Rtf1-mediated eukaryotic site-specific replication termination.

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Abstract:

The molecular mechanisms mediating eukaryotic replication termination and pausing remain largely unknown. Here we present the molecular characterization of Rtf1 that mediates site-specific replication termination at the polar *Schizosaccharomyces pombe* barrier *RTS1*. We show that Rtf1 possesses two chimeric myb/SANT domains, one is able to interact with the repeated motifs encoded by the *RTS1* element as well as the elements enhancer region, while the other shows only a weak DNA binding activity. In addition we show that C-terminal tail of Rtf1 mediates self-interaction, and deletion of this tail has a dominant phenotype. Finally, we identify a point mutation in Rtf1 domain I that converts the *RTS1* element into a replication barrier of the opposite polarity. Together our data establish that multiple protein-DNA and protein-protein interactions between Rtf1 molecules and both the repeated motifs and the enhancer region of *RTS1* are required for site-specific termination at the *RTS1* element.
Introduction

DNA replication is a highly complex process whereby genetic information and epigenetic chromatin states are duplicated, sister chromatid cohesion is established and DNA damage repair is performed. Although there is a general understanding of the factors and mechanisms by which eukaryotic DNA replication is initiated, very little is known about the molecular processes underlying replication pausing and termination. Most replication termination occurs randomly when converging replication forks meet in termination zones between active origins (Santamaria et al. 2000). However, at special genetic elements, site-specific replication termination or pausing is deliberately induced. One class of such elements is the barriers present in the polymerase I-transcribed rDNA arrays from yeasts to metazoans (reviewed by Codlin and Dalgaard 2003; Hyrien 2000). At these replication barriers, a family of transcription termination factors mediate site-specific termination of replication forks moving in one direction while allowing replication forks moving in the other direction to pass unhindered; the factors include TTF1 (mouse and human; Gerber et al. 1997; Lopez-estrano et al. 1998), Reb1 (Schizosaccharomyces pombe; Sanchez-Gorostiaga et al. 2004), as well as the unrelated protein Fob1 (Saccharomyces cerevisiae; Brewer and Fangman 1988; Linskens and Huberman 1988; Kobayashi and Horiuchi 1996). While the biological function(s) of the Reb1/TTF1 barriers have not been established experimentally, the Fob1 barrier has a dual function: It acts i) to prevent collision between replication and polymerase I transcription machinery, which otherwise leads to genetic instability, by ensuring that the two types of forks move in the same direction within the polymerase I transcriptional unit (Takeuchi et al. 2003); ii) to induce recombination and establishment of cohesion between sister-chromatids in order to prevent unequal crossovers and genetic instability (Kobayashi and Horiuchi 1996; Huang et al. 2006).

Interestingly, the S. pombe RTS1 element located in the mating-type region is closely related to the rDNA barriers: i) RTS1 is polar, acting on replication forks moving in the cenII-distal direction, and its biological function is to optimize the replication-coupled recombination event that underlies mating-type switching (Fig. 1A; Dalgaard and Klar 2001). ii) Replication forks stalled at RTS1 induce recombination (Ahn et al.
iii) The cis-acting sequences are related (Fig. 1B). Firstly, RTS1 region B contains four repeated ~60 bp motifs each possessing polar barrier activity (Codlin and Dalgaard 2003). Similar rDNA motifs, which in the metazoan system are called SAL-boxes, are required for barrier activity (Gerber et al. 1997; Lopez-Estrano et al. 1999; Sanchez-Gorostiaga et al. 2004). For the S. pombe Reb1 and the metazoan TTF1 factors, these rDNA barrier motifs have been shown to act as binding sites in vitro (Lopez-estrano et al. 1998; Melekhovets et al. 1997; Sanchez-Gorostiaga et al. 2004; Zhao et al. 1997). iv) In addition, a ~ 60 bp enhancer called region A, characterized by a purine-rich upper and a pyrimidine-rich lower strand has been defined for RTS1. Region A does not possess any independent barrier activity, but mediates in vivo a four fold enhancement of region B activity by promoting a functional interaction between the motifs (Codlin and Dalgaard 2003). Similarly, for the metazoan rDNA elements, in vitro experiments have established the presence of a GC-rich sequence flanking one of the SAL-boxes, which is required for contrahelicase activity. Like the RTS1 region A, this GC-rich sequence is characterized by an asymmetrical distribution of purines and pyrimidines on the two DNA strands (Putter and Grummt 2002). v) Both the S. pombe rDNA barrier and RTS1 require Swi1 and Swi3 factors for activity, while the S. cerevisiae Fob1 rDNA barrier depends on the homologues, Tof1 and Csm3 (Mohanty et al. 2006). Finally, it should be noted that recently a Reb1-independent, but putatively Sap1-dependent barrier where replication pausing is observed was defined within the S. pombe rDNA barrier (Krings and Bastia 2005; Krings and Bastia 2006; Mejia-Ramirez et al. 2005). Interestingly, Sap1 has also been shown to bind in the mating-type region (Arcangioli and Klar 1991), but the smt-0 deletion that removes the cis-acting Sap1 binding sites does not affect the replication barriers in the mating-type region (Dalgaard and Klar, 2000).

