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Eukaryotic Replication Barriers: How, Why and Where Forks Stall

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1. Introduction

Maintaining genetic fidelity is paramount for all living organisms. The process of replicating DNA is especially dangerous for cells. Not only must the genetic sequence be replicated precisely by the replicative polymerases, but stalled replication forks and single-stranded DNA present at the forks increase risks of chromosome breakage leading to rearrangements. Also, once the cell commits itself to the replication process it has to be fully completed before chromosomes can be disentangled and condensed prior to their proper segregation in the subsequent mitosis. Many processes have evolved that ensure the precision and stability of the replication process; helicases remove bound proteins in front of the fork, topoisomerases ensure that topological entanglements generated during replication are resolved; checkpoint activation in response to stalled replication forks controls an array of molecular responses, repair polymerases and proteins to be recruited to stalled replication forks to allow replication restart; moreover, origin firing is controlled such that firing of origins is delayed in response to the replication checkpoint and dormant origins can be activated if replication is not completed. It is at first hand therefore surprising that at specific loci in the genome, molecular mechanisms exist where deliberate pausing or termination of the replication fork occur. This wonder is further confounded by the fact that several studies have shown that these replication barriers cause genetic instability (see MacFarlane, Al-Zeer and Dalgaard, Chapter 16). What the evolutionary benefits of these replication barriers might be remains a major question. More and more evidence is accumulating that indicates many replication barriers have opposing effects on genome stability; on one hand they promote genetic stability though a controlled stalling of the replication fork at specific sites or situations, however, in doing so they potentially cause fork collapse and genetic instability. In many cases these barriers "coordinate" transcription and replication, preventing collisions between the two types of enzymatic complexes, suggesting that such collisions are more detrimental to the stability of the genome than the instability induced by stalling at a replication barrier (references are given in the main text). Thus, one might argue that most replication barriers evolved to promote genetic stability while allowing "controlled" genetic instability, although other functions of replication barriers are also

evident. Here, we review what different types of natural replication impediments are known, how they prevent replication fork progression, and what potential biological function they have.

2. Epstein-Barr virus protein EBNA-1

Epstein-Barr virus or human herpes virus 4 DNA is replicated once per cell-cycle in latently infected cells. Here the DNA binding EBNA-1 protein plays several important roles for viral replication. First, EBNA-1 binds to inverted repeats at the *cis*-acting *OriP* sequence, where it acts to recruit cellular ORC proteins and as a consequence, other replication factors required for replication. Binding to the *OriP* sequence occurs at a region with dyad symmetry containing four low-affinity binding sites for EBNA-1 (Ambinder, et al. 1990). However, EBNA-1 also interacts with another region within *OriP* called *FR* (family of repeats), which contains twenty 30-bp high-affinity sites for the EBNA-1 dimer (Rawlins, et al. 1985). When replication is initiated at *OriP* it proceeds in a bi-directional manner but the replication fork moving toward *FR* is stalled by the bound EBNA-1, thus converting the bi-directional replication process into an uni-directional replication one. Reducing the number of *FR* repeats from 20 to 15, 6, 2 or 0 showed that 6, 15 or 20 copies promoted barrier activity (Dhar & Schildkraut, 1991). The *FR* region with bound EBNA-1 acts both as a barrier for the cellular MCM replicative helicase during viral replication as well as the SV40 large T-antigen for SV40 plasmids. The latter barrier activity has been observed both *in vitro* and *in vivo* (Dhar & Schildkraut, 1991, Ermakova, et al. 1996, Aiyar, et al. 2009). EBNA-1 also prevents strand unwinding by both the SV40 large T-antigen 3′ to 5′ helicase and the *E. coli* dnaB 5′ to 3′ helicase (Ermakova, et al. 1996). Interestingly, FR/EBNA-1 complexes containing 20 or 40 repeats also act as a barrier to RNA polymerase II transcription, and since a viral transcript (although catalysed by RNA polymerase III; Howe & Shu, 1989; Howe & Shu, 1993) is oriented toward *FR*, the *FR*/EBNA-1 barrier could have a role in preventing collisions between transcription and replication forks (Aiyar, et al. 2009). In addition to its role in replication, the *FR*/EBNA-1 element is also required for maintenance and partitioning of viral DNA. The element tethers the viral episome to a cellular chromosome, thereby allowing appropriate segregation into the daughter cells (Marechal, et al. 1999; Sears, et al. 2003; Sears, et al. 2004). Interestingly, the FR/EBNA-1 element also has a negative effect on plasmid maintenance; puromycin resistance encoding plasmids containing 20 or more copies of the element are not efficiently maintained in cell culture (Aiyar, et al. 2009). Thus, the *FR*/EBNA-1 replication barrier element might have both negative and positive effects on viral copy number.

3. rDNA replication barriers

Most organisms share the same basic arrangement of the rDNA, consisting of one or more arrays of a genetic unit, where each unit contains a RNA polymerase I transcribed pre-curser rRNA encoding the 25-28S large rRNA, the 16-18S small rRNA as well as the 5.8 S rRNAs. The latter is separated from the origin of replication by a non-transcribed spacer (NTS). This NTS contains one or more replication barriers that pause or stall replication forks, thus preventing them from entering the polymerase I transcribed unit. Such barriers have been described in many different organisms, including fission yeast (*Schizosaccharomyces pombe*), budding yeast (*Saccharomyces cerevisiae*), ciliated protozoa (*Tetrahymena thermophila*), Pea

(*Pisum sativum*), frog (*Xenopus laevis*), mouse (*Mus musculus*) and human (see below for references). Generally these barriers are thought to prevent collisions (and therefore genetic instability) between the polymerase I transcription bubbles and the replication forks moving in opposite directions, but data suggests that they have both a positive and a negative effect on genome stability (see below).

3.1 Pea rDNA barriers

2D-gel analysis of the rDNA of *P. sativum* detected several replication barriers in the NTS, located just downstream the RNA polymerase I transcript. The *P. sativum* replication barrier region maps to a 27 base pair direct repeat region with the consensus sequence TCCGCC(T/A)CTTGT-ATTCGTTGCGTTG(A/C)A that is either present in 9 or 3 copies in two different classes of arrays (Hernandez, et al. 1988; Hernandez, et al. 1993; Lopez-Estrano, et al. 1999). This repeated sequence motif shows some similarity with the sequence that mediates barrier activity in *S. cerevisia* (Hernandez, et al. 1993), and mobility shifts indicate that an unknown transacting factor(s) can bind to the repeats (Lopez-Estrano, et al. 1999).

3.2 Ciliate rDNA barriers

In *T. thermophila* the rDNA barriers are developmentally regulated. In the germ line micronucleus the 10.3 kb rDNA is present in a single copy, while in the differentiated macro nucleus the rDNA has been excised from the genome, arranged into an inverted repeat (the two polymerase I transcripts arranged in opposite directions), telomeres are added and the repeat is amplified 10000 fold (Reviewed in Tower, 2004). This amplification occurs within one cell cycle. Interestingly, here the 5′ NTS contains three replication barriers that pause the replication fork in a polar manner (MacAlpine, et al. 1997). These barriers are located between the site of replication initiation (that occurs at two sites flanking the centre of the inverted repeat) and the polymerase I transcript. Thus, here the barriers are upstream of the RNA polymerase I transcript. The consensus sequence of the three *cis*-acting sequences is 5′ A(A/T)TTTCANNNNNNNNNNNNNNNNNA(A/G)TTTCATTCANNNNNNNNNTTT TTTTT 3′. These replication pause sites are active both during vegetative growth and when amplification occurs. In addition to the three pause sites, an additional replication barrier is present which only acts during amplification and not during vegetative growth. This barrier is present in the middle of the palindrome and acts to stall the fork until a converging replication fork initiated at the other side of the palindrome arrives to promote termination (Zhang, et al. 1997). Interestingly, this central barrier element is required for both proper excision of the rDNA before amplification in the macronucleus, as well as for maintaining genetic stability at the unamplified rDNA gene in the micronucleus (Yakisich & Kapler, 2006). In the absence of the barrier element breakage occurs at the loci leading to loss of the chromosome arm, which again has a dominant effect on the stability of the homologues chromosome present in the diploid nucleus.

3.3 Frog rDNA barriers

Similarly, developmentally regulated replication barriers have been described in *X. laevis*. Firstly, a barrier is present at the RNA polymerase I termination region. This barrier can be detected in cell culture and tissues where the rDNA is highly transcribed, but not in early embryos and in egg extracts where transcription is low or absent (Hyrien & Mechali, 1993; Wiesendanger, et al. 1994; Hyrien, et al. 1995). Secondly, 15 weaker pause sites distributed

over the rDNA unit appear during the midgastrula stage, for then to disappear again at the neurula stage (Maric, et al. 1999). The appearance of these pause sites was proposed to reflect chromatin remodelling associated with the developmental regulation of polymerase I transcription.

