Recombination at DNA replication fork barriers is not universal and is differentially regulated by Swi1

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DNA replication stress has been implicated in the etiology of genetic diseases, including cancers. It has been proposed that genomic sites that inhibit or slow DNA replication fork progression possess recombination hotspot activity and can form potential fragile sites. Here we used the fission yeast, *Schizosaccharomyces pombe*, to demonstrate that hotspot activity is not a universal feature of replication fork barriers (RFBs), and we propose that most sites within the genome that form RFBs do not have recombination hotspot activity under nonstressed conditions. We further demonstrate that Swi1, the TIMELESS homologue, differentially controls the recombination potential of RFBs, switching between being a suppressor and an activator of recombination in a site-specific fashion.

fission yeast | genome stability | TIMELESS

Evolution is driven, in part, by genetic events that result in global changes to genomic structure. However, gross genetic rearrangements in human cells can result in genetic disease states, including cancers (1, 2). Such potentially harmful rearrangements can be mediated by distinct pathways, including homologous recombination, creating new chromosomal structures (3, 4).

Some processes, such as the conjoining of homologous chromosomes during meiosis I (5, 6), V(D)J recombination in mammals (7), and mating type switching in yeast (8, 9), require the initiation of recombination in a highly programmed fashion, where the sites of recombination and the partner choice are governed to produce specific functional outcomes. However, sites that aberrantly mediate gross chromosomal rearrangements are not programmed to initiate recombination; rather, they have some inherent instability or become unstable because of exogenous factors, generating an unscheduled recombinogenic potential (3, 10). Studies in yeast aimed at identifying naturally occurring fragile chromosomal regions have found that such sites can be complex in nature and can consist of aggregates of distinct genetic elements, including transposons, LTRs of transposons, and tRNA genes (11, 12). Interestingly, these sites exhibit higher levels of instability when DNA replication is compromised (11–13), suggesting that the inherent instability of these sites is related to DNA replication. This hypothesis is consistent with the proposal that perturbations in DNA replication may be among the primary oncogenic stresses in tumor formation (14). It is proposed that these regions provide particularly poor substrates for the replication machinery, making them more susceptible to failures of DNA replication fork progression that ultimately could increase the frequency of the formation of recombination-initiating lesions. Consistent with this model, tRNA genes, which are located in some DNA replication-related fragile sites, have been shown to have potent replication fork barrier (RFB) activity that is thought to arise from the replication machinery, the replisome, colliding head-to-head with RNA polymerase III, which mediates tRNA gene transcription (15).

Transcription-associated recombination (TAR) initiated via collisions of RNA polymerase with the replisome has also been suggested as a potential source of instability for genes transcribed by RNA polymerase II (16, 17); however, such encounters between RNA polymerases and the replisome are relatively common occurrences within S-phase of the cell division cycle, so highly effective mechanisms must exist to prevent the generation of recombinogenic lesions. The facts that replisome stalling is not normal when RNA polymerase II collides head-to-head with the replisome in the genome and that eukaryotic RNA polymerase II-associated TAR has been reported only for plasmid-based transcription support the existence of such mechanisms (17–19). In budding yeast, Mec1 checkpoint kinase activity has been shown to be required to prevent so-called "replication slow zones" from becoming unstable (20). This function of the Mec1 signaling pathway seems to be conserved, because loss of mammalian Ataxia Telangiectasia and Rad3 related (ATR) (the mammalian Mec1 orthologue) increases the instability of fragile sites (21). Mec1 and, by extension, ATR are required to promote replication fork progression, indicating that these proteins are a fundamental part of genome duplication during normal proliferation (22–24).

In the fission yeast, it has been demonstrated that a defined RFB, the *RTS1* element from the *mat* locus (25, 26), can serve as a mitotic recombination hotspot in a RFB-dependent fashion (27, 28), providing firm evidence that RFBs in mitotically dividing cells can drive genetic change. The RFB activity of the fission yeast *RTS1* element has been shown to be dependent on a number of *trans*-acting proteins, 2 of which, Swi1 and Swi3, are the homologues of human TIMELESS and TIPIN, respectively (25). When Swi1 function is lost, so too is the RFB activity of the *RTS1* element (25). Loss of Swi1 also results in the loss of the *RTS1* RFB-mediated recombination activity, consistent with the existence of a direct link between RFB activity and recombination (27, 28). Although this observation might suggest that Swi1 serves as a mediator of recombination, this view is countered by the fact that loss of Swi1 function results in an elevation of recombination in an assay system devoid of a specific, strong RFB (29).