Here we characterize the trans-acting factor Rtf1 that is required for RTS1 function. Rtf1 is a paralogue of the S. pombe Reb1 protein required for rDNA replication barrier activity as well as polymerase I transcription termination, and thus it is a new member of the Rtf1/Ttf1/Reb1 protein family. We address the molecular mechanism by which Rtf1 mediates site-specific replication termination at RTS1.
Materials and methods:

UV mutagenesis: Logarithmically growing cells (strain JZ183) were plated on either sporulation (PMA+) or rich (YEA) media-containing plates and directly irradiated with UV (24 µJoule; 55% survival) using a Stratalinker (Stratagene). PMA+ plates were incubated at 30˚C for five days and then stained with iodine vapor for identification of mutants. YEA plates were incubated for four days at 30˚C, replicated to PMA+, followed by two days incubation at 30˚C, and then stained with iodine vapor. Iodine staining was performed as described by Moreno et al, 1991. The genetic screen used for identification of the dominant rtf1 mutant was done in a similar fashion, except that rtf1+-plasmid pBZ136 had been introduced in the strain JZ183. Strain construction and isolation: Strains were constructed using methods described by Moreno et al, 1991. The genotypes of the strains are described in supplementary data. 2D-gel analysis of replication intermediates: Strains were grown either in YEA or AA-Leu (plasmid-containing strains) media. DNA from logarithmically growing cells was isolated as described by Huberman et al. 1987. Replication intermediates were enriched using BND-cellulose (Sigma; Kiger and Sinsheimer 1969), digested with restriction enzymes and analyzed on two-dimensional agarose gels (Brewer and Fangman 1987). A probe specific to the 0.8 Kb RTS1 fragment (Dalgaard and Klar 2001) was used for the Southern analysis. Signals were quantified using a phosphorimager and Quantity One software (Biorad). For each gel the intensity of ascending part of the Y-arc was used for normalizing the pause- and termination-signal intensities. The quantification method is described in full in Codlin and Dalgaard 2003. Protein expression, purification and gelshift assays: Domain II (AA 244-466) and I + II (AA 94-466) are expressed using the Studier expression systems (Studier and Moffatt 1986). Domain I (AA 94-256) was expressed using the pMAL expression system (New England Biolabs). Partial purification was done using an amylose column or a Ni²⁺ column (domain I+II) followed by an amylose column (di Guan et al. 1988; Petty 1996). Gel-shifts were obtained as described by Sambrook and...
Russell 2001. For each figure, all lanes displayed in a given panel were run on the same gel. For a more complete description refer to supplementary text. **Two-hybrid analysis:** Rtf1 segments were cloned into *S. cerevisiae* two-hybrid vectors, pGADT7 and pGBK7 (MATCHMAKER Gal4 Two-hybrid system3, BD Biosciences Clontech). The analysis was performed as described (Bartel *et al.* 1993) using *S. cerevisiae* strain AH109.

**Results**

**Identification of Rtf1.** The mating-type locus *mat1* has to be replicated in a specific direction for imprinting and mating-type switching to occur (Dalgaard and Klar 1999; Dalgaard and Klar 2001). We have utilized the dependence of the imprinting process on the replication direction in a genetic screen for *trans*-acting factors involved in site-specific termination of replication at *RTS1* (Dalgaard and Klar 2000). Transposition of *RTS1* in the inverted orientation to the cen-distal side of *mat1* changes the direction by which the *mat1* locus is replicated, and therefore leads to the inhibition of imprinting, mating-type switching, mating and sporulation (Dalgaard and Klar 2001; Fig. 1C, line drawing). The strain’s decreased ability to sporulate can be assayed by iodine staining (Fig. 1C; strain JZ183). Iodine stains starch that is produced in the spores of this yeast. Similarly, a reduction in *mat1* imprinting can be quantified by Southern analysis (Fig. 1D; lanes 2 & 3). The assay utilizes the efficient conversion of the *mat1* imprint into a double-stranded break (DSB) by some DNA purification methods (Arcangioli 1998; Dalgaard and Klar 1999). In our genetic screen we utilized that *trans*-acting mutations that abolish replication termination at *RTS1* will partly restore the wild-type direction of fork progression at *mat1*, and as a consequence allow an increased number of cells to switch mating-type, mate and sporulate. (Fig. 1C, lower line drawing & insert). Originally, mutations in three complementation groups, named replication termination factors (*rtf*), were isolated in this screen (Dalgaard and Klar 2000). The majority of the mutations, 28 out of 30, belong to the *rtf1* complementation group described here. The sporulation levels observed for the identified *rtf1* mutants varied from 31% to 61%, compared to 4.8% observed in the parental strain (JZ183) and 65% in the wild-type *h*<sup>90</sup> control strain (JZ1). Importantly, haploid meiosis is not observed in these strains,
establishing that de-repression of the silenced donor loci, mat2P and mat3M, does not occur (data not shown). Furthermore, Southern analysis of the mat1 region of these strains detected increased levels of mat1 DSB, as expected from a partial restoration of the mat1 imprint (Fig. 1D). Subsequently, sub-cloning and complementation studies identified rtf1 as the open reading frame SPAC22F8.07C defined in the S. pombe genome project (supplementary data). A complete rtf1 null-mutation was constructed by replacing the rtf1 open reading frame with the ura4+ gene (strain SC7). Analysis of the chromosomal as well as the plasmid-borne RTS1 shows that \( \Delta rtf1 \) abolishes RTS1 function (Fig. 1E & F).