3.4 Budding yeast rDNA barriers

The replication barrier in the rDNA of *S. cerevisiae* was the first to be described (Brewer & Fangman, 1987; Linskens & Huberman, 1988). Like the other barriers it is located in one of two NTS regions downstream of both the coding regions of the polymerase I transcribed 35S rRNA and the RNA polymerase III transcribed 5S rRNA. However, unlike the other eukaryotic systems, the barrier activity is not mediated by the Reb1 factor involved in Polymerase I transcription termination (*S. cerevisiae* Reb1 is related to Mammalian TTF1 and *S. pombe* Reb1; see below) (Reeder, et al. 1999). Instead the barrier activity is mediated by an unrelated *S. cerevisiae* factor Fob1 that binds to the DNA at a region closer to the origin (Kobayashi & Horiuchi, 1996) and about 90% of replication forks are stalled at this barrier (Brewer et al. 1992). Barrier activity is independent of transcription (Brewer, et al. 1992) and Fob1 interacts with three sites, RFB1-3, where the latter two are the minor barrier sites (Brewer, et al. 1992; Gruber, et al. 2000; Ward, et al. 2000; Kobayashi, 2003). The *cis*-acting sequences show phylogenetic conservation between *Saccharomyces* species (Ganley, et al. 2005). Fob1 possesses a Zn^{2+}-finger domain and a domain with similarity to integrases (Dlakic, 2002); mutations in the former affect DNA binding activity, barrier activity and HOT1 (HOT1 is a recombination hot spot in the rDNA) activity, whilst a mutation of the putative catalytic residue D291A of the integrase domain has no effect (Kobayashi, 2003). Using Atomic Force Microscopy (AFM) it was shown that Fob1 interacts with the barrier sequence in a fashion where the DNA is wound around the protein (Kobayashi, 2003). Moreover, the same data suggest that Fob1 acts as a dimer interacting with two sequences at the same time. The position of the stalled replication fork has been precisely mapped (Gruber, et al. 2000); the 3′ end of the leading-strand and the 5′ end of the lagging-strand map three nucleotides apart, 41 and 38 nucleotides in front of the sequences required for pausing at RFB1, respectively. However, weaker signals due to fork stalling were also observed in a region between RFB1 and RFB2 (Figure 1).

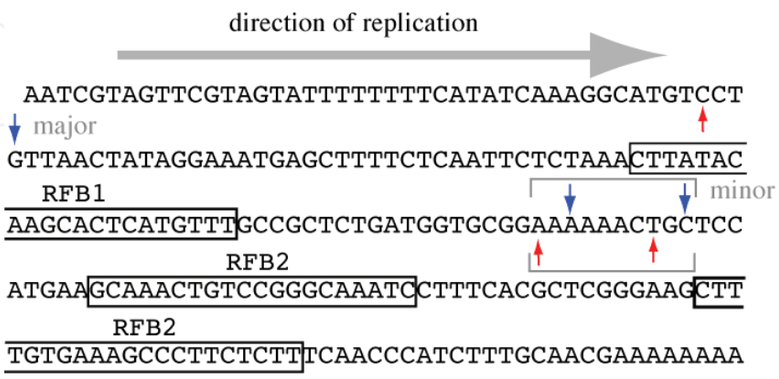

Fig. 1. Positions of the sites of replication stalling for the *S. cerevisiae* rDNA barrier. Positions of stalling of the leading-strand polymerase (red arrows) and 5′-ends of the last lagging-strand Okazaki fragment (blue arrows) are shown relative to the binding sites of Fob1.

AFM analysis also shows that the RNA primer at the lagging-strand has been removed in the stalled replication complex. (Kobayashi, 2003). Stalling of the replication fork at the Fob1 barrier depends on Tof1 (*S. pombe* Swi1/Human TIMELESS) and Csm3 (*S. pombe* Swi3/ Human TIPIN), but not Mrc1 (*S. pombe* Mrc1/ Human Claspin) (Mohanty, et al. 2006). When the replication fork is stalled at the Fob1 barrier located at an ectopic site (Calzada, et al. 2005) the replisome (Mrc1, Tof1, MCM-Cdc45, GINS and DNA polymerases α and ε) is maintained intact, thus allowing the replication fork to restart. The stability of the stalled replication fork was also shown not to depend on replication checkpoint kinases Mec1 and Rad53 or the Sml1 factor (See Section 6.0), nor does the replication restart depend on the Rad52 recombinase. Stalling leads to the recruitment of the Rrm3 helicase, which was suggested to mediate restart. These data and suggestions were later verified by a study that showed that replication stalling was dependent on Tof1 and Csm3, but partly restored in *Δtof1 Δrrm3* and *Δcsm3 Δrrm3* mutants (Mohanty, et al. 2006). It was proposed that Tof1 and Csm3 mediate stalling by counteracting Rrm3, but since Rrm3 is required for efficient replication past many non-histone DNA binding proteins (See Section 10.0), the effect could be unspecific (Mohanty, et al. 2006). Similarly, the requirement for two other helicases, Sgs1 and Srs2, was tested in the absence of Tof1 but neither affected barrier activity. Another study looked at Sgs1, Top3, Dnl4 and Rad52 with again no major effects on barrier activity, although in all the mutants there was an increase in the amount of single-stranded DNA at the fork measured using electron microscopy (Fritsch, et al. 2010). However, increased barrier activity was observed in a Dna2 mutant, a helicase implicated in Okazaki fragment maturation, suggesting that events at the lagging strand affect the stalled fork (Weitao, et al. 2003a; Weitao, et al. 2003b). The biological function of the Fob1 barrier has been an area of intense research and resulted in some key findings. Firstly, Fob1 barrier activity promotes recombination between repeats in the rDNA array and has a role in repeat expansion through induction of recombination and unequal sister-chromatid exchange (Kobayashi & Horiuchi, 1996; Kobayashi, et al. 1998; Mayan-Santos, et al. 2008; Ganley, et al. 2009). Double stranded breaks have been detected at the barrier and related to replication fork pausing, potentially due to fork collapse (Weitao, et al. 2003a; Weitao, et al. 2003b; Fritsch, et al. 2010). Secondly, barrier activity acts to prevent collisions between the DNA replication fork and the polymerase I transcription forks, leading to fluctuations in copy numbers and formation of extra chromosomal rDNA circles (ERCs) (Takeuchi, et al. 2003). Thirdly, Fob1 barrier activity has also been implicated in ageing as its fork barrier activity leads to formation of ERCs that accumulate in the mother cell, as well as in an increased loss of heterozygosity of markers distal to the rDNA array on chromosome XII (Defossez, et al. 1999; Lindstrom, et al. 2011). However, recent data suggest that age related replication stress underlies the ageing process, and not the formation of ERCs (Lindstrom, et al. 2011). Forthly, Fob1 also has a role in silencing of the rDNA through the recruitment of the regulator of nucleolar silencing and telophase exit (RENT) complex that includes Net1, Sir2, CDC14, Tof2, Lrs4 and Csm1 as well as Cohesin (Huang & Moazed, 2003), but this role is independent of the replication barrier activity of the protein (Bairwa, et al. 2010). The RENT complex inhibits polymerase II transcription and represses recombination (Kobayashi, et al. 2004; Kobayashi & Ganley, 2005). Lastly, Fob1 also regulates the activity of Topoisomerase I, as Fob1 dependent but replication independent topoisomerase I catalysed nicks have been mapped within the replication barrier region (Burkhalter & Sogo, 2004; Di Felice, et al. 2005).

3.5 Mammalian rDNA barriers

Replication barriers have also been identified in human and mouse rDNA arrays (Little, et al. 1993b; Langst, et al. 1998; Lopez-estrano, et al. 1998b). The barrier signals were detected by 2D-gel analysis of replication intermediates and map to the binding sites of the TTF-I transcription factor within the NTS region located downstream of the 38S rRNA polymerase I transcribed regions. The TTF-1 transcription factor belongs to the same family of proteins as *S. pombe* Rtf1 and Reb1 and *S. cerevisiae* Reb1 (see Figure 2). TTF-I mediates termination of polymerase I transcription, but also has additional roles in polymerase II termination as well as both polymerase I transcription activation and silencing (Langst, et al. 1998; Wang & Warner, 1998). TTF-I binds to ten 18 base-pair long or eleven 11 base-pair long *Sal-boxes* in mouse and human cells, respectively, which are located within the NTS region of the rDNA. TTF-I binding mediates polar polymerase I transcription termination (Grummt, et al. 1985a; Grummt, et al. 1985b; Lang, et al. 1994; Reeder & Lang, 1994). However, TTF-I *Sal*-box binding also promotes replication barrier activity. 2D-gel analysis of replication intermediates isolated from the human cell cultures suggests that the rDNA replication barriers are bi-polar, stalling forks moving in both directions (Little, et al. 1993a). A similar analysis of the mouse barriers showed that in this system the TTF-I dependent barriers are polar, mediating replication stalling at each of the four clusters of *Sal-boxes* of replication forks moving in the opposite direction to that of the flanking RNA polymerase I transcription (Lopez-estrano, et al. 1998a). Finally, an *in vitro* study suggests that only *Sal-box 2* acts as a strong replication barrier (Gerber, et al. 1997). Using the SV40 *in vitro*

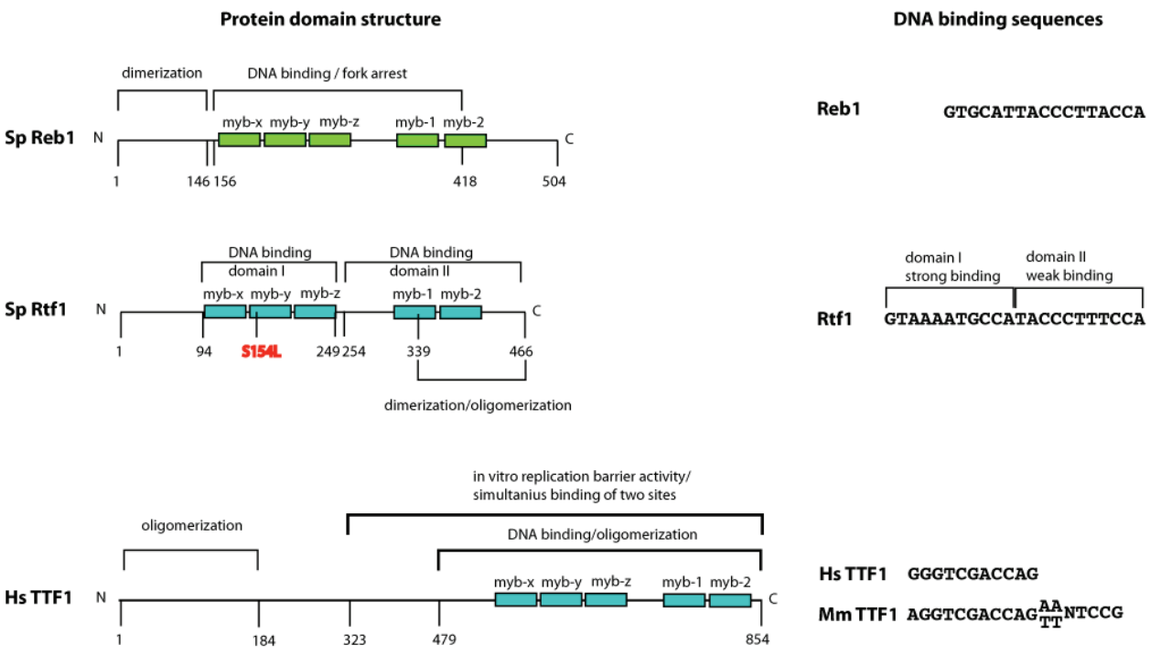

Fig. 2. Protein domains and DNA interaction sequences of the related TTF-I, Reb1 and Rtf1 factors. Left, the positions of the structural myb DNA-binding motifs identified by a hidden Markov model analysis are shown (Eydmann, et al. 2008); Domains with defined functions are indicated by square horizontal brackets. The position of the Rtf1-S154L mutation that changes the polarity of the *RTS1* element is indicated in red. Right, DNA recognition sequences of Reb1 and Rtf1 and Human/Mouse TTF-I.