The Swi1 and Swi3 homologues in *Saccharomyces cerevisiae*, Tof1 and Csm3, respectively, have been shown to be intimately associated with the replisome in a complex known as the "replisome progression complex" (RPC) (30). This association suggests a model in which Swi1 (Tof1/TIMELESS) functions within the RPC to monitor the status of the chromosomal traffic ahead of the replisome so as to modulate by some means the response of the replisome to potentially problematic factors. Consistent with this view, Swi1/Swi3 have been shown to maintain replication fork stability and S-phase checkpoint activation (29, 31, 32).

In this study we used the fission yeast model to demonstrate that different eukaryotic RFBs have different recombinogenic potential and to show that the RPC component, Swi1, functions

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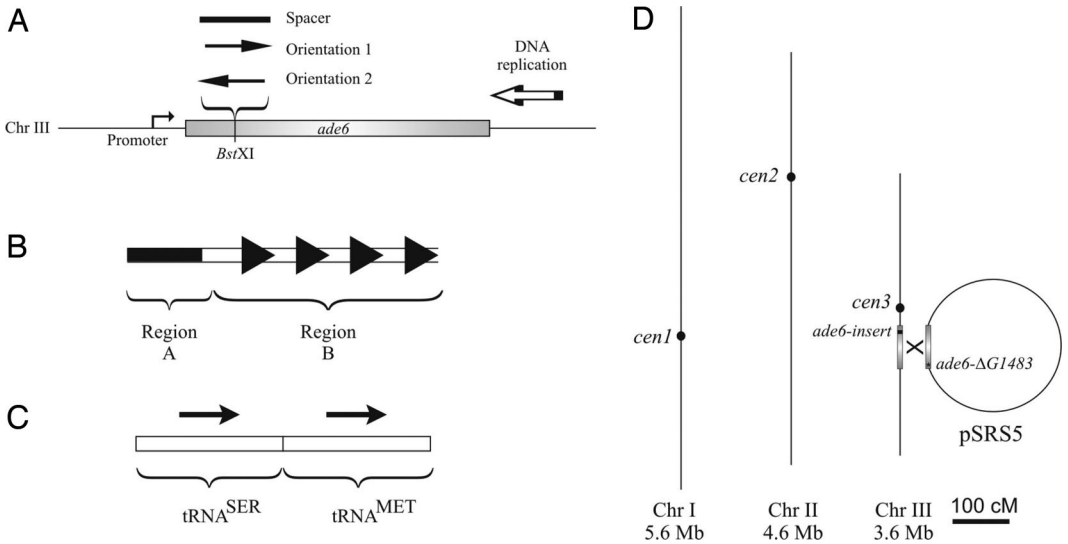

Fig. 1. Schematic representation of the systems used to monitor the recombination potential of distinct genetic elements. (*A*) Genetic elements *RTS1*, *sup3-e*, or $tRNA^{GLU}$ were inserted into the *ade6* ORF (*open rectangle*) at the BstXI site. Elements were inserted into this site in both orientations independently, as indicated by the black arrows above the BstXI site. Two distinct spacer controls, consisting of origin-free stretches of the *his3* ORF, were inserted independently at this site. The *ade6* ORF is expressed from left to right; the angular arrow indicates the promoter. The large open arrow indicates the predominant direction of DNA replication. (*B*) A schematic representation of the *RTS1* element (25). The element consists of 2 regions, region A, which interacts with Rtf2 protein, and region B, which is made up of 4 repeats (*black arrowheads*) and interacts with Rtf1 proteins. Both Rtf1 and Rtf2 are required for RFB activity (25). The direction of the black arrowheads indicates the polarity of the *RTS1* barrier. An RFB is generated when the replication fork approaches region A first (i.e., from left to right in the diagram). (*C*) A schematic representation of the *sup3-e* element. This element is made up of 2 tandemly arranged tRNA genes, $tRNA^{SER}$-$tRNA^{MET}$. They are co-transcribed using the regulatory elements of the $tRNA^{MET}$ gene, and a mature suppressor, $tRNA^{SER}$, is produced. Black arrows indicate the direction of transcription. Orientation 1 would be expected to generate a head-to-head collision between the replisome and RNA polymerase III. (*D*) Plasmid-by-chromosome intermolecular recombination assay. The 3 chromosomes of *S. pombe* are represented by the thin vertical lines. The wild-type *ade6* locus is located at a centromere (*cen1*) proximal position on chromosome III. The inserts generated in the *ade6* ORF (depicted in *A*) are located at this position on the chromosome in distinct strains. A second *ade6* allele, *ade6-Δ1483*, was created within the plasmid (pSRS5). This *ade6* allele has a mutation at a 3′ position within the gene distal to the BstXI site into which the test elements were inserted (see *Materials and Methods*). Gene-conversion events between the plasmid borne *ade6* allele and the chromosomal borne *ade6* allele (the genetic element being tested) result in adenine prototrophs. The frequency of prototroph production represents recombination frequency.