**Definition of functional Rtf1 domains.** The large number of isolated rtf1 alleles allowed us to define the functional domains of the Rtf1 protein. The alleles include 10 single amino acid (AA) substitutions, 6 frame-shifts (one in an intron splice junction), and 4 nonsense mutations (Fig. 2A & B; Suppl. Fig. S1). All the mutants isolated in the initial screen were recessive (data not shown). The distribution of point mutations suggested that in addition to the known myb-motif, an additional functional domain might be present, thus, we employed bioinformatics for its identification. The Rtf1 sequence (CAF31329; SpRtf1) and related sequences were used to search a non-redundant protein sequence database through the World Wide Web interface to the PSI-BLAST program (default parameter settings). A ~400 AA Rtf1 segment showed statistically significant similarity to proteins from a variety of species (E-value << 0.05) and was retained for further analysis. Previously, a ~200 AA conserved segment (here domain II) encompassing the two myb/SANT motifs was identified in *Mus musculus* TTF1 (MmTTF1), *S. cerevisiae* Reb1 and *M. musculus* c-myb (Evers *et al.* 1995, which refers to the two myb/SANT motifs as domain I and II). The myb motif is a ~50 AA sequence which folds into a domain consisting of three helices characterized by tryptophan (Trp) residues essential for DNA binding. In the case of this protein family, mutation of Trp668 to Lys (W668K) in MmTTF1 was found to abolish binding of the dsDNA recognition sequence (Evers *et al.* 1995). In addition, a subclass of the myb motifs called the SANT motif has been shown to interact with histone tails (Boyer *et al.* 2004). Interestingly, the two domain II c-myb motifs of Rtf1 are identified on the sequence level to belong to this sub-class. A more careful examination of the PSI-BLAST
output revealed that the conserved domain II, present in the second half of the protein, displayed similarity to a putatively related domain in the first half, i.e., the ~400 AA Rtf1/Reb1 conserved segment can be divided into two structurally related regions both predicted to interact with DNA via myb-like folds (Fig. 2A & B; domain I & II). A careful computational analysis, using a hidden Markov model, the Conserved Domains Database and the PhD structural predictions establishes that this family of proteins possesses two chimeric putative DNA-binding domains, both displaying an overall similarity to metazoan c-myb. These two domains potentially contain in total five structural myb-motifs, two of which might also be SANT motifs (supplementary text; Fig. 2A & B).

**Rtf1 domain I can bind to RTS1 region A & B.** To characterize the DNA-binding specificities of the two domains, fusion proteins between a 6xHis-tagged maltose binding protein (MBP) and Rtf1 segments encompassing domain I, domain II and the chimeric domains (domain I+II) were purified (Suppl. Fig. S2A). Using the domain I, gel-shift assays were performed with a labeled dsDNA oligonucleotide corresponding to motif 4 from region B (Codlin and Dalgaard 2003; Fig. 3A). The analysis detected several sharply defined mobility shifts characteristic of protein binding, and potentially of more than one molecule. It should be noted that Western analysis of shifted material verifies that the shift is due to binding of domain I (Fig. S2B), and that binding can be out competed with excess cold specific competitor (Fig. S2C). Furthermore, gel-shifts with dsDNA oligonucleotides resembling three shorter segments of motif 4 establish that domain I binds to the middle third of the motif (Fig. 3B; left panel). A linker scanning mutagenesis of motif 4 has earlier defined two linker substitutions that abolish motif 4 barrier activity *in vivo* (Codlin and Dalgaard 2003). We used the five dsDNA oligonucleotides synthesized for that study to further identify sequences within motif 4 required for Rtf1 domain I binding. Interestingly, none of the substitutions completely abolished binding (data not shown; Codlin and Dalgaard 2003). However, gel-shift assays using the rep4-mut3 substitution, which *in vivo* abolishes barrier activity, leads to a marked reduction in the amount of shifted material (Fig. 3B; right panel). Together these experiments establish that the main domain I binding site is located in the middle third of motif 4.
Interestingly, in this part of motif 4, purines and pyrimidines are distributed asymmetrically between the two strands. As mentioned in the introduction the RTS1 element possesses an enhancer region characterized an asymmetric distribution of pyrimidines and purines. We decided to investigate if domain I also displays an affinity for region A dsDNA (Fig. 3A: right panel). Again, gel-shift assays detected DNA binding. The binding could somewhat be out competed with poly I:C but not poly G:C, thus displaying some specificity. Western analysis of shifted material verifies that the shift is due to binding of domain I (Fig. S2B), and that binding can be out competed with excess cold specific competitor (Fig. S2C). However, the domain I displays a lower affinity for region A (Kd=3467 nM) than for motif 4 dsDNA (Kd=549 nM; Fig. 4). Importantly, assays with the segment containing the chimeric domains detected similar binding specificities as observed for domain I only; shifts of a slightly reduced intensity are observed for all four region B motifs as well as for the enhancer region A (Fig. 3C, domain I+II).