replication system, this study also defined the *Sal-box 2 cis*-acting sequence requirements for site-specific replication termination, and verified the TTF-I dependence for barrier activity. When bound to *Sal-box 2,* TTF-I counteracts the strand displacement activity of the SV40 large-T antigen 3'-5' helicase (Putter & Grummt, 2002b). Three *cis*-acting elements are required for full activity of *Sal-box 2*. Firstly, the *in vitro* barrier activity depends on the *Sal-box 2* sequences that mediate TTF-I binding (Grummt, et al. 1985a; Putter & Grummt, 2002b). Secondly, this binding site is flanked by a GC-rich box that consists of 20 cytosine residues followed by a GC-rich stretch at the origin-proximal side. Introduction of point mutations within this region, shortening the stretch of cytosines (dC stretch), or inverting this region relatively to the *Sal-box,* abolished replication barrier activity and contra-helicase activities (Gerber, et al. 1997; Putter & Grummt, 2002a). The 20 base pair long dC stretch potentially forms a secondary structure, a poly dG-dG-dC triple helix that can act as a barrier for the progressing helicase or polymerase (Putter & Grummt, 2002a). Thirdly, flanking the GC rich sequence is a stretch of 26 thymidines that acts as an enhancer of the barrier activity; deletion of the thymidines causes a ~30% reduction in activity (Putter & Grummt, 2002a). The position of the *in vitro* leading-strand replication termination site has been mapped to 28 nucleotides from the *Sal-box* just in front of the long stretch of dC residues (Gerber, et al. 1997).

Several studies of the 883 amino acid long TTF-I factor have been performed. Two regions within the protein have been implicated in polymerization of the protein (Sander, et al. 1996a; Gerber, et al. 1997), (Figure 2). A 323 N-terminal truncated version of TTF-I is fully active for both *in vitro* transcription and replication termination, while a 445 amino acids N-terminal truncation leads to loss of both activities. Neither of these truncations affect the DNA binding of the protein, however, the region between residue 323 and 445 is required for polymeric TTF-1 to interact simultaneously with two DNA sites (Sander & Grummt, 1997; Evers & Grummt, 1995; Sander, et al. 1996b; Gerber, et al. 1997). Similar to the other replication barriers described below, the data suggest that passive binding of TTF-1 is not sufficient to cause replication barrier activity, but that in addition specific interactions with replication fork proteins must occur. Furthermore, dimerization or polymerization of TTF-I might be important for replication termination as observed recently for *S. pombe* Reb1 (see 3.6). Interestingly, TTF-I binds both in the promoter region and, as described above, in the transcription termination region of the polymerase I transcribed element, and a 3C analysis shows that these two regions interact by a mechanism that depends on TTF-I (Nemeth, et al. 2008). This interaction has been proposed to be important for regulation of transcription initiation; TTF-1 recruits the chromatin remodelling complex NoRC to the promoter region through a direct interaction in the N-terminal part of TTF-I to silence rDNA transcription (Nemeth, et al. 2004). The N-terminal domain of TTF-I has a negative effect on DNA binding through an interaction with the DNA binding domain. This inhibition is relieved through the interaction in *trans* with NoRC (Nemeth, et al. 2004). The described interaction between TTF-I molecules bound at the promoter and termination regions, also opens up the possibility that there might be coordination between transcription initiation at the promoter and replication barrier activity at the transcription termination region.

The proteins Ku70 and Ku86 have also been implicated in replication barrier activity at the mammalian rDNA (Wallisch, et al. 2002). Using affinity purification with a bait that consisted of the GC-rich region that flanks the *Sal box* 2, a protein fraction was isolated which stimulated *in vitro* replication termination. The stimulating activity could be depleted from the HeLa cell extracts using an oligonucleotide sequence containing the GC rich region

bound to DYNA beads, and subsequently the depleted extracts could be complemented by addition of recombinant Ku70/Ku86. Thus, Ku70/Ku86 binding promotes replication termination at the *Sal-box 2*, potentially involving the formation of secondary structures when the DNA is unwound by the helicase or replicated by the polymerase.

3.6 Fission yeast rDNA barriers

The rDNA barrier region of *S. pombe* is more complex than the other systems described, in that four different barrier elements have been defined; *RBF1-4*. These barrier elements are clustered downstream of the coding region of the 25S rRNA gene in the NTS. Again the elements act as polar barriers for replication forks initiated at the origin and moving toward the RNA polymerase I transcribed unit, thus preventing collisions between the two types of enzymatic complexes. Two different *trans*-acting factors have been identified that serve as barriers at these sites, Reb1 and Sap1.

Sap1 is responsible for the barrier activity at the *RFB1* site, which in one study was delineated to a 21 bp region (Krings & Bastia, 2005) and in another to a 30 bp region (Mejia-Ramirez, et al. 2005). Sap1 is an essential DNA binding protein involved in chromatin formation, checkpoint activation and maintenance of genome stability (Arcangioli & Klar, 1991; Ghazvini, et al. 1995; de Lahondes, et al. 2003; Noguchi & Noguchi, 2007). Loss of Sap1 causes chromosomal segregation defects, while overexpression causes toxic DNA replication dependent chromosome fragmentation and abnormal mitosis. Due to the fact that Sap1 is essential, the evidence for Sap1 binding at the *RFB1* site is indirect. Firstly, Sap1 was purified from crude extracts as a factor that binds the *cis*-acting sequences at *RFB1* (Mejia-Ramirez, et al. 2005). Secondly, *RFB1* point mutations that affect Sap1 binding *in vitro* also affect barrier activity *in vivo* (Krings & Bastia, 2005). Lastly, supershifts can be achieved with antibodies against tagged-Sap1 in EMSA experiments (Krings & Bastia, 2005). Binding of the dimeric Sap1 protein to the *RFB1* site causes a slight bending of the DNA *in vitro* (Krings & Bastia, 2005). Replication fork stalling at *RFB1* is dependent of the *trans*-acting factors Swi1 and Swi3 (Mejia-Ramirez, et al. 2005). Sap1 also binds the *SAS1* sequence required for mating-type switching (Arcangioli & Klar, 1991), but does not cause barrier activity at this locus (Kaykov, et al. 2004; Krings & Bastia, 2005; see Section 8.1). A comparison of the interactions between Sap1 and these two *cis*-acting sequences showed that the Sap1 dimer bound differently to the two sites; the interaction of the Sap1 protein with *RFB1* covered successive major grooves, had translational symmetry and occurred with higher affinity; while the interaction with SAS1 was a minor groove interaction, occurred with a relatively lower affinity and had rotational symmetry (Krings & Bastia, 2006).

Reb1 was identified as mediating barrier activity at the two *cis*-acting sites *RFB2* and *RFB3* (Sanchez-Gorostiaga, et al. 2004). Reb1 also mediates Polymerase I termination at the same sequences (Melekhovets, et al. 1997). Reb1 belongs to the same family of factors as Human/Mouse TTF1, *S. cerevisiae* Reb1 and *S. pombe* Rtf1, which are characterized by the presence of a repeated myb domain (Eydmann, et al. 2008) Figure 2). Reb1 acts as a dimer that dimerizes through a 146 amino acid long N-terminal domain (Biswas & Bastia, 2008). This dimerization allows the dimeric protein to interact with two recognition sites at the same time (Singh, et al. 2010). When the two sites are in *cis* the intervening DNA is looped out, however, the dimeric protein can also interact with two sites in *trans*. In the latter case, "chromosome kissing" was observed between a Reb1 dependent barrier on chromosome 2, Ter344314, and two sites on chromosome 1, Ter4257637 (Cyp8) and Ter4680236 (Srw1/Ste9)

(Singh, et al. 2010). Furthermore, using weakened binding sites at the Ter344314 and Ter4680236 sites it was shown that this "chromosome kissing" was important for barrier activity. Only the middle 156-418 AA Section of Reb1 is absolutely required for barrier activity. Barrier activity at *RFB2* and *RFB3* depends on both Swi1 and Swi3, however, a Swi1 mutation (*swi1-rtf*) that abolishes barrier activity at the *RTS1* element does not affect barrier activity at the *RFB1-4* (see Section 8.0; Krings & Bastia, 2004). Interestingly, when the 156-418 AA Reb1 segment was expressed in *S. cerevisia*, it was unable to act as a barrier even though it was binding to the RFB3 sequence (Biswas and Bastia, 2008). Finally, Reb1 has also been shown to be important for gene regulation; Reb1 binding at the promoter of *Ste9* is required for transcriptional activation and G1 arrest (Rodriguez-Sanchez, et al. 2011). Reb1 also acts as a replication barrier at this site.