differentially to control the recombinogenic potential of different sites in a site-specific fashion.

Results

Distinct RFBs Have Different Recombination Potentials. The fission yeast *RTS1* element is a polar RFB, and previous work has demonstrated it can function as a polar, intrachromatid mitotic recombination hotspot (27). To test whether a single *RTS1* RFB can drive intermolecular homologous recombination, we established a plasmid-by-chromosome recombination reporter system based on a system we have used previously (33). Briefly, we introduced the *RTS1* element into the ORF of the *S. pombe ade6* gene at the BstXI site (Fig. 1*A*), thereby rendering the strains auxotrophic for adenine. The *RTS1* element (Fig. 1*B*) was inserted in both orientations in distinct strains. A third strain was created with a spacer control that comprises an origin-free stretch of DNA. The predominant direction of DNA replication through the *ade6* locus is from a centromere-distal origin, so the majority of passive DNA replication of this locus is from this direction (Fig. 1*A*) (34–36). Consequently, only *ade6*::*RTS1-orientation-2* should result in an *RTS1*-mediated RFB, because of the polar nature of *RTS1* (Fig. 1*B*), which we shall refer to as the "barrier orientation." Second, we transformed these strains with a plasmid, pSRS5 that carries an *ade6* allele with a mutation engineered at the 3′ end of the gene, distal to the site into which the *RTS1* element was inserted (Fig. 1*D*). (pSRS5 also has an *ars1* element enabling it to replicate autonomously in fission yeast.) The 3 strains generated were then subjected to fluctuation analysis to obtain a recombination frequency (adenine prototrophs per million viable cells).

Fig. 2*A* shows that the spacer control and *RTS1* inserted in the orientation that does not create a RFB (orientation 1) gave similar levels of intermolecular recombination. However, *RTS1* inserted in the barrier orientation (orientation 2) resulted in considerable stimulation of intermolecular recombination. We then carried out 2D gel electrophoresis on total cellular DNA to determine whether we observe the expected RFB for *RTS1* in the barrier orientation (orientation 2) but not for *RTS1* in the opposite orientation or for the spacer control. Fig. 2*D* (*Left pair*) shows that a strong RFB is observed for the barrier orientation (orientation 2) but not for the opposite orientation (orientation 1). Quantification of triplicate gel sets demonstrated that *RTS1* in the barrier orientation generated a RFB with a pause intensity of more than 1 order of magnitude greater than the nonbarrier orientation (orientation 1) (Fig. 2*E*, *2 Left columns*).

Previously, it has been shown that tRNA genes can generate strong polar RFBs (15). To test the universality of RFB-mediated recombination hotspot activity for other RFBs, we generated new strains with a tRNA gene cassette, *sup3-e*, in place of the *RTS1* element within the *ade6* gene (Fig. 1*A*). *sup3-e* is a double-tRNA gene that consists of tandemly arranged serine and methionine tRNA genes that can suppress UGA (opal) codons by insertion of a serine residue (Fig. 1*C*) (37), and it has been used previously to demonstrate that tRNA genes maintain genomic sequence conservation via intergenic gene conversion (38–40). The use of the suppressor tRNA gene permitted us to check for tRNA gene expression by monitoring the suppression of a stop codon in a marker gene. The *sup3-e* cassette was inserted in both orientations into the BstXI site within the genomic *ade6* locus, as done for the *RTS1* constructs (Fig. 1*A*).