**Rtf1 domain I binds region B dsDNA.** As mentioned above, a TTF1 domain II mutation which abolishes dsDNA binding has been identified (Evers et al, 1995). We therefore tested if the purified domain II displays an affinity for region A or motif 4 dsDNA oligonucleotide. No domain II binding was detected using the region A dsDNA oligonucleotide (data not shown), however, a weak shift is observed for motif 4 dsDNA oligonucleotide (Fig. 3D). Importantly, the shift is only observed in the absence of unspecific competitor poly I:C DNA suggesting that the interaction either is sequence unspecific or that the domain also can interact in a sequence unspecific manner (data not shown). We therefore proceeded to test whether the motif 4 linker substitutions described above affected binding, and found that when the rep4-mut4 mutation is introduced, the shift is abolished, showing that the detected interaction is sequence-specific (data not shown; Fig. 3C). This substitution, which also abolishes motif 4 barrier function in vivo (Codlin and Dalgaard 2003), affects the sequence which shows similarity to the binding sequence defined for *S. pombe* Reb1 (Melekhovets *et al.* 1997). Thus, the observations are consistent with Rtf1 domain II interacting with the motif’s Reb1-like recognition sequence (Codlin and Dalgaard 2003).
Importantly, we have previously established that a single motif can act as a weak replication barrier, and that in the absence of region A, the introduction of additional motifs has an additive effect on the overall barrier activity (Codlin & Dalgaard, 2003). The datasets are therefore consistent with Rtf1 molecules binding each of the four repeats present in region B in vivo. We also establish that domain I, but not domain II, can interact specifically but with a lesser affinity with region A dsDNA. This potentially allows at least five Rtf1 molecules to act at RTS1 (see discussion).

**Domain I is involved in establishment of the polarity of the RTS1 barrier activity.** To gain further insight into the mechanism of Rtf1-mediated replication termination at RTS1, we decided to investigate the in vivo activity of mutant rtf1 alleles, containing AA substitutions. The analyzed domain II point mutations either strongly reduced or abolished barrier activity (mutations rtf1-S340F, rtf1-R293K and rtf1-M343R; data not shown). However, while abolishment of the wild-type barrier activity is observed in the six mutant domain I alleles, a novel barrier signal could be observed in some; the signal is the strongest in the rtf1-S154L genetic background (Fig. 5A), is detectable in the rtf1-L162Y strain (Suppl. Fig. S2D), barely detectable in the rtf1-P136L strain and is absent for rtf1-L129F and rtf1-G183E (data not shown). When the SacI-PstI fragment is analyzed, the wild-type signal is located close to the apex on the ascending part of the Y-arc (Fig. 5A, insert), however, the novel signal is located on the descending part (Fig. 5A, middle panels). This novel barrier signal is strongest when only the cis-acting region B is present; for unknown reasons the presence of region A causes a reduction of the signal intensity (compare Fig. 5A and B). There are two possible explanations for the appearance of this novel barrier signal; either the forks replicate through the RTS1 sequence and pause at a de novo site outside the element, or the RTS1 barrier activity has inverted its polarity now pausing replication forks moving in the opposite direction (Note; we conclude that only replication pausing occurs as we do not observe any termination signal). To discriminate between the two possibilities, we first excluded that
replication forks were stalling at a different position within the plasmid DNA. An analysis of an empty plasmid detected no barrier signal (Suppl. Fig. S2E), thus, the RTSI cis-acting sequence is still required for Rtf1-S154L-mediated pausing. We also verified that the novel Rtf1-S154L barrier is dependent on swi1+ and swi3+ activities (Suppl. Fig. S2F), and that the novel signal could be observed when the element was cloned in both orientations within the plasmid (Fig. 5A, B & D). Again in the presence of region A, the barrier intensity of the signal is lower and only clearly visible when located close to the middle of the fragment (Fig. 5D; also a relative difference in intensity is observed for the wild-type barrier in the two orientations, Fig. S2G; left panel). Finally, we excluded that the novel barrier is due to “collisions” with polymerase II transcription initiated at the flanking nmt1 promoter, similar to the collisions recently observed between transcription forks initiated by polymerase III and replication forks (Krings and Bastia 2006). Changes between repressed “low-level” and induced “high-level” nmt1-promoter mediated polymerase II transcription has no effect on the wild-type RTSI activity (Suppl. Fig. S2G). However, while we observed no effect of polymerase II transcription on Rtf1-S154L barrier activity, when the transcription forks move in the same direction as the paused replication forks (Fig. 5C), a reduction of the barrier activity is observed when the transcription occurs in the opposite direction (Fig. 5E). A possible explanation is that transcription displaces Rtf1-S154L molecules bound to the DNA. We then investigated the second possibility; the polarity of the RTSI barrier has changed in the Rtf1-S154L genetic background. We utilized the method where the polarity of a replication barrier can be established by analyzing overlapping restriction fragments of replication intermediates such that the position of the barrier is moved from one end of the DNA fragment to the other. This analysis was done for plasmids containing RTSI derived elements in both orientations, and it verified that the polarity of the Rtf1-S154L barrier is inverted (Fig. 5A & D). To investigate the possibility that the change in polarity was due to the S154L mutation affecting domain I DNA binding, we purified the mutant domain
and analyzed it’s binding to motif 4 and region A dsDNA. We observe gel-shift signals using the S154L-domain I at lower concentrations than observed with the wild-type domain I (Kd= 264 nM and 343 nM for motif 4 and region A, respectively; Fig. 6), establishing that the mutant domain is binding with a greater affinity than the wild-type domain. However, at the lower protein concentrations we also observe a smaller Hill coefficient in both cases; 1.0 and 0.71 versus 1.41 and 1.14 for motif 4 and region A, respectively (Fig. 4 & 6). At higher protein concentrations there is no linear fit but a stronger negative cooperativity. Thus, the mutation affects the domains ability to form mutimeric complexes with both region A and motif 4.