The RFB4 barrier is the weakest of the four barriers, and has been proposed to be generated by collisions between the polymerase I transcription machinery and the DNA replication machinery (Krings & Bastia, 2004). The intensity of the RFB4 barrier signal increases in the absence of Swi1, Swi3 or Reb1, potentially because more replication forks are colliding with the transcription machinery. Also, RFB4 does not act as a replication barrier when the region is moved onto a plasmid.

4. Centromeric and telomeric replication barriers

Replication pause sites have been described at both the *S. cerevisiae* telomeres and centromeres. At the Y' elements of the telomeres the replication fork pauses at internal $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric sequences as well as at the terminal $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats. The internal $C_{1\text{-}3}A/TG_{1\text{-}3}$ sequences promote stalling independent of the orientation relative to of the progressing replication fork, and the replication pausing is intensified in absence of the Rrm3 helicase (Ivessa, et al. 2002; Makovets, et al. 2004, Makovets, 2009). In the *rrm3* mutant strain, pausing can also be observed at an inactive *ARS* element in the subtelomeric region. Insertion of *Tetrahymena* telomeric repeats in the subtelomeric region of *S. cerevisiae* did not lead to pausing suggesting that it is the binding of a *trans*-acting factor that leads to the barrier activity and not the repeat sequences themselves (Makovets, et al. 2004). However, mutation of the sub-telomeric binding sites of Tbf1 and Reb1, deletion of the *Rif1, Rif2*, *Sir2* or *Sir3* genes, or introduction of a C-terminal truncated version of Rap1, do not affect the replication pause (Makovets, et al. 2004; Makovets, 2009). Tbf1 and Reb1 act at chromatin barriers in the subtelomeric region, while Sir2 mediates silencing at the telomeres and Rap1 binds directly the telomeric repeats when they are double stranded. The C-terminal truncated version of Rap1 is unable to interact with the Rif proteins and deficient in the recruitment of Sir proteins to the telomeres, although DNA binding to the telomeric repeats is unaffected. Thus, it is argued that it is most likely Rap1 binding per se, potentially through the interaction with other unknown protein(s), which mediate the pause activity. Since the strength of the replication pause is dependent on the length of the telomeres, a potential role of the pause is to regulate the time in which the telomeres can be elongated; short telomeres do not cause pausing and are therefore replicated faster, thus giving telomerase longer time for elongation.

Several replication pause sites have also been observed at the sub-telomeric regions of *S. pombe*, however, it is not known what proteins mediate pausing at these sites (Miller, et al. 2006). In addition, a protein that binds the telomeric repeats, named Taz1, has been attributed an interesting role; in the absence of Taz1 replication defects are observed at the

telomeric repeats, leading to loss of telomeric sequences and chromosome entanglement. In addition, in the absence of Taz1, replication pausing is observed at the junction between the telomeric repeats and the sub-telomeric region, as well as at repeats located internally within the chromosome. In the latter case, the requirement is independent of the orientation of the repetitive sequence (Miller, et al. 2006). One possibility is that Taz1 has a role in recruiting replicative helicases that act to aid fork progression through the repeats. With respect to this, it is interesting to note that the human homologues of Taz1, TRF1 and TRF2, have also been shown to affect telomeric replication, although in a different manner (Ohki & Ishikawa, 2004). Using the SV40 *in vitro* replication system, it was shown that addition of recombinant TRF1 and TRF2 lead to stalling of the replication fork at the telomeric region of the linear SV40 DNA. Similarly, overexpression of TRF1 in HeLa cells, leads to an increase of replication foci that overlap with telomeric signals, suggesting an increase of replication forks stalled at telomeres.

Replication pausing is also observed at the *S. cerevisiae* centromeres *CEN1*, *CEN3* and *CEN4*, and presumably replication pausing occurs at all centromeres (Greenfeder & Newlon, 1992). Interestingly, pausing at the centromeric DNA is bipolar and thus occurs independently of the direction by which the replication fork enters the centromeric DNA. A mutational analysis of the *cis*-acting sequences showed that the barrier activity is dependent on the ability of the centromeric DNA to form a nuclease resistant core protein structure, suggesting that it is the interaction with centromeric proteins that causes the pause to replication fork progression (Greenfeder & Newlon, 1992). It is not known whether replication pausing is important for centromere function. Interestingly, recent papers describing the genome-wide localization of phosphorylated histone H2A show accumulation at the centromeric regions of both *S. cerevisiae* and *S. pombe,* thus, potentially replication stalling occurs at centromeres in both yeasts (see Section 11.0).

5. Replication barriers at tRNA genes, retrotransposons and LTRs

Early work identified replication pause sites at *Ty1-LTRs* and tRNA genes in *S. cerevisiae* (Greenfeder & Newlon, 1992, Deshpande & Newlon, 1996). These tRNA gene replication barrier activities were shown to be polar only stalling replication forks moving in one direction, that opposite to the direction of Polymerase III transcription. *Cis*- and *trans*-acting mutations that reduce or abolish the efficiency of transcription initiation correspondingly reduced or abolished replication barrier activity. Indeed, a temperature sensitive mutation in the large subunit of RNA polIII, that affects transcription initiation but not the formation of the initiation complex consisting of TFIIIC and TFIIIB at the tRNA gene also abolished barrier activity. Therefore, the replication barrier activity most likely results from a direct interaction between the transcription machinery and the progressing replication fork complex, although a build up of supercoiling between the approaching transcription and replication forks was also proposed as a potential mechanism for fork pausing (Deshpande & Newlon, 1996). Importantly, a later study showed that barrier activity is abolished in a *Δtof1* mutant (*S. pombe* Swi1/Human TIMELESS), but is restored in the *Δtof1 Δrrm3* double mutant (Mohanty, et al. 2006). In the same study, increased stalling was observed at the tRNA gene in the absence of the Rrm3 helicase.

S. pombe $tRNA^{GLU}$ and *sup3-e* tRNA genes have also been shown to pause replication forks. However, in this system the tRNAs act as bi-polar barriers stalling replication forks moving in both orientations. Furthermore, the tRNA gene barrier activity is independent of Swi1

function (McFarlane & Whitehall, 2009; Pryce, et al. 2009). Similarly, polar replication pausing has been observed at *S. pombe* retrotransposons *Tf2 LTRs* (Zaratiegui, et al. 2011). Interestingly, replication pausing at these elements is abolished by the *sap1-c* mutation. The *sap1-c* allele was isolated as a spontaneous mutation that restored growth and improves viability to a double mutant strain of the two CENP-B homologues Abp1 and Cbh1. The Δ*abp1 Δcbh1* double mutant has poor viability due to increased levels of unreplicated regions and/or recombination structures, and the *sap1-c* allele was isolated as a spontaneous mutation that restored growth and viability. The *sap1-c* mutation reduces the Sap1 proteins ability to bind DNA. Thus, Abp1 and Cbh1 have roles preventing genetic instability and replication defects induced by Sap1 barrier activity. Δ*abp1* and Δ*cbh1* single mutants slightly increase the intensity of the Sap1 dependent barrier signal, and in the Δ*abp1* Δ*cbh1* double mutant recombination intermediates can also be observed by 2D-gel analysis of replication intermediates (Zaratiegui, et al. 2011). Abp1 also localizes to tRNA genes suggesting that it might have a role in maintaining genome stability at these replication barriers as well. Abp1 interacts with Mcm10 that has been shown to have primase activity (Locovei, et al. 2006), thus Abp1 might promote replication restart after pausing through a priming event.

6. Replication slow zones

Replication slow zones have been described in *S. cerevisiae* and are characterized by increased amounts of replication intermediates as measured by 2D-gel analysis (Cha & Kleckner, 2002). These zones are regularly spaced throughout the genome between active origins, except at the centromere. The replication slow zones were identified as regions of genetic instability in the *mec1* mutant background. Mec1 is the homologue of Human ATR and *S. pombe* Rad3, and has multiple roles in DNA replication, replication checkpoint activation, DNA damage repair and recombination. Interestingly, the genetic instability is suppressed by a *Δsml1* mutation, suggesting that the instability is due to low levels of dNTPs. Sml1 is an inhibitor of ribonucleotide reductase, and the lack of Sml1 leads to an increase in dNTP levels. Similarly, the *Δrrm3* mutation partly suppresses the genetic instability observed at replication slow zones, which is correlated with a decrease in the Sml1 protein level (Hashash, et al. 2011). Thus, the data suggest that low levels of dNTPs cause replication forks to slow down even in an unperturbed S-phase, and that Mec1 is important for maintaining the stability of these slow moving forks, potentially via the function of Mec1 in regulating the nucleotide pools through inhibition of Sml1 and in intra-S and G2-M checkpoint activation. Whether replication slow zones are important for genome stability in higher organism has yet to be established.