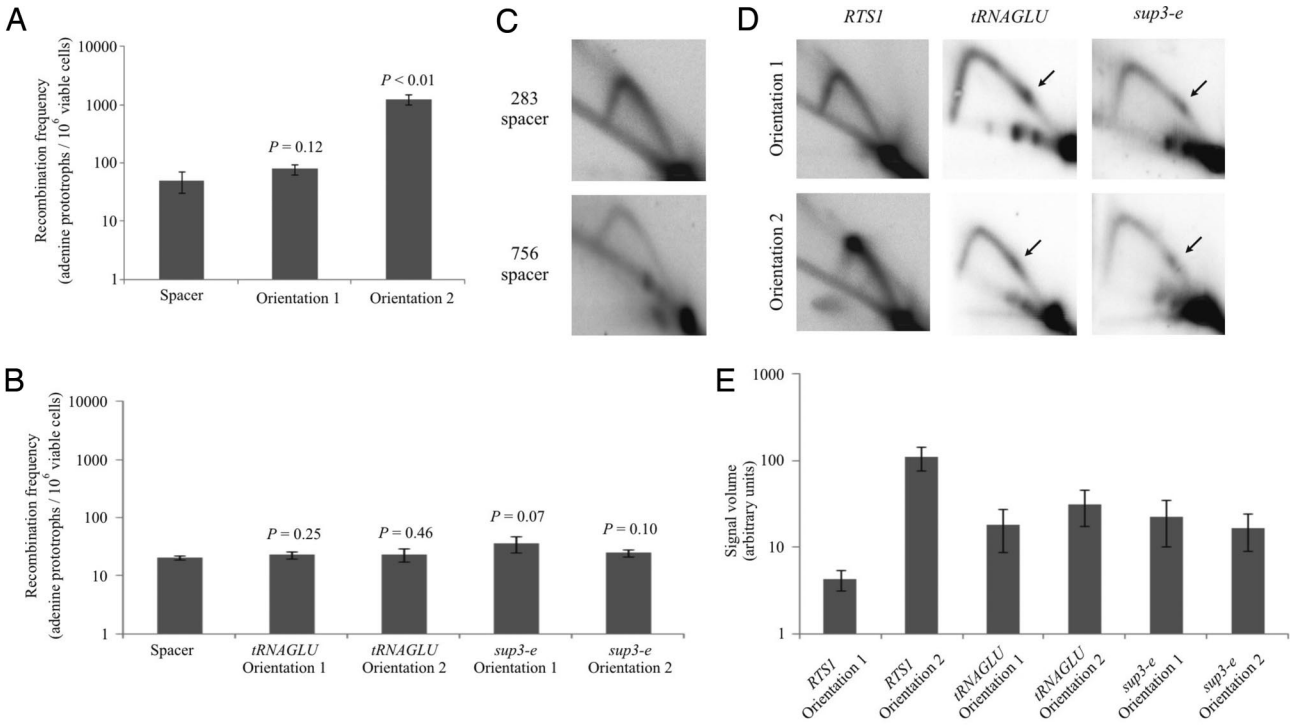

Fig. 2. Differential mitotic intermolecular recombination hotspot activity of DNA replication fork barriers. (*A*) *RTS1* is an orientation-dependent intermolecular mitotic recombination hotspot. *RTS1* in orientation 2 generates a recombination frequency almost 2 orders of magnitude higher than in either orientation 1 or the space control. (*B*) tRNA genes do not generate mitotic intermolecular recombination hotspots. Mean recombination frequencies for $tRNA^{GLU}$ and *sup3-e* in both orientations are indistinguishable from the mean recombination frequency obtained for the spacer control. *P* values are derived from Student's *t* test of pairwise comparisons of the spacer control and the individual elements. (*C* and *D*) RFB activity of *RTS1* and tRNA genes in a $swi1^+$ background. Two-dimensional DNA gel electrophoresis and Southern blotting were used to analyze DNA replication intermediates for the *ade6* locus of strains with the genetic elements inserted. (The strains used for this analysis did not carry the plasmid pSRS5.) Neither control element ($his3'_{283}/his3'_{756}$) generates an RFB. Both *RTS1* (orientation 2) (*D*, *Bottom left*) and the tRNA gene elements (*D*, *Middle* and *Right*) generate RFBs (*arrows*). (Note: the restriction enzymes used in the analysis of the *RTS1* and tRNA gene elements differed because of the different sequences of the element. This difference results in the RFB being located at distinct positions on the replicative Y arc, as is also the case in Fig. 3*D*.) *P* values are obtained from Student's *t* test of pairwise comparisons between the values for the spacer control and individual elements ($n \geq 3$ in all cases). (*E*) Quantification of RFB intensity. *RTS1* barrier orientation (orientation 2) results in an RFB of significantly greater intensity than the nonbarrier control (*Extreme left*). All tRNA gene elements generate RFBs of uniform intensity that are significantly less intense than the *RTS1* orientation 2 RFB. Values are obtained from 3 independent gels. Error bars represent SD.