**The Rtf1 C-terminal region is required for function and can mediate dimerization/polymerization.** Finally, a genetic screen for dominant mutants was conducted. A multi-copy plasmid carrying the *rtf1* gene was transformed into the JZ183 strain, and the obtained strain was mutagenized. One mutant with increased iodine staining was isolated. Analysis of *RTS1* replication intermediates verified that there is a complete loss of replication barrier activity in this mutant (supplemental data; Fig. S2J). By crossing the isolated mutant strain with the Δrtf1 strain (SC8), and observing that no crossovers occurred in 27 tetrads analyzed, it was established that the mutation is closely linked to Rtf1 (data not shown). Sequence analysis of the *rtf1* gene detected a mutation introducing a nonsense codon at position 346, leading to a 120 AA truncation of the Rtf1 protein. Transformation of the strain with an *rtf1*+ plasmid (pBZ136) verified that the isolated strain carried a partially *rtf1*+-dominant mutation (Fig. 7A; strain ES8). One possible model for the partially dominant effect of this truncation is that it inhibits a functionally important dimerization or oligomerization of the Rtf1 molecules. To test this hypothesis, we employed a two-hybrid analysis. A self-interaction could be detected with the 127 AA C-terminal region of Rtf1 that includes one of myb-sant domains (Fig. 7B). However, this interaction was masked by the presence of DNA-binding domains,
probably because the fusion proteins could bind at other positions in the *S. cerevisiae* genome with greater affinity than at the reporter genes used for the assay. Thus, our genetic analysis shows that the Rtf1 C-terminal region is required for RTS1 function, and the two-hybrid results establish that this is through a role in Rtf1 dimerization or polymerization.

**Discussion**

The analysis presented here allows us to propose a model for Rtf1-mediated impediment of replication fork progression at RTS1 (Fig. 8A). In summary, the presented data suggest that at least five Rtf1 molecules can bind to the double-stranded RTS1 element through interactions involving both of the protein’s myb domains but mainly promoted by domain I (Fig. 2, 3, 4 & 8A, upper panel). Importantly, Rtf1 is able to interact both with the repeated region B motifs and the enhancer region A.

The Rtf1 binding to the *cis*-acting sequences might be stabilized through protein-protein interactions between Rtf1 molecules involving the Rtf1 C-terminal domain (Fig. 6). One possibility is that Rtf1 DNA binding at multiple sites within the RTS1 in combination with interactions between Rtf1 molecules acts as a topological constraint for DNA unwinding by the replicative helicase (Fig. 7A). Such a constraint could be augmented by DNA looping, a property which already has been observed for c-myb; the c-myb and c/EBP transcription factor complex together mediate DNA looping required for transcriptional activation (Tahirov *et al.* 2002). In addition, binding of multiple Rtf1 molecules within region B combined with the interaction between Rtf1 molecules could act to recruit Rtf1 to the lower affinity site within the region A dsDNA. Indeed, the dominant phenotype of the Rtf1 allele lacking the C-terminal region (Fig. 7) combined with the observation that region A has no intrinsic barrier activity but mediates a cooperative enhancement of the region B activity (Codlin and Dalgaard 2003) strongly support a role of C-terminal domain’s self-interaction in recruitment of Rtf1 to the enhancer region A. One possibility we are investigating is that the protein interacts with
single stranded DNA formed at region A when the DNA is unwound by the replicative helicase (Eydmann and Dalgaard; unpublished observation).