7. Replication barriers mediated by DNA structures or repetitive sequences

Inverted repeats and micro repeats, through formation of triplexes and G-quartets have all been shown to inhibit DNA polymerase progression *in vitro* (for a review see Mirkin & Mirkin, 2007). Similarly, there is growing *in vivo* evidence that structures and repetitive sequences in the DNA are difficult templates, which promote replication fork stalling and as a consequence genetic instability. Since formation of structures distinct to the double helix are not energetically favoured, especially in front of the replication fork where there is supercoiling, it is most likely that the structures are formed in the lagging-strand template (Mirkin & Mirkin, 2007). Sequences that have been shown to mediate fork stalling include

inverted repeats as well as (CAG)n/(CTG)n, (CGG)n/(CCG)n, and (GAA)n/(TTC)n repeat sequences. In the case of the inverted repeats, a very elegant recent study showed that while two Alu sequences oriented as direct repeats did not affect replication fork progression, the same sequences oriented as inverted repeats caused fork stalling in *E. coli*, *S. cerevisiae* and a mammalian cell line (Voineagu, et al. 2008). In *E. coli* and the mammalian cell lines the ability of the inverted repeats to mediate stalling was dependent on the homology between the inverted sequences, and it gradually decreased with decreasing homology, thus supporting the idea that structures formed at the sequences were responsible for the pause. Furthermore, by varying the distance between the inverted sequences the authors were able to show they were most likely due to formation of hairpins in the lagging-strand template and not by cruciforms formed in front of the replication fork. The foundation of this conclusion was the fact that similar barrier activity was observed even in the presence of a 12 bp spacer, which would either reduce or abolish the ability of the repeated sequence to form a cruciform structure. Interestingly, *S. cerevisiae* Tof1 and Mrc1 (homologues of *S. pombe* Swi1 and Mrc1 and Human Timeless and Claspin) are required for efficient passage through the repeats and mutation of these factors leads to an increase in the intensity of the replication pause signal, an effect which is opposite to that observed at protein-mediated barriers. The repetitive sequences d(CGG)n, d(CCG)n d(CTG) and d(CAG) are also thought to form hairpin structures with both Watson-Crick and nonWatson-Crick base pairs, and d(CGG) sequences can form quartets (Chen, et al. 1995, Gacy, et al. 1995, Zheng, et al. 1996, Mariappan, et al. 1998). Both (CAG)n/(CTG)n and (CGG)n/(CCG)n repeats have been shown to stall replication forks in *S. cerevisiae* and mammalian cells, while (GAA)n/(TTC)n have been shown to stall forks in *S. cerevisiae* (Pelletier, et al. 2003; Krasilnikova & Mirkin; 2004a, Krasilnikova & Mirkin, 2004b; Kim, et al. 2008). The barrier activity was length dependent, although there were differences between systems; 10 (CGG)/(CCG) repeats were sufficient to stall replication forks in *S. cerevisiae* but 40 were required in mammalian cells (Voineagu, et al. 2009). Similarly, 60 (GAA)/(TTC) repeats do not cause any barrier activity, while increased barrier activity can be observed with increasing number of repeats (120, 230 and 340 units). There are also variations in whether the orientation of the repetitive sequences are important for barrier activity; in *S. cerevisiae* (GAA)n/(TTC)n barrier activity is orientation-dependent, whilst (CGG)n/(CCG)n repeats pause the replication fork in both orientations (Pelletier, et al. 2003; Kim, et al. 2008): In mammalian cells (CGG)n/(CCG)n repeats act as a barrier in both orientations (Voineagu, et al. 2009). Again, both *S. cerevisiae* factors Tof1 and Mrc1 were required for efficient replication through the repeat sequences as observed for an inverted repeat. Interestingly, a mutant Mrc1 protein ($Mrc1^{AQ}$) that can not be phosphorylated by the checkpoint kinases did not affect the barrier activity, thus the authors concluded that it is not the checkpoint function of Mrc1, but this factor's role in stabilizing stalled replication forks that is required (Voineagu, et al. 2009). Instability of stalled replication forks at repeat sequences is thought to underlie a range of Human diseases including fragile X-syndrome, Fraxe, Huntinton's disease and myotonic dystrophy (reviewed in Pearson, et al. 2005).

8. Cellular differentiation involving replication barriers: Mating-type switching in fission yeast

In the fission yeast *S. pombe,* a program of mating-type switching is mediated by a replication-coupled recombination event. Three different replication barriers are involved in

setting up this cellular program of differentiation, where the expressed mating-type specific cassette at the *mat1* locus is replaced with a gene cassette expressing the information of the opposite mating-type. The information is copied from one of the two transcriptionally silenced centromere-distally located donor loci, *mat2P* and *mat3M,* into to the expressed *mat1* locus. In order for this program of cellular differentiation to occur, the *mat1* locus has to be replicated in a centromere-distal direction. The unidirectional replication of the *mat1* locus is maintained by the *RTS1* element, which is located at the centromere-proximal side of *mat1* and which acts as a polar replication terminator. Replication forks that move in the centromere-distal direction are terminated at the *RTS1* element, while forks moving in the centromere-proximal direction are allowed to pass through unhindered (Dalgaard & Klar, 2001). At the sequence level the *RTS1* element consists of two *cis*-acting regions that cooperate for function (Codlin & Dalgaard, 2003); a 446 base pair region named region B that contains four repeated ~55 bp long motifs as well as a 64 bp enhancer region called region A of similar length. Each of the repeated motifs of region B contributes to the overall barrier activity. A linker substitution analysis of region-B-motif-4 established that only a 20 bp region within the 55 bp long repeat is required for activity. This 20 base pair region shows similarity to the *S. pombe* Reb1 recognition site (Figure 2). Region A on the other hand is characterized by an uneven distribution of purines and pyrimidines on the two strands. In the absence of region A, the presence of each of the repeated motifs of region B has an additive effect on overall barrier activity. In the presence of region A, the region B motifs cooperate for function leading to a four-fold increase in overall barrier activity. Individually, region A does not possess any barrier activity. A recent study showed that the factor Sap1 binds to the enhancer region A (Zaratiegui, et al. 2011), however, it is not known whether Sap1 binding contributes to enhancer activity. Several factors have been identified that are required for efficient replication termination at the *RTS1* element. Rtf1 is a member of the family of factors that include *S. cerevisiae* Reb1, *S. pombe* Reb1 and human/mouse TTF-I (Eydmann, et al. 2008, see Sections 3.4 & 3.5; Figure 2). Deletion of the *rtf1* gene abolishes *RTS1* barrier activity. This protein family is characterized by the presence of two myb-domains that respectively contain three and two myb DNA interacting motifs. Each of the two Rtf1-myb domains have been expressed and purified separately and have been shown to have DNA binding activity; Rtf1-domain I binds *RTS1* DNA *in vitro*, interacting both with the repeated motifs of region B and the enhancer region A (Eydmann, et al. 2008). The Kd for the interaction with region A is 3467 nM, while the interaction with the repeated motif is somewhat stronger with a Kd for the interaction at 549 nM. A ten base pair substitution that abolishes barrier activity of the region B motif 4 *in vivo* strongly reduces binding of the Rtf1-domain I *in vitro*. Rtf1-domain II on the other hand only interacts weakly with the region B motif 4. A 10 bp substitution of the region flanking the binding site of domain I, that abolishes barrier activity of motif 4 *in vivo*, also abolishes binding of the Rtf1-domain II *in vitro*. Amino acid substitutions have been identified in both Rtf1-domain I and II that abolish barrier function, establishing genetically that they are of functional importance (Eydmann, et al. 2008). In addition, a point mutation has been identified in Rtf1-domain I (S154L) that changes the polarity of the *RTS1* barrier, such that instead of terminating replication forks moving in the centromere-distal direction, it acts as a pause site for replication forks moving in the centromere-proximal direction. The Rtf1-domain I-S154L mutation slightly enhances the domain affinity for region A and motif 4, such that the Kd is now 343 nM for region A and 265 nM for the motif 4. This observation suggests that the Rtf1-S154L protein is binding

the *RTS1* element, but that it is unable to stall the replication fork, thus a protein-protein interaction(s) between Rtf1 and the progressing replication fork may be important for barrier activity. In addition, a dominant Rtf1-mutation has been identified that abolishes termination of replication. This non-sense mutation truncates the Rtf1 protein such that 120 amino acids of the C-terminus are missing. Two-hybrid analysis of this 120 AA C-terminal Rtf1 tail shows that it can interact with itself. This discovery suggests that Rtf1 self-interactions are required for barrier activity and that the tail-less Rtf1 allele interferes with the action of the wild-type protein at *RTS1* (Eydmann, et al. 2008).

In addition to DNA binding proteins other factors have been shown to be required for *RTS1* function (Inagawa, et al. 2009). Rtf2 is required for efficient termination of DNA replication at the *RTS1* element. An epistasis analysis of the enhancer region A deletion and the *Δrft2* mutation suggest that Rtf2 acts through the region A deletion. In the absence of Rtf2 replication forks pause in an Rtf1-dependent manner, but are restarted again. This replication restart is dependent on the Srs2 helicase, but not the Rqh1 helicase. Potentially, Srs2 acts by removing Rtf1 from the DNA in front of the replication fork, in a manner similar to its role in preventing recombination by removing Rhp51/Rad51 from single-stranded DNA (Krejci, et al. 2003; Veaute, et al. 2003). Rtf2 is the defining member of a family of proteins that are conserved from *S. pombe* to humans, which are characterized by the presence of a novel type of C2HC2 ring finger motif that potentially only binds one Zn^{2+} atom. A similar Ring finger motif, named the SP motif, with only one Zn^{2+}-atom binding site, is found in many E3 SUMO ligases including *S. cerevisiae* Siz1, Siz2; *S. pombe* Pli1, Nse2; human PIAS1, PIASx*β*, PIAS3, PIASy, Mms21 (Watts, et al. 2007; Yunus & Lima, 2009) and an epistasis analysis suggests that Rtf2 and SUMO (*pmt3*) might act together in the same pathway (Inagawa, et al. 2009). However, Rtf2 also seems to have a role that is independent of SUMO, as slow moving replication forks are present at the *RTS1* element in the Rtf2 single mutant that are absent in the SUMO single mutant. In addition, Rtf2 interacts with proliferating cell nuclear antigen (PCNA) and might be travelling with the replication fork. Sumorylation and ubiquitination of PCNA at residues K127 and K164 has in *S. cerevisiae* been shown to affect molecular events at stalled forks (Stelter & Ulrich, 2003). Of these residues only K164 is conserved in *S. pombe* PCNA (gene *pcn1*). Interestingly, when lysine K164 is mutated to an alanine, it has no effect on barrier activity measured by genetic assays, which utilize efficiency of sporulation as the readout (Figure 3B). Thus most likely, Rtf2 targets either other residues of PCNA or other replication proteins for SUMOylation. Finally, both Swi1 and Swi3 are required for barrier activity at the *RTS1* element (Dalgaard & Klar, 2000). Swi1 and Swi3 travel with the replication fork as part of the Replication Progression Complex (RPC) and genetic evidence suggests that Swi1 might interact directly with Rtf1 to mediate replication barrier activity; a point mutation in Swi1, *swi1-rtf3* G2785A, has been identified that abolishes termination of *RTS1* but does not affect other replication barriers such as the rDNA barrier and the *mat1* pause site *MPS1* (Dalgaard & Klar, 2000; Krings & Bastia, 2004). Recent work has demonstrated that *in vitro* the hetromeric complex of Swi1 and Swi3 can interact with double-stranded DNA (Tanaka, et al. 2010). In addition, a super-shift can be achieved through an interaction with purified Mrc1, a replication checkpoint protein that is also traveling with the RPC. Furthermore, data suggested that the *swi1-rtf3* G2785A mutation affects the super-shift caused by Mrc1 binding, thus providing a possible mechanism for the loss of barrier activity at *RTS1* (Tanaka, et al. 2010). However,