A new spacer control insert was generated also, because the *sup3-e* element is smaller than *RTS1*. These strains were also transformed with the plasmid carrying a recombination marker allele of *ade6*, pSRS5, and were subjected to fluctuation analyses (Fig. 2*B*). Unlike *RTS1, sup3-e* does not generate an intermolecular recombination hotspot (Fig. 2*B*). Because this observation is distinct from that observed for *RTS1*, and to dispel the possibility that *sup3-e* is unique and not representative of more general RNA polymerase III transcribed elements, we created a further strain pair in which a single tRNA gene, $tRNA^{GLU}$, was inserted individually in both orientations into the BstXI site within the *ade6* ORF. (The spacer control is the same as that used for *sup3-e*, because the single tRNA gene was inserted within an identically sized DNA fragment.) The $tRNA^{GLU}$ gene behaved in a fashion identical to *sup3-e*, with no measurable recombination hotspot activity in either orientation. Next we subjected these strains to 2D gel electrophoresis and found that we could detect RFBs of uniform intensity (as quantified from triplicate gels) for both *sup3-e* inserts and both $tRNA^{GLU}$ gene inserts (Fig. 2 *D* and *E*), indicating that the tRNA genes generate nonpolar RFBs, although the intensity of these RFBs was significantly less than that of *RTS1* in the barrier orientation (Fig. 2*E*).

Swi1 Is an Element-Specific Regulator of DNA Replication-Associated Intermolecular Recombination. *RTS1* RFB activity is dependent on Swi1. To determine whether Swi1 is required for RFB-associated intermolecular recombination, we tested plasmid-by-chromosome recombination levels in a Swi1-deficient strain. When Swi1 function is lost, the orientation-dependent stimulation of intermolecular recombination by *RTS1* is lost (Fig. 3*A*), consistent with the data for intramolecular recombination (27). Also, as reported previously (25), the strong RFB activity of *RTS1* is lost in the Swi1-deficient strain (Fig. 3*D*, *left-hand pair*).

We then constructed *ade6*::*sup3-e* and *ade6*::$tRNA^{GLU}$ strains that were Swi1-deficient and carried the test plasmid. We carried out fluctuation analyses on these strains to quantify the recombination frequency. Fig. 3*B* shows that when Swi1 function is lost, *sup3-e* and $tRNA^{GLU}$ generate orientation-independent mitotic intermolecular recombination hotspots that are not apparent in the wild-type strain. This observation demonstrates that, when the RPC is perturbed, tRNA genes become a source of genetic instability.

No Correlation Between Recombination Levels and RFB Intensity. The observation that Swi1 functions to suppress tRNA gene-induced recombination hotspot activity led us to conclude that Swi1 functions to permit the replisome to replicate through sites of RNA polymerase III transcription without generating substrates for recombination. We hypothesized that in the absence of Swi1 function(s), DNA replication would be disrupted significantly when RNA polymerase III collision with the replisome results in a significantly enhanced RFB. To test this hypothesis, we carried out 2D gel electrophoresis analysis of DNA extracted from