Finally, we identify a domain I mutation that changes the polarity of RTS1 (Fig. 5). When the Rtf1 domain I mutations that cause this inversion of the barrier’s polarity, are superimposed on the known structure of c-myb in complex with its dsDNA-binding site, it is evident that the mutation is not located on the DNA-binding surface (Fig. 8B). However, when the initial Kd is estimated for this domain, we find that it is lower than that of the wild-type domain, suggesting a stronger DNA affinity, however, at the higher protein concentrations we observe a decreased affinity and a Hill coefficient below one. Thus while the mutation does not significantly affect the initial complex formation, the mutant protein does display a decreased ability to form multimeric complex. However, the characteristics of this rtfl allele add some support to the model that unknown protein-protein interaction(s) involving domain I and replication protein(s) are affected by the mutation. Among the replication proteins, the replicative helicase (MCMs, reviewed by Takahashi et al. 2005), as well as Rtf2 (Codlin and Dalgaard 2003), Swi1 and Swi3 factors are likely candidates. Swi1 and Swi3 travel with the replication fork (Katou et al. 2003; Noguchi et al. 2004) and act at MPS1 to coordinate pausing of leading-strand replication in response to a lagging-strand signal (Fig. 1A; Vengrova and Dalgaard 2004). The identification of a swi1-rtf mutation, which only affects termination of replication at RTS1 but not at other replication barriers establishes that such RTS1-specific interactions involving replication fork proteins do occur (Codlin and Dalgaard 2003; Krings and Bastia 2004). This parallels the situation in E. coli, where the transacting factor Tus is thought to mediate replication termination through direct interactions with the replicative helicase DnaB (Mulugu et al. 2001). Importantly, the observation that in the Rtf1-S154L genetic background there is a loss of replication termination activity affecting the forks moving in one direction, but a gain of replication pausing activity acting on forks moving in the other, shows that the proposed Rtf1
domain I interactions are of importance when the element is replicated in both directions: Wild-type Rtf1 domain I interactions are required for efficient replication termination of the forks moving in one direction, but also must act to prevent pausing of the forks moving in the opposite.

Finally it should be noted that the identification of two DNA binding domains within the Rtf1 protein could have implications for understanding the molecular mechanisms underlying wide range of activities attributed to the Reb1/TTF1/Rtf1 protein family; polymerase II transcription activation (Carmen and Holland 1994; Graham and Chambers 1994; Packham et al. 1996; Wang and Warner, 1998), polymerase I transcription activation/repression (Wang et al. 1990) and termination (Lang et al. 1994; Lang and Reeder 1993; Mason et al. 1997; Melekhovets et al. 1997; Zhao et al. 1997), as well as chromatin insulator function (Fourel et al. 2001). Interactions with double-stranded DNA as well as dynamic changes in these interactions could play an important role for all these molecular processes.

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Mejia-Ramirez, E., A. Sanchez-Gorostiaga, D. B. Krimer, J. B. Schwartzman, and P. Hernandez, 2005 The mating type switch-activating protein Sap1 Is required for


Figure 1. Isolation of rtf1 mutants. A) Line drawing displaying the wild-type mat1 region on chromosome II. The positions of the imprint (filled circle), the RTS1 element (triangle) and the MPS1 (horizontal bracket) are given. Gray arrows indicate the directions by which the replication forks are moving within the mat1 region, as well as the polarity of the RTS1 replication barrier. B) Graphic outline of the RTS1 sub-elements. Region A (box) and the four repeated region B motifs (triangles; rep1, 2, 3, 4) are shown. C) Graphic outline of the genetic screen used for isolation of the rtf1 mutants. Top: The rearranged mating-type region of the JZ183 strain; the site-specific terminator RTS1 has been deleted at the cen-proximal side of mat1 and inserted at the cen-distal side in the inverted orientation. Top insert: Colonies of strain JZ183 stain yellow with iodine vapor. Bottom: Mutagenesis (vertical arrow) of rtf (replication termination factor) genes abolishes RTS1 function and leads to a partial restoration of the wild-type direction of replication at mat1 (gray arrows). Thus, imprinting (black circle) and mating-type switching are partly reestablished. Bottom insert: Colonies of rtf1 strains stain black with iodine vapor (strain JZ184, Fig. 2 legend). D) rtf1 mutations partly restore mat1 imprinting. Southern analysis of HindIII-digested chromosomal DNA (Dalgaard and Klar 1999). A probe specific to the mat1P HindIII fragment was utilized. Signals that
correspond to \textit{mat1}, \textit{mat2P} and \textit{mat3M} fragments are indicated. The \textit{mat2P} and \textit{mat3M} are detected due to partial homology. \textit{mat1} imprinted DNA is fragile during purification, where hydrolysis at the imprint leads to the formation of a double stranded break (DSB). The generated fragments are indicated in the panel. The difference in the molecular sizes of the DSB fragments from wild-type (lane 1) and mutant strain (lane 3) is due to the transposition of \textit{RTS1}. E) Rtf1 is required for \textit{RTS1} function. 2D-gel analysis of replication intermediates at the wild-type \textit{RTS1} locus in wild type (JZ1) and \textit{Δrtf1} (SC11) strains. The genomic position of the \textit{NsiI} restriction fragment analyzed is indicated in panel A. Stall (S) and termination (T) signals observed for wild-type replication intermediates are indicated. Note that the \textit{RTS1} element is replicated in both directions, however, we have earlier shown that while the majority of replication forks move in the permissive direction, the small fraction of replication forks in the non-permissive direction is stalled and terminated (Codlin and Dalgaard 2003). F) 2D-gel analysis of plasmid-borne \textit{RTS1} from wild-type (SC1) and \textit{Δrtf1} (SC46) strains. Earlier published experiments have established that the \textit{RTS1} element at this position in the plasmid is replicated in both orientations (Codlin and Dalgaard, 2003). Thus, stalling and termination signals are observed originating from forks moving in the direction where the barrier is active, while a normal Y-arc is formed by replication forks moving in the other direction. Stall (S) and termination (T) signals observed for wild-type replication intermediates are indicated.

