainly dependent on the known pathways of recombination in *S. pombe*. Furthermore, they indicate that strand invasion by *Rhp51* is required for the majority of deletion events at blocked replication forks.

Rqh1 constrains recombination induced by the RTS1 RFB

To see if Rqh1 limits recombination induced by the *RTS1* RFB, we compared the frequency of Ade⁺ recombinants in wild-type and *rqh1Δ* strains containing *RTS1* at sites A or B (Figure 4A and Table I). In *rqh1Δ* strains with *RTS1* in orientation 1, at either site A or B, the frequency of recombinants is ~3-fold higher than in the equivalent wild-type strain, and the percentage of these recombinants that are conversion-types is either the same or reduced compared to wild-type (Figure 4B and Table I). These differences are similar to those observed in strains without *RTS1* (Doe *et al*, 2000). However, with *RTS1* in orientation 2 the already high frequency of Ade⁺ recombinants is dramatically increased by *rqh1Δ* mutation. In the strain with *RTS1* at site A, this increase is >12-fold over the equivalent wild-type strain, while the increase is >20-fold with *RTS1* at site B. Again the percentage of these recombinants that are conversion-types is reduced significantly in a *rqh1Δ* mutant (Figure 4B and Table I). No difference in the growth/viability of *rqh1*[−] strains with or without *RTS1* in either orientation 1 or 2 was detected (data not shown). The increased frequency of deletion-types is therefore not due to a detrimental effect on growth of *RTS1* in orientation 2 in the absence of Rqh1. Instead, these data show that Rqh1 limits the recombination that is induced by the *RTS1* RFB, and in particular it limits the formation of deletion-type recombinants.

Stalled/blocked replication forks can reverse, either spontaneously or by an enzyme-driven reaction, to form a double-stranded tail of nascent DNA onto which recombinases can load and promote recombination (McGlynn and Lloyd, 2002). One way in which RecQ DNA helicases are thought to limit recombination at stalled/blocked replication forks is by resetting reversed forks (Doe *et al*, 2000; Karow *et al*, 2000). Reversed forks have recently been observed by 2-D gel analysis at the replication fork pause site within *mat1* of *S. pombe* (Vengrova and Dalgaard, 2004). These reversed forks appear as a cone-shaped signal displaced up from the Y-arc at about the position of the replication pause site (Figure 4C). This signal was observed to increase in a *rqh1Δ* mutant consistent with the idea that Rqh1 resets reversed forks (Vengrova and Dalgaard, 2004). To see if forks blocked at *RTS1* were undergoing reversal, we performed a 2-D gel analysis of genomic DNA from a *rqh1Δ* mutant that contained *RTS1* in orientation 2 at site A (Figure 4D). Consistent with our other analyses of DNA from strains with *RTS1* in this position, a strong RFB is detected. In addition, a triangular-shaped signal emanating from the descending portion of the Y-arc can be seen. Previous studies have shown that this signal represents a mixture of double-Y- and X-shaped molecules formed by converging replication forks and, in the case of the X-shaped molecules, by recombination. However, we are unable to detect a cone-shaped signal indicative of fork reversal. Therefore, if fork reversal occurs at the *RTS1* RFB, it is below the detection limits of our assay. It should be noted that previous 2-D gel analyses of the *RTS1* RFB have also not detected evidence of fork reversal in wild-type cells (Dalgaard and Klar, 2001; Codlin and Dalgaard, 2003).

Detection of one-sided DSBs at the RTS1 RFB in rqh1Δ strains

It has been suggested that in the absence of Rqh1, stalled/blocked replication forks might be more susceptible to being

Figure 4 Effect of *rqh1Δ* mutation on the frequency and type of recombinants induced by replication fork blockage at *RTS1*. (A) Bar chart showing the frequency of Ade⁺ recombinants in wild-type and *rqh1Δ* strains containing *RTS1* at site A or B in orientation 1 or 2 as indicated. In order of presentation, the strains are MCW1262, MCW1433, MCW1443, MCW1447, MCW1256, MCW1260, MCW1445 and MCW1438. (B) Bar chart showing the percentage of Ade⁺ recombinants in 'A' that are conversion-types. (C) Schematic of a 2-D gel showing the expected position of the cone-shaped signal that would be indicative of fork reversal. (D) 2-D gel analysis of replication intermediates detected by probe A in the *Eco*NI DNA fragment from a *rqh1Δ* mutant containing *RTS1* at site A in orientation 2 (MCW1447).