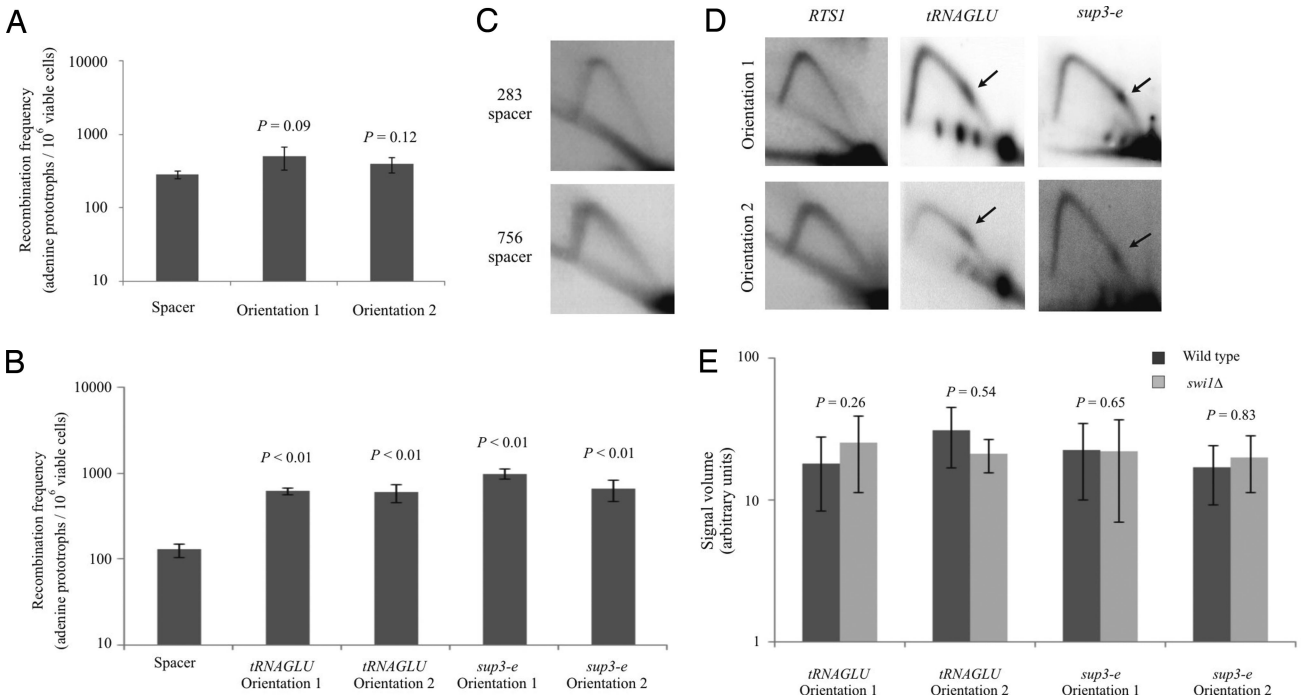

Fig. 3. Swi1 differentially regulates the recombination potential of distinct RFBs. (*A*) Intermolecular recombination frequencies for *RTS1* in a *swi1*Δ background. The orientation-dependent recombination hotspot activity for *RTS1* is lost when *swi1* is mutated, indicating that *swi1* is required for RFB-associated hotspot activity. (*B*) Loss of Swi1 function results in tRNA genes becoming orientation-independent, intermolecular mitotic recombination hotspots. In a *swi1*Δ mutant background all tRNA gene elements become mitotic recombination hotspots. *P* values are obtained from Student's *t* test of pairwise comparisons between the values for the spacer control and individual elements ($n \geq 3$ in all cases). (*C*) Spacer controls do not exhibit any RFB activity in a *swi1*Δ mutant background. (*D*) The polar RFB activity of *RTS1* is lost in the *swi1*Δ mutant (*Bottom left*). Conversely, tRNA gene-containing elements retain RFB activity in the *swi1*Δ mutant (*arrows*). (*E*) RFB intensities for tRNA genes do not alter in the *swi1*Δ mutant. Quantification of RFB activity for all tRNA gene elements demonstrates no significant difference between *swi1*$^+$ and *swi1*Δ strains. All values have bee derived from 3 independent gels. *P* values are obtained from Student's *t* test of pairwise comparisons between *swi1*$^+$ and *swi1*Δ for each element. Error bars represent SD.

Swi1-deficient strains containing *sup3-e* and *tRNA*^{GLU} elements (Fig. 3*D*, *Middle and Right pairs*). Quantification of the replication pauses did not identify any significant change in RFB intensity for either *sup3-e* or *tRNA*^{GLU} in a *swi1*Δ mutant background (Fig. 3*E*), indicating that the increase in recombination is not concomitant with an increase in pause intensity (Fig. 3 *B* and *E*) and suggesting that there is no direct relationship between RFB intensity and recombination.

Discussion

Eukaryotic chromosomes do not have uniform levels of stability along their length, and there are fragile sites that are more susceptible to initiating genetic change, a feature that is, paradoxically, conserved (10). However, a uniform feature of genomes that directly affects their stability is that they must be replicated correctly. To ensure this outcome, the replication machinery must be capable of responding to different features of the genomic landscape. In this study we have demonstrated that in fission yeast the TIMELESS homologue, Swi1, a component of the RPC, functions differentially to regulate genome stability, switching between a stimulatory and inhibitory role for replication-associated recombination in a site-specific fashion. We demonstrate that DNA replication-associated fragile sites are not determined simply by replication perturbation alone.