**Figure 2. Bio-informatics analysis of the Rtf1 amino acid sequence.** A) Graphic outline of the position of the two c-myb like domains (blue boxes) and their structural motifs (white ellipses) as well as identified mutations. In the gelshift experiment presented below domain II encompasses the C-terminal tail. The positions of missense and frameshifts/nonsense mutations are given above and below, respectively. The position of the dominant mutation (strain ES8, R346*) is highlighted (red arrow). The strain names and identified mutations are: JZ184, \textit{W405G}; JZ185, \textit{S340F}; JZ221,
**Figure 3. DNA-binding specificities of the two Rtf1 c-myb-like domains.** A key above panels defines the experimental conditions used. Unbound (gray arrows) and shifted material (black arrows) are indicated for each panel. Unless otherwise stated, the experiments were done in the presence of 100 µg/ml poly G:C. The DNA oligonucleotides...
utilized are given under each panel. Western analysis of shifted material is provided for panel A and B as supplementary data (Fig. S2F). A) Gel-shift assays using purified domain I protein and a dsDNA oligonucleotide resembling motif 4 (ds-rep4; left panel) and region A (ds-regA; right panel). The sequences of the “upper” strands of the dsDNA oligonucleotides are displayed. Both panels; DNA binding challenged by addition of 50 µg/ml nonspecific competitors (given) does not abolish the observed shifts. However, binding can be efficiently out-competed by addition of specific competitors constituted by unlabeled substrates (Suppl. Fig. S2G). B) Definition of the domain I’s binding site within the motif 4 sequence. Left panel, three gel-shift assays using three different segments of the motif 4. The sequences of the upper strands of the three dsDNA oligonucleotides, 1, 2, and 3, are displayed. Right panel, gel-shift assay utilizing dsDNA oligonucleotides ds-rep4 or ds-mut3. The different mobility’s observed for unbound wild-type and mutant dsDNA oligonucleotides are due to the presence of a GATC overhang on the ds-mut3 oligonucleotide. C) Comparison of domain I’s and the chimeric domain’s affinities to the five different dsDNA oligonucleotides constituting region A (ds-regA) and each of the four repeated region B motifs (ds-rep1, 2, 3, 4). Unbound and shifted material is indicated to the left of the panels with gray and black arrows, respectively. The names of the utilized oligonucleotides are shown above the panels. It should be noted, that the retardation observed using the chimeric domains is greater than that observed for the individual domains. Since the increased retardation reflects the increased molecular size the observation establishes independently that the gel-shifts are due to binding of the purified domain(s). D) Domain II interacts weakly with motif 4. Gel-shift assay using purified domain II protein and dsDNA oligonucleotides ds-rep4 and ds-mut4. A weak gel-shift is only observed with the wild-type sequence (ds-rep4) but not the mutant (ds-mut4). Importantly, we do not see this shift in the presence of unspecific poly I:C competitor DNA (data not shown), suggesting that while the result obtained using the ds-mut4 oligo indicates that the interaction is sequence specific the interaction must be
weak as it can be out-competed with a unspecific competitor. Also, the smear observed in the top section of lanes 2,3,5,6 is due to the domain II interacting with single-stranded oligo DNA (see below).

**Figure 4.** Characterization of domain I binding to double stranded region A and repeat 4. A) Gelshift of ds repeat 4 DNA while titrating domain I. B) Gelshift of ds region A DNA while titrating domain I. C) Hill plot of the data points obtained above. The binding to repeat 4 and region A DNA fit a simple model with a Hill coefficient of 1.45 and 1.16, respectively, characteristic of low or no synergistic binding. The dissociation konstant Kd for domain I binding to repeat 4 DNA is determined to 549 nM. Binding to the region A is slightly weaker than to repeat 4 with a Kd of 3467 nM.

**Figure 5.** A) The domain I point mutation, S154L, changes the polarity of the RTS1 replication barrier activity. Pause signals are indicated by blue arrows. Determination of the polarity of the rtf1-S154L region B replication barrier using 2D-gel analysis of replication intermediates. The polarity is determined by analyzing overlapping fragments where the position of region B is shifted from one end of the analyzed fragment to the other. The polarity of the barrier activity can be determined by comparing the position of the barrier signal on the arc constituted by Y-structures between the three panels. The position on the Y-arc relative to the 1N and 2N signals shows how far the replication fork has traveled into the analyzed fragment before it was paused. The polarity of the replication barrier determined by the analysis is given below (red arrows). The polarity and position of the stalled fork is displayed above each panel. Insert, 2D-gel analysis of the wild-type region B, SacI-PstI fragment. A 2D-gel analysis of the genomic RTS1 element in the rtf1-S154L genetic background, verifies the loss of the wild-type barrier activity, but fails to detect an activity with inverted polarity suggesting that the novel barrier is only observed when RTS1 is located on a plasmid (Fig. S2I). B) The rtf1-
**S154L** barrier activity is not enhanced by region A. Analysis of region A and B, cloned in the same orientation as region B shown in panel B. The pause signal is indicated by a blue arrow. The barrier signal is only clearly visible on the analyzed PacI-KpnI fragment. 