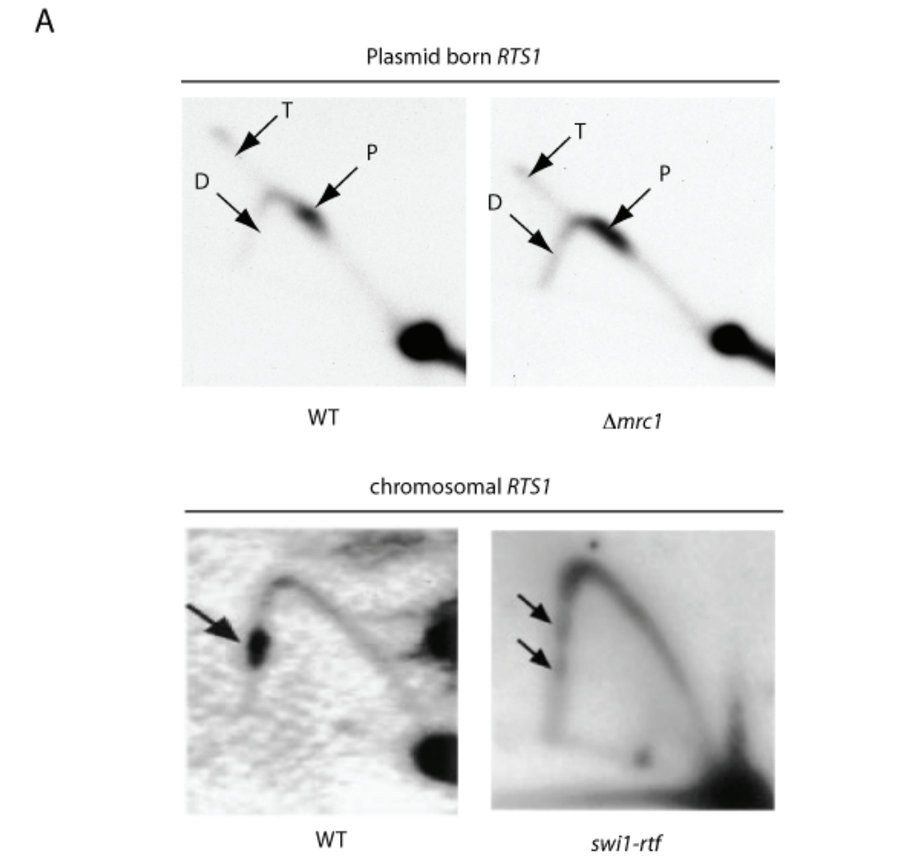

Fig. 3. A. Comparison of the effect on barrier activity of the *Δmrc1* mutation and the *swi1-rtf* mutation. The upper two panels show replication intermediates that have been digested with *Sac*I and *Pst*I and separated on a 2D-gel as described earlier. T is a termination signal, D the descending arc and P the pause signal. The analysed *RTS1* element is present on a plasmid (pBZ142) (Method is described in Codlin & Dalgaard, 2003). Below, as comparison, the effect of the *swi1-rtf* mutation on the *RTS1* element at it wild-type genomic position is shown (reproduced from (Dalgaard & Klar, 2000). B. Sporulation assays used for identifying effects on replication pausing at the *MPS1* element (left two panels) and replication termination at the *RTS1* element (right two panels). In the first case a reduction of replication pausing will lead to reduced sporulation, while in the second case reduced termination will lead to increased sporulation (For a description of the assay see Codlin & Dalgaard, 2003) .

our analysis of a Δ*mrc1* strain shows that this mutation does not affect the overall *RTS1* barrier activity, although the region of stalling does seem to be slightly expanded and the intensity of the descending arc is slightly more intense suggesting an increase of replication restart (Figure 3A). Thus, the *swi1-rtf3* G2785A mutation must affect other protein-protein interactions required for barrier activity at *RTS1,* the most likely candidate for the interacting partner being Rtf1. A model for the possible mechanism of replication termination at *RTS1* is given in Figure 4.

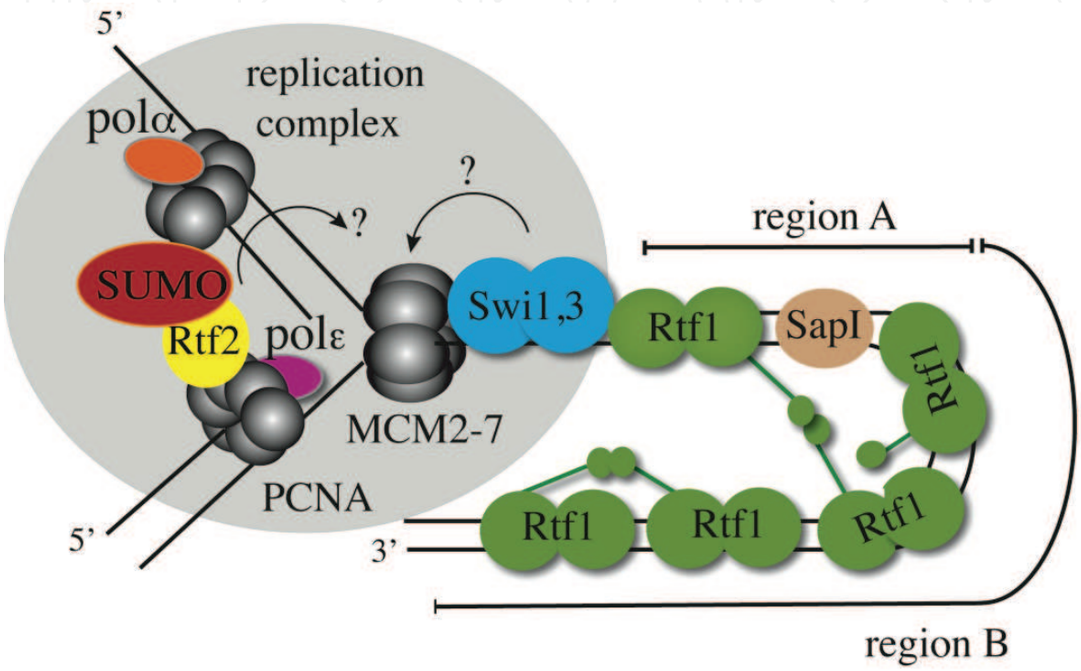

Fig. 4. Model for the molecular mechanism of replication termination at the *RTS1* element. Rtf1 molecules interact with the repeated motifs present in the *RTS1* element as well as the enhancer region A. Potentially, C-terminal interactions of Rtf1 are important for stabilizing the interactions and can provide additional constraints when the DNA template is unwound by the approaching helicase. The function of the interaction of Sap1 with the enhancer region (region A) is unknown. When the replication complex approaches the *RTS1* element, protein-protein interactions stall the progression. These protein-protein interactions are most likely between Rtf1-domain I and Swi1. The interactions potentially lead to inhibition of DNA unwinding by the MCM2-7 replicative helicase. The stalled replication fork is stabilized by the action of Rtf2, potentially by SUMOylation of other replication factors (Inagawa, et al. 2009) .

8.1 Molecular differentiation of sister chromatids through replication pausing

Another replication barrier required for *S. pombe* mating-type switching is the *MPS1* site required for imprinting at the *mat1* locus (Dalgaard & Klar, 1999; Dalgaard & Klar, 2000; Vengrova & Dalgaard, 2004). *mat1* imprinting is required for mating-type switching. At *MPS1* the replication forks are paused but then all re-started again. All *cis*- and *trans*-acting mutations that abolish replication pausing at *MPS1* also abolish imprinting, suggesting a mechanistic role between imprinting and replication pausing. Also, inversion of the *mat1*

locus relative to the *RTS1* element so that it is replicated in the opposite orientation, abolishes both pausing and imprinting (Dalgaard & Klar, 1999). The *cis*-acting sequences that are required for pausing at the *MPS1* are named the *abc* region (Sayrac, et al. 2011). Replication pausing can be observed both in P and M cells and interestingly the required sequences are located within the two Plus (P) and Minus (M) DNA cassettes that are swapped during the switching process (Dalgaard & Klar, 2000; Vengrova & Dalgaard, 2004). Thus, different *cis*-acting sequences mediate barrier activity in the two cell-types. Generally there is no sequence similarity between the P and M cassettes, however, within the *abc* region there is some sequence similarity (Sayrac, et al. 2011). The part of the *abc* region that is required for pausing is about 60 bp long and is located approximately 30 nucleotides from where the imprint is introduced. Both the *P-* and *M-abc* regions act as pause sites for the replication fork when they are located on a plasmid. Furthermore, competition experiments suggest that a *trans*-acting factor is binding to the *abc* region to mediate pausing; introduction of two multi-copy plasmids each containing 10 copies of the *M-* or *P-abc* regions cause a 30-40% reduction in sporulation (the sporulation efficiency is dependent on the efficiency of mating-type switching and mating). Importantly, the data does suggest that the factor(s) binding to the *abc* region is present in the cells in a significant number of molecules. Interestingly, the *abc* region does not mediate replication pausing at the transcriptionally silenced donor *loci*, even though *mat2P* is replicated in the correct orientation for pausing. This observation is important as it establishes a mechanism by which replication barriers can be regulated in other systems through the regulation of heterochromatin formation.