Different Genomic Elements Possess Different Recombinogenic Characteristics. Previous studies have shown that DNA replication-associated fragile sites can contain tRNA genes, implicating these elements in the generation of recombinogenic lesions (11, 20). Also, tRNA genes use gene-conversion mechanisms to maintain their genome-wide sequence uniformity, suggesting they have recombination-initiating potential (38–40). Because tRNA genes have been demonstrated to generate RFBs (15), it is tempting to speculate that collisions of RNA polymerase III and the replisome result in instability of the replication fork. In the case of *sup3-e* and *tRNA*^{GLU}, however, we observed no stimulation of recombination in the wild type, despite clearly measurable RFB activity (Fig. 4 *C* and *D*). Because RNA polymerase III is responsible for the transcription of many different species of RNA from the genome template (41), this finding is consistent with a model that proposes that RNA polymerase III generally does not serve as a significant recombination-initiating factor during S-phase.

This observation is in stark contrast to the high levels of recombination observed for the *RTS1* element (Fig. 4*A*) and indicates that different RFBs have very different recombinogenic potential. Moreover, these data demonstrate that there is not a simple, linear relationship between RFB intensity and recombination.

The Replisome Progression Complex Is a Regulator of the Relative Fragility of a Genomic Locus. Here we have demonstrated 2 fundamentally opposing activities of the RPC in response to distinct RFBs (Fig. 4). On the one hand, in *RTS1* the RPC component Swi1 is absolutely required for RFB activity and the associated recombination (Fig. 4 *A* and *B*). For *sup3-e* and *tRNA*^{GLU}, however, Swi1 functions either to prevent the RFB being processed into recombination-initiating lesions (i.e., stabilization of replication forks) or to prevent any such lesions being processed into recombination products (Fig. 4 *C* and *D*). The function of Swi1 that determines this distinction between different RFBs remains unknown, but this protein has been

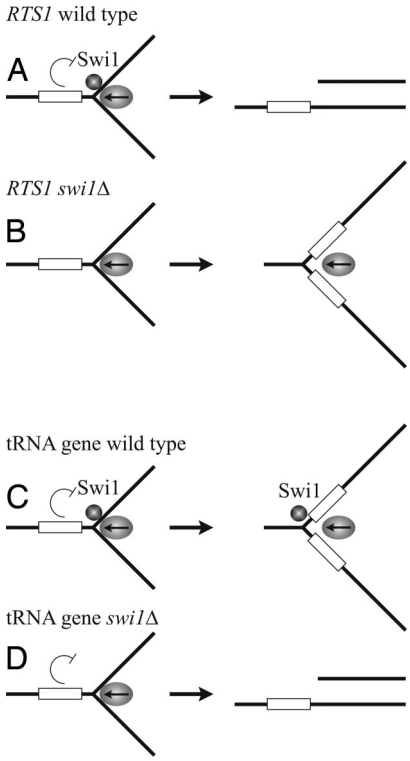

Fig. 4. Model for differential regulation of distinct RFBs. (*A*) *RTS1* barrier orientation requires Swi1 function (*small sphere*) for barrier activity (*half circle*) and the generation of recombinogenic lesions (most likely 1-sided double-stranded break; ref. 27). The open rectangles represent the appropriate *cis* element. The ovals containing an arrow represent the replisome, with the arrows indicating the direction of replisome progression. (*B*). On loss of Swi1 function, *RTS1* barrier activity is lost, and recombinogenic lesions are not stimulated. (*C*) tRNA genes generate a lower-intensity Swi1-independent RFB. RFB activity is transient, and no stimulation of recombinogenic lesions is apparent. Swi1 is required to prevent the RFB from creating recombinogenic lesions. (*D*) Loss of Swi1 function does not impair the replisome's ability to pause in response to the tRNA gene, but it does result in the pause becoming genetically less stable, with elevated levels of recombinogenic lesions being generated. (The nature of these lesions remains unknown; a 1-sided double-stranded break is shown for illustration.)

implicated in the maintenance of replication fork stability and S-phase checkpoint function. The observation that recombination is increased at tRNA genes on loss of Swi1 function is similar to the observation that loss of Mec1/ATR checkpoint activity increases instability at fragile sites (20), perhaps suggesting that there is a link to a Swi1 checkpoint function (29, 31, 32). Links between genotoxic stress regulation and circadian control have been known for some time (42). Given the homology between Swi1 and TIMELESS, our findings indicate an association between genome instability induced by DNA replicative stress and circadian regulation control, consistent with the fact that human TIMELESS has previously been associated with the DNA replication and checkpoint machinery (reviewed in ref. 42).