cleaved by an endonuclease like Mus81-Eme1 (Kaliraman *et al*, 2001; Doe *et al*, 2002). This kind of endonucleolytic cleavage would detach one arm of the fork to produce a one-sided DSB. To see if this happens at the *RTS1* RFB, genomic DNA from wild-type and *rqh1Δ* strains, which contain *RTS1* at site A, was digested with *Pst*II, separated on a 1-D gel, and analysed by Southern blotting using radiolabelled probes A and B (Figure 5). Genomic DNA was prepared and restricted in agarose plugs to prevent its shearing. Probes A and B are

Figure 5 Detection of one-sided DSBs in a *rqh1Δ* mutant containing *RTS1* at site A in orientation 2. (A) Schematic of the recombination substrate showing the position of *PstI* sites and DNA probes. The region between 7.0 and 7.6 kb from the right-hand *PstI* site, in which the DSB maps, is also marked. (B) Analysis of genomic DNA, prepared in agarose plugs and restricted with *PstI*, on a 1-D gel using radiolabelled probe B. The strains are MCW1262 (lane a), MCW1443 (lane b), MCW1433 (lane c) and MCW1447 (lane d). (C) The same as 'B' except that probe A was used instead of probe B.

specific to regions that flank *RTS1* at site A (Figure 5A). Both probes detect the full-length *PstI* fragment from wild-type and *rqh1Δ* DNA (Figure 5B and C). The full-length band is ~9.2 kb but runs slightly slower (~9.5 kb) than this when compared to the DNA ladder (lane e). This is probably because its migration is retarded by the agarose plug. An additional faster migrating band, which is indicative of a DSB, is detected with probe B in DNA from the *rqh1Δ* strain containing *RTS1* in orientation 2 (i.e. the orientation in which replication fork blockage is detected) (Figure 5B, lane d). This band is ~7.6 kb based on a comparison with the DNA ladder. However, as noted above, it is likely that the actual size of this band is a few hundred base pairs shorter. As this band is not detected by probe A (Figure 5C, lane d), the DSB site can be mapped approximately to the *RTS1* element. If this was a two-sided DSB then a ~2 kb band would be detected

by probe A. However, only the full-length *PstI* fragment is detected with this probe (Figure 5C, lane d). These data indicate that DSBs are formed at or near *RTS1* in the replicated arms of the blocked replication fork in the absence of Rqh1. Quantification of the bands in Figure 5B lane d indicates that ~1.9% of the genomic DNA is broken in this way.

To provide further evidence that one-sided DSBs are formed at the *RTS1* RFB in *rqh1Δ* strains, we performed a similar analysis as above on DNA from strains containing *RTS1* at site B (Figure 6A–C). In this case, the DNA was digested with *Bsu36I* and *EcoRV*, and analysed using probes C and D (Figure 6A). Like above, both probes detect the full-length *Bsu36I*–*EcoRV* genomic DNA fragment (~8.2 kb) (Figure 6B and C, lanes b and c). Probe C also detects a band of ~2.5 kb, which represents a portion of the normal

Figure 6 The detection of one-sided DSBs in a *rgh1Δ* mutant containing *RTS1* at site B in orientation 2. (A) Schematic of the recombination substrate showing the position of *Bsu36I* and *EcoRV* sites, and DNA probes. The region between 5.1–5.3 kb from the *EcoRV* site, in which the DSB maps, is also marked. (B) Analysis of genomic DNA from the *rgh1Δ* mutant strains MCW1438 (lane b) and MCW1445 (lane c). Genomic DNA was prepared in agarose plugs and restricted with *Bsu36I* and *EcoRV* before analysis on a 1-D gel using radiolabelled probe C. (C) The same as 'B' except probe D was used instead of probe C. (D) 1-D gel analysis of genomic DNA from wild-type (MCW1260) and *rgh1Δ* (MCW1438) strains carrying plasmids as indicated. Strains were grown in EMM without thiamine for ~20 h to allow expression of RusA from the *ntm1* promoter in pREP41. RusA and RusAD70N carry C-terminal green fluorescent protein tags so their expression in the wild-type and *rgh1Δ* strains was confirmed by fluorescent microscopy prior to harvesting the cells for the preparation of genomic DNA. The amounts of the ~5.3 kb band in lanes e–g relative to the total probed DNA are 2.2% (lane e), 4.5% (lane f) and 3.6% (lane g). Repetitions of this experiment show that these differences in the amount of the ~5.3 kb band are not significant. (E) The same as 'D' except probe D was used instead of probe C.

his3 locus on chromosome 2 that has not been deleted in our strains (Figure 6B, lanes b and c; and D, lanes b–g). A faint nonspecific band of ~2.5 kb is also detected by probe D

(Figure 6C, lanes b and c; and E, lanes b–g). All of these bands are detected in both wild-type and *rgh1Δ* strains, irrespective of the orientation of the *RTS1* element.