As mentioned above, replication pausing at *MPS1* is required for introduction of an imprint that marks switchable cells of *S. pombe*. This imprint has been shown to consist of two ribonucleotides incorporated into the DNA (Vengrova & Dalgaard, 2004; Vengrova & Dalgaard, 2005; Vengrova & Dalgaard, 2006). Several *cis*-acting regions have been identified that are required for the introduction of the imprint. First, there is a small *cis*-acting sequence located distal to *mat1* that is named *SAS1* (Arcangioli & Klar, 1991). *SAS1* mediates binding of the *trans*-acting factor Sap1 that is required for barrier activity at the rDNA and *LTRs* (see Sections 3.6 & 5.0). However, the deletion of a 264 bp region (*Msmt0*) that includes *SAS1* does not affect replication pausing at *MPS1*, suggesting that Sap1 has another role during imprinting (Dalgaard & Klar, 2000). A study of the interaction between Sap1 and its binding sites *SAS1* and in the rDNA suggests that the protein might be interacting differently with the DNA at the two sites and that this might cause the difference in whether the protein mediates barrier activity (see Section 3.6). Another *cis*-acting sequence that is required for the introduction of the imprint is a 204 bp spacer region that is located centromere-proximal to the *abc* region and the site of imprinting (Sayrac, et al. 2011). Deletion of this region leads to abolishment of imprinting but only a small decrease in the intensity of the *MPS1* signal. Replacing the region with a randomized sequence only has a small effect on both imprinting and pausing. Similarly, gradually reducing the length of the spacer region gradually reduces imprinting. High-resolution Southern blot analysis of replication intermediates from the strain carrying the spacer deletion mapped the position both of the 3′ end of the leading-strand and the 5′ end of the lagging-strand to the imprinting site, suggesting that the imprint consists of ribonucleotides that originate from the priming of an Okazaki fragment. Furthermore, the high-resolution Southern blot analysis also detected a centromere-proximal lagging-strand priming site about 350 nucleotides from the site of the imprint in the wild-type strain, which also previously has

been detected by RIP mapping (Vengrova & Dalgaard, 2004; Sayrac, et al. 2011). This priming site is absent in the spacer deletion strain (instead a diffuse set of priming sites are observed closer to the imprint), but restored when the spacer is replaced by a random sequence (Sayrac, et al. 2011). The analysis also showed that while the sequences within the *abc* region are required, there is no sequence requirement for the region where the imprint is introduced. The data suggest that the imprint is formed in response to a site-specific priming event induced by replication pausing, and that the position of subsequent priming sites for subsequent replication fork restart is important for the formation of the imprint. Potentially, topological restraints could prevent access of factors if the priming site chosen after the release of the fork is too close to the imprinting site. This is the first example of a *cis*-acting region affecting the position of priming sites and suggests that chromatin could affect primer localization during lagging-strand replication. Importantly, the data provide a mechanism by which replication barriers can act to differentiate sister-chromatids for cellular differentiation.

The *mat1* imprint/ribonucleotides are maintained in the DNA for one cell-cycle, potentially through the binding of a *trans*-acting factor to flanking *cis*-acting sequences and act themselves as a replication barrier in the S-phase of the next cell-cycle (the 3'-end of the leading-strand was mapped to the nucleotide preceeding the ribonucleotides), thus leading to induction of the replication-coupled recombination event that drives mating-type switching (Vengrova & Dalgaard, 2004). Ribonucleotides have been shown to frequently be incorporated during DNA replication (Nick McElhinny, et al. 2010a; Nick McElhinny, et al. 2010b) and to stall DNA polymerases when present in the replication template *in vitro* (Vengrova & Dalgaard, 2004). Interestingly, only a single ribonucleotide present in a DNA template has been show to act as a barrier for DNA polymerase ε (Nick McElhinny, et al. 2010). However, RNA can template DNA repair *in vivo* and both *S. cerevisiae* polymerases α and δ can use templates containing four ribonucleotides in a row, although with decreased efficiency (Storici, et al. 2007).

9. Interference between RNA polymerase II transcription and the DNA replication machinery

In *S. cerevisiae,* RNA polymerase II transcription has been shown to interfere with DNA replication fork progression. Transcription associated recombination (TAR) increased when the orientation of polymerase II transcribed genes was head-on to the progressing replication fork (Prado & Aguilera, 2005). Using cell-cycle specific promoters they also showed that this increase was dependent on the S-phase. The study also detected a replication barrier by 2D-gel analysis of replication intermediates within the recombination substrate that was dependent on polymerase II transcription. The intensity of the replication barrier signal was increased in an Rrm3 mutant. Importantly, more recent data suggest that it is the formation of RNA-DNA hybrids (R-loops) that are the cause of TAR and not the collision of the two types of forks (Aguilera & Gomez-Gonzalez, 2008; Gonzalez-Aguilera, et al. 2008). Also, several mutations affecting the maturation of mRNPs increase TAR. While these experiments were done using a *CEN*-plasmid, a genome wide study identified 96 sites where there were high levels of DNA polymerase binding (Azvolinsky, et al. 2009). A significant number of these were genes highly transcribed by RNA Polymerase II. However, there was no correlation between the direction of replication and transcription at these sites. The sites also correlated with high occupancy of the Rrm3 helicase, but the absence of Rrm3

did not lead to an increase in the DNA polymerase occupancy. Similarly, 2D-gel analysis of replication intermediates detected replication fork barriers at some of these sites, but the absence of Rrm3 did not lead to an increase in pausing at these barriers. R-loops have also been proposed to act as barriers for replication fork progression in human cells (Tuduri, et al. 2009; Tuduri, et al. 2010). Topoisomerase 1 (Top1) together with ASF/SF2, a splicing factor of the SR family, act to suppress the formation of DNA-RNA hybrids during transcription, thus preventing these R-loops from interfering with the progression of replication forks. In Top1 deficient cells γH2AX, a phosphorylated specialized histone (see Section 11.), accumulates at genes that are highly expressed during S-phase such as histone genes. The Top1 deficiency might affect fork progression in two ways; through Top1's role in releasing super-coiling between two types of converging forks, and through Top1's role in regulation of mRNP assembly, presumably by binding and phosphorylating splicing factors of the SR family (Rossi, et al. 1996; Soret, et al. 2003; Malanga, et al. 2008). It has long been known that in bacterial genomes highly-expressed genes are oriented such that transcription does not interfere with replication and it has been proposed that this might also be true for a large fraction of the human genome (Huvet, et al. 2007).

10. The Rrm3 helicase mediated replication progression at non-nucleosomal protein-DNA barriers

The *S. cerevisiae* Rrm3 5′ to 3′ helicase has been shown to have an important function at replication barriers. Rrm3, which is a member of a family conserved from yeast to humans (Zhou, et al. 2002), was originally identified because its absence caused an increase in recombination and formation of extra chromosomal circles at the rDNA array (Keil & McWilliams, 1993; Ivessa, et al. 2000). Rrm3 travels with the replication fork, interacts *in vivo* with Pol2 (the catalytic subunit of DNA polymerase ε) and has a role in replication at all the yeast chromosomes (Azvolinsky, et al. 2006). Importantly, in the absence of Rrm3 replication pausing/stalling is observed (or increased) at an estimated 1400 sites in the genome, including centromeres, tRNA genes, inactive replication origins, and the silent mating-type loci, as well as telomeric and rDNA sites (Ivessa, et al. 2003). Potentially, Rrm3 is required for proper replication through all stable, non-nucleosomal protein-DNA complexes. Replication through the rDNA is generally impaired in a *Δrrm3* mutant leading to replication stalling at several sites including the polymerase III transcribed 5S rRNA gene, at inactive origins and at the beginning and end of the RNA polymerase I transcription unit (Ivessa, et al. 2000). In addition, the intensity of the Fob1-dependent replication barrier significantly increased and more replication termination was observed at the barrier. Rrm3 also affects replication at the telomeres and internal tracts of $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric DNA; in the absence of Rrm3 replication slowing at the repeats were increased and in addition replication stalling was observed at multiple sites within the sub-telomeric regions including in active origins (Ivessa, et al. 2002). At the silent mating-type regions and at the tRNA genes the Rrm3-dependent stalling was shown to be dependent on the presence of the associated protein complexes (Ivessa, et al. 2003). Also, loss of the ATPase function of Rrm3 has the same effect as deletion alleles, establishing that the catalytic activity of the helicase is needed for this function. Due to the increased genetic instability of Rrm3 mutants, their viability is dependent on *mrc1*, *mre11, rad50*, *sgs1*, *srs2*, *top3*, *xrs2* and *dia2*, genes involved in activation of the inter-S phase checkpoint and replication fork restart (Torres, et al. 2004; Morohashi, et al. 2009). Interestingly, Dia2 is an F-box protein (E3 ubiquitin ligase) that also

travels with the replication fork and might have a role at stalled DNA replication forks at protein-DNA barriers, perhaps by interaction with key substrates (Mimura, et al. 2009; Morohashi, et al. 2009). However, a recent study looking at the Fob1-dependent barrier using 2D-gel analysis of replication intermediates did not detect any effect on intensity of the barrier signal in a Δ*dia2* mutant (Bairwa, et al. 2011).