The fact that Swi1 functions very differently when the replisome encounters tRNA genes might suggest that the more widespread function of Swi1 is to suppress unwanted recombination at RFBs throughout the genome and that the function at *RTS1* is a unique activity that has evolved to mediate events at a highly specialized site. An alternative view is that *RTS1* generates such a strong RFB that a tipping point has been reached beyond which Swi1 no longer has the ability to maintain replication fork stability, a proposal supported by the relative intensities of the distinct RFBs (Fig. 2*E*). This idea might provide an attractive explanation of why some *S. cerevisae* fragile sites contain multiple elements capable of blocking DNA replication, including tRNA genes (11). Some of these sites, however, become fragile only when the checkpoint pathways are perturbed (20), indicating that complex sites can be endured during normal S-phase and that the relationship between the level of the replicative blockage and fragility is not simple. A more general role for Swi1 in suppressing recombination at milder RFBs is supported by the observation of elevated intramolecular recombination in the absence of specified RFBs in a *swi1*Δ mutant (29).

Previous studies have demonstrated that tRNA genes provide a polar RFB (15). Here we show that both *sup3-e* and $tRNA^{GLU}$ function as orientation-independent, nonpolar RFBs (Figs. 2*D* and 3*D*). This finding again indicates further complexity, because distinct elements have distinct RFB characteristics, and chromosome context may influence this difference.

Finally, others have previously reported Swi1-/Swi3-independent RFB in the rDNA locus of *S. pombe* (43). Here we show that *sup3-e* and $tRNA^{GLU}$ located within the *ade6* ORF provide further examples of Swi1-independent RFBs that function as recombination hotspots in the absence of Swi1 function.

Closing Remarks. Previously, it has been reported that RFBs can serve as mitotic recombination hotspots. Here we demonstrate that this function is not a universal feature of all eukaryotic RFBs. Indeed, we propose that RFBs generated widely throughout the genome may not serve as mitotic recombination hotspots and that limited RFB activity is not sufficient to create a fragile site capable of mediating recombination events under normal cellular conditions. Consistent with this idea, natural fragile sites in the budding yeast genome are complex and are not generated by simple RFB elements alone. Last, we demonstrate that a key component of the RPC, Swi1 (*Sc*Tof1/*Hs*TIM), functions differentially to regulate the outcome of an encounter between a RFB and the replisome in a site-specific fashion. These findings expose a new level of complexity to the study of genome instability associated with perturbation of DNA replication.

Materials and Methods

Yeast Strains and Plasmids. Table S1 lists the strains used in this study. Culture media, strain storage, and *S. pombe* transformation were as described in by Moreno et al. (44). For a detailed description of the construction of the modified *ade6* alleles and the plasmid pSRS5, see SI *Materials and Methods*.

Determination of Recombination Frequency. Recombination frequency was determined using fluctuation analysis. Briefly, cells were plated on appropriate solid medium at a dilution that gave well-dispersed single colonies. Colonies were permitted to grow to no greater than 1 mm in diameter. At this stage the whole colony was placed in 5 ml of appropriate liquid medium and incubated with shaking at 30 °C until the early stationary phase. Serial dilutions of cultures were made and plated out onto yeast extract agar (YEA) and YEA containing 20 mg/ml guanine (pH 6.5), which prevents the uptake of adenine because of purine antagonism (45), or onto selective pombe minimal glutamate medium (with and without adenine). Plates were incubated at 33 °C for 3 days before counting. Recombination frequencies were determined from colony counts. For each strain to be tested, the recombination frequencies of 7 independent cultures were measured, and the median value was used. This process was repeated a minimum of 3 times for each strain to be tested, and mean values of the median values were generated. *P* values were generated by pairwise comparisons using Student's *t* test.

Analysis of DNA Replication Intermediates. For a detailed description of DNA purification and 2D gel electrophoresis protocols, see SI *Materials and Methods*.

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