However, probe C also detects a ~5.1 kb band that is specific to DNA from *rqh1Δ* strains containing *RTS1* in orientation 2 (Figure 6B, lane b). This band is indicative of a DSB, and, as it is not detected by probe D, it can be mapped to the *RTS1* element. Furthermore, the failure to detect a band of ~2.9 kb in this DNA sample with probe D indicates that the DSB is one-sided (note that the ~11.3 kb band is formed by deletion-type recombination) (Figure 6C, lane b). These data are consistent with those for *RTS1* at site A, and indicate that a significant portion of replication forks that are blocked at *RTS1* are cleaved in the absence of Rqh1. Quantification of the blot in Figure 6B reveals that ~1.3 % of the genomic DNA contains a one-sided DSB at *RTS1* at site B, which is similar to the amount of breakage detected at *RTS1* at site A.

An HJ resolvase does not increase fork breakage at the *RTS1* RFB

One way in which a blocked replication fork can give rise to a one-sided break is if it reverses to form an HJ that is then cleaved by an HJ resolvase (Seigneur *et al*, 1998). Although our 2-D gel analysis had provided no evidence of fork reversal at the *RTS1* RFB in an *rqh1Δ* mutant (Figure 4D), we looked to see whether the expression of an HJ resolvase would cause an increase in the amount of one-sided DSBs in these strains. For this, we used a recombinant form of the bacterial HJ resolvase RusA that has previously been shown to suppress both *rqh1Δ* and *mus81Δ* mutant phenotypes (Doe *et al*, 2000, 2002). Genomic DNA was prepared from wild-type and *rqh1Δ* strains containing *RTS1* at site B in orientation 2, and the plasmid pREP41 or derivatives of pREP41 that express RusA (pREP41-rus) or a D70N mutant form of RusA that can bind but not cleave HJs (pREP41-rusD70N) (Doe *et al*, 2000). The genomic DNA was digested with *Bsu36I* and *EcoRV* and analysed using probes C and D as described in the previous section. No DSBs were detected in DNA from the wild-type strain containing either pREP41 or pREP41-rus (Figure 6D and E, lanes b and d). Curiously, a band of ~6.5 kb was detected with probe C in DNA from the wild-type strain expressing RusAD70N (Figure 6D, lane c). We are currently uncertain of the significance of this band. Similar to the data in Figure 6B, a band of ~5.3 kb was detected with probe C in each of the *rqh1Δ* DNA samples (Figure 6D, lanes e–g). This band is indicative of a one-sided DSB occurring within the *RTS1* element. The slight discrepancy in size between it and the ~5.1 kb band detected in Figure 6B, lane b is probably due to differences in the way the two gels have run. The amount of one-sided DSB product is approximately the same in each of the DNA samples (Figure 6D, lanes e–g). These data therefore provide no evidence for HJ formation by fork reversal at the *RTS1* RFB in an *rqh1Δ* mutant.

Discussion

Direct repeats of mutant heteroalleles are widely used to monitor the frequency of inter/intra-chromatid recombination in mitotic cells (Klein, 1995). We have further developed this system here to monitor the effect of a site-specific RFB on recombination. Previous studies on the effects of RFBs on recombination in eukaryotes have concentrated on the RFB at the rDNA locus in *S. cerevisiae*. However, the rDNA is an atypical locus, making extrapolations to the wider genome uncertain. Furthermore, it contains other factors that stimu-

late recombination, namely the *HOT1* site and collisions between replication forks and RNA polymerases (Voelkel-Meiman *et al*, 1987; Takeuchi *et al*, 2003), which complicate analysis of the contribution that the RFB makes to recombination. Our system avoids these complications and uncertainties by using the *RTS1* RFB positioned at a 'typical' genomic locus. With this we have confirmed that replication fork blockage by a programmed RFB can induce deletion recombination. It also strongly induces conservative gene conversion events, and we believe that this is the first time that this has been shown for a site-specific RFB. Recombination that is stimulated by replication fork blockage at *RTS1* depends mainly on known pathways, which are catalysed by the Rhp51 and Rad22 recombinases (Doe *et al*, 2004). Furthermore, this recombination is controlled by the Rqh1 DNA helicase. In particular, Rqh1 greatly limits the formation of deletion recombinants seemingly by preventing replication fork breakage.