11. γ-H2A.X formation at stalled replication forks

Stalling of replication forks generally leads to the activation of the protein kinases of the PI(3) kinase-like kinase (PIKK) family, *S. pombe* Rad3, *S. cerevisiae* Mec1 and Mammalian ATR. One function of the activation of these kinases is to stabilize replication forks to prevent their collapse (Desany, et al. 1998; Lopes, et al. 2001). The PIKK mediated phosphorylation of a specialized histone called H2A.X (mammalian) or H2A (yeast) might help stabilize the stalled fork (Cobb, et al. 2005; Papamichos-Chronakis & Peterson, 2008) but also recruits DNA damage repair proteins (Mammalian Mdc1 and *S. pombe* Crb2 and Brc1; Du, et al. 2006; Williams, et al. 2010). Two studies have utilized this molecular beacon for identifying sites of replication stalling genome wide (Szilard, et al. 2010; Rozenzhak et al. 2010). In *S. cerevisiae,* **γ-**H2A (the phosphorylated form of H2A) enriched loci are concentrated at the rDNA locus, telomeres, DNA replication origins, *LTRs*, tRNA genes and centromeres, all of which are known replication barriers, but also at actively repressed protein-coding genes (Szilard, et al. 2010). In the latter case, the analysis showed that actively repressed genes, which are notably enriched for the transcription factors Sum1 and Ume6 that are known to recruit the two Hst1 and Rpd3 histone deacetylases (HDACs) (Kadosh & Struhl, 1997; Xie, et al. 1999; Robert, et al. 2004). This observation suggests that hetero-chromatin may pose an obstacle to progression of DNA replication forks. Importantly, loss of Hst1 or Rpd3 histone deacetylase activity abolished the **γ-**H2A enrichment at genes specifically regulated by Hst1 or Rpd3. Generally, **γ-**H2A enrichment was depended on both Mec1 and Tel1 (the latter is activated by double-stranded breaks), suggesting that both replication fork stalling as well as collapse occurred at the identified loci. Also, increased **γ-**H2A enrichment was observed in a *Δrrm3* mutant background, suggesting a decreased ability of replication forks to pass through the barriers, thus leading to an increase in **γ-**H2A accumulation. A similar genome wide study in *S. pombe* identified **γ-**H2A enriched loci that corresponded well with those observed in *S. cerevisiae,* including the mating-type locus (including the *RTS1* element, the region containing *MPS1* and the imprint, and the IR elements that flank the transcriptionally silenced donor loci), the rDNA loci (including the gene coding region and the replication barriers), and all heterochromatin regions, including the centromeres (at the *otr* elements, but not the *cnt* or *imr* elements nor at the flanking inverted repeats) and telomeres, both Tf2-type retrotransposons and wtf elements and finally in a subset of gene coding sequences that were characterized by the presence of repetitive sequences (Szilard, et al. 2010). Contrary to what was observed in *S. cerevisia* **γ-**H2A accumulation was almost exclusively dependent on Rad3 and only at the telomere (in the absence of Rad3) on Tel1. In the mating-type region (the *RTS1* element and *MPS1*), **γ-**H2A accumulation was dependent on Swi1 and Swi3 function in pausing and termination, while at the hetrochromatic regions **γ-**H2A accumulation is associated with the presence of Clr4-dependent heterochromatin and partially depends on Swi6. Several **γ-**H2A sites found in budding yeast were absent in fission yeast, including tRNA genes, *LTRs* (in

the absence of the transposon) and replication origins. The absence of γ-H2A accumulation at tRNA genes and *LTRs* is interesting, as fork stalling is observed at these sites by 2D-gel analysis (see Section 5.), and might reflect that either different types of stalled fork exist or that the duration of the stall is important for γ-H2A accumulation.

12. Concluding remarks

It is evident that many types of replication barriers have been defined. Whilst there are differences between these elements, there are also similarities. At some barriers replication forks only pause and then restart again without fork collapse. However, at others the replication fork is stalled until an approaching fork arrives from the other side for mediation of replication termination. Different molecular responses and levels of genetic instability are observed at the barriers. What determines the fate of a stalled replication fork at a barrier is still generally unknown. However, it is evident that helicases, such as *S. cerevisiae* Rrm3 and *S. pombe* Srs2 promote replication through protein mediated barriers (Section 8. & 10.) and Tof1 and Mrc1 through barrier caused by "structure" in the template (Section 6.), while *S. pombe* Rtf2 acts to stabilize the stalled fork for replication termination (Section 8.). It is also evident, that many different proteins can act as replication impediments. Generally, these proteins do not promote barrier activity through the formation of "stable" complexes, although in the absence of *S. cerevisiae* Rrm3 barrier activity stalling at stable protein-DNA complexes can be observed (Section 9.). Barrier activity is most likely generated via direct interaction(s) with the progressing replisome. For example, most protein-mediated barriers are polar, only stalling replication forks when encountered from one side, while for *S. pombe* Sap1 acts as a barrier at some *cis*-acting sites but not others (Sections 8. & 3.6). It should be mentioned that strong replication barriers often consist of several closely spaced *cis*-acting sequences where one or more *trans*-acting factors mediate the replication barrier. Also, these *trans*-acting factors have the ability to dimerize or polymerize, potentially increasing the efficiency of interaction, but more likely providing additional topological constraints when the DNA is unwound by the replicative helicase. Also, it is common for known protein-mediated barrier activity to depend on the *trans*-acting factors Tof1/Csm3 (*S. cereviaise*) and Swi1/Swi3 (*S. pombe*), although there are some notable exceptions (for example, see Pryce et al. 2009). Putatively, the *S. cerevisiae* Tof1/Csm3 or *S. pombe* Swi1/Swi3 heteromeric complexes slide along the double-stranded DNA in front of the replicative helicase and senses the presence of barrier proteins. It has been shown earlier that in the absence of *S. cerevisiae* Tof1/Csm3 an uncoupling of the replicative helicase from the replicative polymerases can occur (Katou, et al. 2003; Nedelcheva, et al. 2005), thus Tof1/Csm3 (and phylogenetic related proteins) could directly inhibit MCM function when barrier proteins are encountered. Consistent with this model, the 3′ end of the leading-stand and the 5′ end of the lagging-strand have been mapped in close proximity about approximately 30-40 bp from the *cis*-acting sequences that mediate the barrier activity both at the *S. cerevisiae* rDNA barrier and at the *S. pombe MPS1* site (Figure 5A; Sections 3.6 & 8.1).

Interestingly, DNA structures in the template can also stall replication fork progression in a site-specific manner. These barrier signals most likely act on the lagging-strand as impediments to polymerase progression (Figure 5B). Interestingly, here *S. cerevisiae* Tof1 and Mrc1 are required for efficient replication through the elements (Mrc1 does not affect barrier activity at protein barriers), but not through the checkpoint activation function of these proteins. Still, the characteristics of these barriers suggest that the mechanism by which this

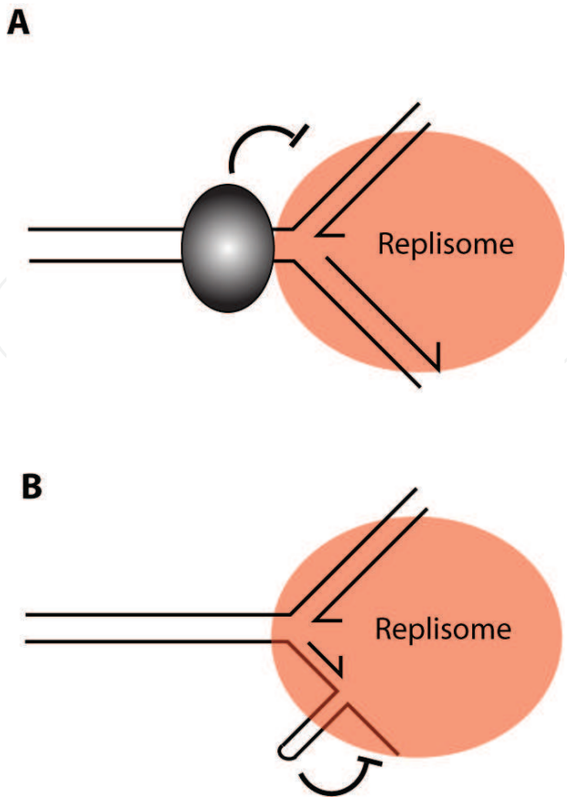

Fig. 5. The two types of replication barriers described. A) DNA bound factors can stall replisome progression, leading to a 3′ leading-strand end and 5′ lagging-strand end a certain distance from the barrier. B) Structure at the lagging-strand template leads to stalling of replisome progression.

type of barriers stalls replication forks is different from the one by which protein barriers act. Potentially, structures in the lagging-strand template strand could also explain by *S. pombe* tRNA genes mediate barrier activity in a Swi1 independent manner.

It is also evident from this comparison that replication barriers both prevent and cause genetic instability and a number of key points highlight this: I) Many of the described barriers have either been shown or are thought to prevent conflicts between progressing RNA polymerases I, II and III and replication forks, thus promoting genetic stability. II) Other barriers are thought to promote telomere addition for maintenance of genetic stability. III) Several barriers have been shown to cause genetic instability, including rDNA barriers (see Section 2.4), the *RTS1* element (Ahn, et al. 2005), transposons (Zaratiegui, et al. 2011), as well as DNA structure in the template (Section 7.). IV) Again others have specific roles in induction of recombination events, including genetic rearrangements important for contraction/expansions of rDNA arrays and cellular differentiation or development in *S. pombe* and *Tetrahymena* (Sections 3.2 & 8.).

It is highly likely that additional biological roles will be defined for replication barriers in the future. Here, research into such genetic elements' roles in cellular differentiation and development in higher eukaryotes would be important. In addition, it will be interesting to understand how replication barriers drive evolution through instability at the stalled forks. It is already evident from studies of fragile sites, genomic rearrangements, repeat

expansion/contraction and mutations that underlie the genetic instability of cancer cells, that replication barriers are likely to have a profound role in disease formation. Thus, the importance of a better understanding of the molecular processes that lead to stalling of replication forks and that control the events at these forks, should not be underestimated.

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The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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