C) Transcription initiated at the flanking nmt-promoter does not affect the rtf1-S154L barrier activity. The pause signal is indicated by a blue arrow. D) Analysis of the polarity of the Rtf1-S154L RTS1 barrier, cloned in inverted orientation. See panel A for the description of symbols. Pause signals are indicated by blue arrows E) Transcription initiated at the nmt1-promoter reduces Rtf1-S154L RTS1 barrier activity when transcription moves in the opposite direction of that of the stalled replication forks. The line drawing below displays the relative orientation of the nmt1 promoter and the RTS1 element within plasmid pBZ143. Insert, displays an enlargement of the apex of the Y-arcs observed when analyzing the Pst1-SacI fragment in the presence and absence (middle autoradiograph in panel D) of transcription. The pause signals are indicated by blue arrows.

**Figure 6.** Characterization of domain I-S154L binding to double stranded motif 4 and region A DNA. A) Gelshift of ds motif 4 and region A DNA while titrating domain I-S154L. B) Hill plot of the data points obtained above, only data points for the five lowest concentrations were used for the linear fit. The Hill coefficient of 1.0 and 0.71 were obtained for motif 4 and region A, respectively. The dissociation constant Kd for domain I-S154L binding to repeat 4 and region A DNA is estimated to 265 nM and 343 nM, respectively.

**Figure 7. The Rtf1 C-terminal tail is required for function.** A) Characterization of the dominant rtf1-R346* mutation. The three left panels display the iodine staining phenotypes of sporulating wild-type, ∆rtf1 and the rtf1-R346* colonies, respectively. The strains carry the RTS1 allele that allows quantification of in vivo barrier activity by iodine staining of sporulating colonies (Fig. 1C & D). In this genetic
background wild-type rtf1+ strains stain yellow, while rtf1 mutants stain black. The two right panels show that the introduction of the rtf1+ plasmid (pBZ136) complements the sporulation phenotype of the recessive ∆rtf1 mutant but not the dominant rtf1-R346* mutation (right panel). Strain names are given in brackets. B) Two-hybrid analysis of Rtf1 amino-acid segments’ ability to interact. Different Rtf1 segments (graphic outline) were fused to the GAL4 activation domain (AD) and GAL4 DNA-binding domain (BD). Interactions between two fusion proteins are detected by increased expression of the two reporter genes GAL1-HIS3 and GAL2-ADE2. Expression allows the ade2 his3 Saccharomyces cerevisiae strain to survive in the absence of histidine and adenine supplements in the media. Only when the Rtf1 C-terminal tail (P6) fused to both the AD- and the BD-domain are combined, cells become histidine and adenine prototrophs.

**Figure 8.** A) Model of Rtf1’s mediated termination of replication at RTS1. B) Model of c-myb in complex with its target DNA. c-myb residues (Ogata et al. 1993) that align with the Rtf-S154L and -L162Y residues are highlighted in red and yellow respectively (Fig. 2B).
A

upper strand sequence
rep 4 5' GACAGAAAGTGGGTTCCGTAATTTCGAGTAATTGCGACATACCCCGTCTCCCAAAATGGGGAG 3'
reg A 5' GGGATGAAATGAGGGTTATACGAGAATGGGAAATAGAAGAAAAGAAATAAAATCCAGTGGTCTG 3'

B

upper strand sequence
rep 4 5' GACAGAAAGTGGGTTCCGTAATTTCGAGTAATTGCGACATACCCCGTCTCCCAAAATGGGGAG 3'
mut 3 5' AGGCAAGTTGGGTTCCGTAATTTCGAGTAATTGCGACATACCCCGTCTCCCAAAATGGGGAG 3'
mut 4 5' AGGCAAGTTGGGTTCCGTAATTTCGAGTAATTGCGACATACCCCGTCTCCCAAAATGGGGAG 3'
1 5' AGGCAAGTTGGGTTCCGTAATTTCGAGTAATTGCGACATACCCCGTCTCCCAAAATGGGGAG 3'
2 5' AGGCAAGTTGGGTTCCGTAATTTCGAGTAATTGCGACATACCCCGTCTCCCAAAATGGGGAG 3'
3 5' AGGCAAGTTGGGTTCCGTAATTTCGAGTAATTGCGACATACCCCGTCTCCCAAAATGGGGAG 3'

C

domain I
- - + + +
domain I+II
- - - - -
domain I
- - + + +
domain I+II
- - - - -

D

domain II
- ++ + ++
A

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B

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AA:  
1-466  
82-466  
232-466  
339-466
A

region A  region B  C-terminal tail

domain I  domain II

DNA-unwinding by helicase

B