Recombination at blocked replication forks

Various agents, which are known to cause replication fork stalling or blockage, induce direct repeat recombination. Induction of recombination generally depends on passage through S-phase thereby linking replication fork stalling/blockage with recombinant formation (Galli and Schiestl, 1999). However, it is unclear to what extent recombination is initiated directly at the stalled/blocked fork as opposed to at DSBs that form as a consequence of it. In the case of HU, it seems that most recombinants are formed only after prolonged exposure when the stalled replication forks have broken (Saintigny *et al*, 2001). In accord with this, it takes ~16 h for HU to induce Rad52 foci in *S. cerevisiae* as opposed to ≤30 min for γ-irradiation, which induces DSBs directly (Lisby *et al*, 2004). This is also consistent with our data showing that very little direct repeat recombination is induced after a 6-h exposure to HU in *S. pombe*, even though all the cells are arrested in S-phase.

Similar to HU, the induction of recombination by both the Tus-Ter RFB in *E. coli* and the rDNA locus RFB in *S. cerevisiae* correlate with the detection of double-strand ends and DSBs, respectively (Bidnenko *et al*, 2002; Weitao *et al*, 2003b). In contrast, replication fork blockage by *RTS1* only results in detectable DSBs in an *rqh1Δ* mutant. Furthermore, whereas a DSB at site A induces almost exclusively deletion-type recombinants (Osman *et al*, 1996), *RTS1* at this site induces mostly conversion-types. Similarly, with a DSB at site B >90 % of conversion-types are *ade6-L469* to *ade*⁺ (Osman *et al*, 1996), compared to ~10 % with *RTS1* at this site. Therefore, much of the recombination that is stimulated by replication fork blockage at *RTS1* is unlikely to stem from DSB formation.

Intriguingly, UV, like *RTS1*, induces a high percentage of conversion-types. These may arise from recombination-mediated bypass of UV-induced lesions and/or the repair of daughter strand gaps postreplication (Rupp and Howard-Flanders, 1968; Higgins *et al*, 1976). Unlike UV, replication fork blockage at *RTS1* is not expected to generate lesion-containing single-stranded gaps, which would necessitate recombination for their repair. However, gaps could be generated by replication fork convergence. The normal merging of replication forks is believed to generate fully replicated but intertwined DNA molecules, which are decatenated by

topoisomerase II (Top2) (DiNardo *et al.*, 1984; Holm *et al.*, 1989). However, Top2 may be unable to decatenate converging replication forks that arrest prematurely. The concerted action of a RecQ helicase and topoisomerase III may be required here to untwine the DNA between the arrested forks and enable gap filling by a DNA polymerase (Wang, 1991). Possibly recombinases can load at these putative gaps before they are filled in. While this might be a tenable model for recombination induced by *RTS1*, we prefer the idea that recombinases load directly at the blocked fork and act before convergence. Is there enough time for this to happen, especially as there is no evidence that replication fork blockage at *RTS1* induces a checkpoint response (unpublished data)? If we assume that the putative replication origins in Figure 2A fire at about the same time then, based on a fork rate of 3600 bp/min, the fork travelling toward *cen3* would encounter *RTS1* at site B, approximately 19 min before the opposing fork. Based on the dynamics of recombination proteins revealed by photobleaching studies in mammalian cells (Essers *et al.*, 2002), and by ChIP analyses in *S. cerevisiae* (Sugawara *et al.*, 2003), it is reasonable to think that this time interval is sufficient for recombination to occur.

The majority of recombinants that are induced by *RTS1* depend on Rhp51. We suspect that Rhp51, aided by Rad22, loads onto regions of single-stranded parental or nascent DNA that become exposed at the blocked fork through the action of helicases and/or exonucleases. Strand invasion between different *ade6⁻* heteroalleles could then lead to gene conversion by mismatch repair and/or DNA synthesis. One way that this could happen is if Rhp51 assembles on the leading nascent strand so that strand invasion generates a 3' end for priming DNA synthesis (Figure 7A, step 3). Exposure of this strand to Rhp51 might depend on the reversal of the replication fork (Figure 7A, steps 1–2). However, we have failed to detect evidence for this by 2-D gel analysis, so, if the leading strand is used, it might be exposed with little or no fork reversal (not shown). Following DNA synthesis or mismatch repair, the invading strand could be unwound by a DNA helicase (possibly Rqh1 or Srs2) thereby generating a conversion-type recombinant (Figure 7A, steps 3–5). Alternatively, the DNA junctions formed by fork reversal and/or strand invasion could be cleaved by an endonuclease, such as Mus81-Eme1, to generate a deletion-type recombinant (Figure 7A, steps 3a–5a).

Figure 7 Hypothetical model for the formation of Ade^+ recombinants following replication fork blockage at *RTS1*. Red boxes are the *ade6* genes. The open circles indicate the position of point mutations within the *ade6* genes. Further details are in the main text.

The role of *Rqh1* in limiting recombination at the *RTS1* RFB

An important finding of this study is the detection of one-sided DSBs at the *RTS1* RFB in an *rqh1Δ* mutant. These are indicative of replication fork breakage and correlate with the increased levels of deletion-type recombinants in this mutant. Similar to *rqh1Δ* mutation, CPT also causes replication fork breakage and induces mainly deletion-type recombinants. These data indicate that one-sided DSBs generally give rise to deletion recombination, and suggest that a failure to prevent replication fork breakage is responsible for the hyper-recombination phenotype of an *rqh1Δ* mutant. The hyper-deletion-type recombination probably occurs by strand invasion from the broken DNA end into the 'wrong' *ade6⁻* heteroallele (Figure 7B). However, there is also some stimulation of conversion-type recombination in an *rqh1Δ* mutant, which cannot be explained by the model in Figure 7B. This may be due to a defect in unwinding DNA junctions that are formed by strand invasion (Figure 7A, steps 3–4), which would make them longer-lived and more prone to give rise to conversion-type recombinants.

Similar to our study, increased levels of one-sided DSBs have been detected at the RFB in the rDNA locus of *S. cerevisiae sgs1Δ* mutants (Weitao *et al*, 2003a). Here, increased breakage is associated with increased pausing at the RFB (Weitao *et al*, 2003a). It is therefore unclear whether the extra breakage is due to a defect in maintaining fork stability, or simply that there are more paused forks available to be broken. In contrast to these data, we have not detected an increase in blocked forks at the *RTS1* RFB in an *rqh1Δ* mutant. We can therefore make a more definite link between DNA breakage and a failure to maintain fork stability. However, we cannot exclude the possibility that the level of breakage in an *rqh1Δ* mutant is the same as in the wild type, and that the breaks are only detected in the mutant because their processing is impaired. This idea is plausible because in *recBC* mutants of *E. coli*, RecQ, together with the single-strand exonuclease RecJ, appear to perform DSB resection (Kuzminov, 1999). Nevertheless, the accumulating evidence that RecQ helicases act directly at stalled/blocked replication forks (Courcelle and Hanawalt, 1999; Cobb *et al*, 2003; Courcelle *et al*, 2003; Hishida *et al*, 2004; Vengrova and Dalgaard, 2004), together with the hyper-deletion-type recombination in an *rqh1Δ* mutant, encourages us to believe that the breaks we observe are a consequence of a failure to protect the blocked fork from nucleolytic attack.

Based on current knowledge of RecQ helicases, Rqh1 could help protect the fork in several different ways, including: promoting the stability of replisome components at the blocked fork (Cobb *et al*, 2003); exposing ssDNA at the blocked fork to enable recombinases to load, which in turn might protect the fork (Courcelle and Hanawalt, 1999; Hishida *et al*, 2004); and by unwinding DNA junctions that might otherwise be cleaved by an endonuclease (Doe *et al*, 2000, 2002; Karow *et al*, 2000). These DNA junctions could include HJs formed by fork reversal, which might be cleaved by an HJ resolvase in the absence of Rqh1 (Doe *et al*, 2000). However, although such reversed forks have been detected at the *mat1* RFB (Vengrova and Dalgaard, 2004), and are observed in *S. cerevisiae rad53* mutants following HU treatment (Sogo *et al*, 2002), we were unable to detect them at the *RTS1* RFB. Furthermore, the expression of *RusA* in either a

wild-type or *rqh1Δ* mutant failed to generate more one-sided DSBs. Possibly fork reversal is not that extensive at the *RTS1* RFB and therefore tends not to form HJs. Instead, the replication fork may be blocked in such a manner to make it suitable for cleavage, with little or no reversal, by an endonuclease like Mus81-Eme1 (Whitby *et al*, 2003). Indeed, replication fork blockage at *RTS1* may be similar to that at the RFB in the rDNA locus of *S. cerevisiae*. Here, the leading strand halts three bases behind the nascent lagging strand (Burkhalter and Sogo, 2004). In theory, Mus81 could cleave this blocked fork. Intriguingly, genetic studies indicate that Mus81-Eme1 shares an overlapping role with Rqh1 for processing DNA junctions (Boddy *et al*, 2000; Doe *et al*, 2002). This makes Mus81-Eme1 a prime candidate for making the breaks that we detect in an *rqh1Δ* mutant. Unfortunately, an *rqh1Δ mus81Δ* double mutant is unviable, and so prevents us from directly testing this idea.

Concluding remarks

As mentioned above, the normal function of *RTS1* is to ensure the directionality of replication across *mat1* (Dalgaard and Klar, 2001). One might have thought, therefore, that forks blocked at *RTS1* would simply be stabilised to await fork convergence. Certainly, this is what appears to happen at the Tus-Ter RFB in *E. coli*, which only provokes recombination if a second round of DNA replication runs into the back of the blocked fork—a reaction that is unlikely to occur in *S. pombe* (Bidnenko *et al*, 2002). It is surprising, therefore, that recombination is stimulated at forks blocked by *RTS1* positioned at an ectopic site. Possibly, *RTS1* acts aberrantly when placed at an ectopic site, and at its normal genomic locus recombination is efficiently suppressed. Alternatively, the action of recombinases at *RTS1* may be designed to help maintain fork stability by promoting sister chromatid interactions, and therefore could occur at both normal and ectopic *RTS1* sites. This would only result in detectable genetic change when the block is within regions of repetitive DNA. However, we have not detected any difference in the growth or viability of recombination-deficient (e.g. *rad22⁻*) strains with or without *RTS1* in orientation 1 or 2 (data not shown). It would seem, therefore, that recombinase action at forks blocked at *RTS1* within the *ade6⁻* direct repeat is unnecessary for growth and survival. Nevertheless, regardless of its *raison d'être*, our data suggests that recombination may be a common feature at programmed RFBs in eukaryotes, especially knowing that *RTS1* shares characteristics with RFBs in mammalian genomes (Codlin and Dalgaard, 2003). Further analysis of the events at *RTS1* may also provide new clues to the actions of recombinases at accidental RFBs. These studies are in progress.

Materials and methods

Strains, plasmids and probes

Details of the strains, plasmids and probes are in Supplementary data.

Media and general genetic methods

Media for growing *S. pombe* and general genetic methods are as described (Gutz *et al*, 1974).

Recombination assays

Spontaneous, UV-induced and *RTS1*-induced recombinant frequencies were measured as described (Osman *et al*, 2000). The

modifications to this procedure used to assess the effect of CPT and HU are described in Supplementary data. Recombinant frequencies are the mean from at least three independent assays where five independent colonies were tested in each assay. Two sample *t*-tests were used to determine the statistical significance of differences in recombination frequencies.

Preparations of *S. pombe* genomic DNA

DNA for 2-D gels was isolated by CTAB extraction (Lopes *et al*, 2003). Following restriction digestion, replicating DNA was enriched by fractionation on a benzoylated naphthoylated DEAE (BND)-cellulose (Sigma) column. The preparation of genomic DNA in agarose plugs was performed using a CHEF genomic DNA plug kit (Bio-Rad) according to the manufacturer's instructions. Further details are in Supplementary data.

2-D and 1-D gels

Neutral/neutral 2-D gels were run as described (Brewer and Fangman, 1987). 1-D gels were either 0.7 or 0.8% agarose and

were run in 1 × TBE at 50–60 V for ~16 h. Gels were Southern blotted and probed with the indicated ³²P-labelled DNA fragments. Blots were analysed by phosphorImaging using a Fuji FLA3000 and Image Gauge software. Further details are in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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