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CENP-B preserves genome integrity at replication forks paused by Retrotransposon LTR

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Abstract

Centromere-binding protein B (CENP-B) is a widely conserved DNA binding factor associated with heterochromatin and centromeric satellite repeats[1]. In fission yeast, CENP-B homologs have been shown to silence Long Terminal Repeat (LTR) retrotransposons by recruiting histone deacetylases[2]. However, CENP-B factors also have unexplained roles in DNA replication[3], [4]. Here, we show that a molecular function of CENP-B is to promote replication fork progression through the LTR. Mutants have increased genomic instability caused by replication fork blockage that depends on the DNA binding factor Switch Activating Protein 1 (Sap1), which is directly recruited by the LTR. The loss of Sap1-dependent barrier activity allows the unhindered progression of the replication fork, but results in rearrangements deleterious to the retrotransposon. We conclude that retrotransposons influence replication polarity through recruitment of Sap1 and transposition near replication fork blocks, while CENP-B counteracts this activity and promotes fork stability. Our results may account for the role of LTR in fragile sites, and for the association of CENP-B with pericentromeric heterochromatin and tandem satellite repeats.

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Author Contributions M.Z., B.A. and R.A.M. designed the experiments presented and wrote the paper. M.Z. performed and analyzed the experiments. M.W.V. provided bioinformatic analysis. D.G. and D.V.I. provided strains. S.W. and J.B. performed additional experiments.

Author Information The sequences from the ChIP-seq experiments are available at the Sequence Read Archive (www.ncbi.nln.nih.gov/sra) with accession number SRA024710.2. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to R.A.M. (martiens@cshl.edu).

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In fission yeast, CENP-B proteins are encoded by three homologs, *autonomously replicating sequence binding protein 1* (*abp1)*, *cenp-B homolog 1* (*cbh1)* and *cbh2*, and were previously characterized as DNA binding factors at origins of replication and centromeric repeats, respectively[3], [5]. *abp1* mutants grow slowly, while double mutants with *cbh1* or *cbh2* have severely stunted growth, abnormal mitosis and morphological defects, and triple deletion mutants are inviable[6], [7]. As a result, double Δ*abp1*Δ*cbh1* mutants form microcolonies on solid media (Fig. 1a and Supplementary Table 1) and exhibit high levels of cell death (Supplementary Fig. 1). We observed the spontaneous appearance of faster growing cells in a culture of Δ*abp1*Δ*cbh1* that grew at rates similar to the Δ*abp1* single mutant, lacked morphological defects (Fig. 1a and Table 1) and showed lower levels of cell death (Supplementary Fig. 1). Genetic analysis revealed the presence of a single essential locus that also suppressed the lethality of the triple mutantΔ*abp1*Δ*cbh1*Δ*cbh2* (not shown). We performed whole genome resequencing in the mutant strain[8] and isolated a missense mutation in the coding sequence of the DNA binding factor Sap1 (*sap1E101D*, henceforth called *sap1-c*; Supplementary Fig. 2) that cosegregated with suppression of slow growth in Δ*abp1*Δ*cbh1* and resulted in lethality in a WT background. Sap1 is a protein with essential roles in chromosome stability[9]. Sap1 has been implicated in a programmed replication fork block in the rDNA monomer that ensures directional replication to prevent mitotic recombination between rDNA repeats[10]–[12].

To test the effects of CENP-B and *sap1-c* mutations on genome integrity, we examined chromosomes by Pulsed Field Gel Electrophoresis (PFGE). While singleΔ*abp1* and Δ*cbh1* mutants had wild-type chromosome lengths, the double Δ*abp1*Δ*cbh1* mutant had a smear of DNA fragments indicating double strand breaks (DSB) in all three chromosomes (Fig. 1b). Treatment of the Δ*abp1*Δ*cbh1* sample plugs with the restriction enzyme NotI allowed migration of the chromosomes into the gel, and detection of telomeric and centromeric sequences (Supplementary Fig. 3), suggesting the presence of scattered unresolved replication or recombination intermediates that interfere with the migration of full length chromosomes, but not with NotI digested DNA, into the pulsed field gel. This indicates that Abp1 and Cbh1 have roles in the maintenance of genome integrity.

Surprisingly, the *sap1-c* mutation restored genome integrity to all chromosomes with Chromosome 3 exhibiting size variability in several isolates of Δ*abp1*Δ*cbh1sap1-c* mutant (Fig. 1b). In fission yeast, Chromosome 3 harbors the ribosomal DNA (rDNA) repeats. Temperature sensitive (ts) alleles of *sap1* exhibit changes in the size of Chromosome 3 attributable to loss of fork barrier activity and an increase in mitotic recombination at rDNA[12], and the changes in chromosome 3 size in the *sap1-c* mutants are associated with altered rDNA copy number (Supplementary Fig. 4). The ts alleles *sap1-1* and *sap1–48*[12] suppressed slow growth in the Δ*abp1*Δ*cbh1* double mutant, mimicking *sap1-c* (Supplementary Fig. 5). Consistent with a reduction in fork barrier activity, a probe containing a canonical Sap1 binding sequence had reduced electrophoretic mobility shift (EMSA) in crude extracts from *sap1-c* mutants (Supplementary Fig. 6). We conclude that the suppression of the Δ*cbh1*Δ*abp1* phenotype is not specific to the *sap1-c* mutation but a result of defective function of Sap1, and therefore that the loss of genome integrity in Δ*abp1*Δ*cbh1* mutants is a consequence of Sap1 activity.

Blocked replication forks are potential sources of genome instability because they can lead to collapse of the replisome and DSB formation[13]. The fact that Sap1 activity leads to DNA damage in the absence of Abp1/Cbh1 suggests that the function of CENP-B is to manage Sap1-arrested replication forks. In the absence of Sap1 loss of replication fork blockage would render Abp1/Cbh1 activity unnecessary and lead to increased genome stability in Δ*abp1*Δ*cbh1* mutants. This model predicts that CENP-B and Sap1 would colocalize to the regions where they acted on the replication fork, and that these regions would engage in homologous recombination (HR) and degrade to DSB in the absence of CENP-B. To test this hypothesis, we performed Chromatin Immunoprecipitation of Sap1, Abp1 and Cbh1 followed by High Throughput sequencing (ChIP-seq). Abp1 has previously been shown to localize and recruit Cbh1 to the LTR of Tf1 and Tf2 retrotransposons, where they play a role in their transcriptional silencing[2]. We demonstrated a strong colocalization of Sap1 with Abp1 and Cbh1 at these LTR as well as at solo LTR scattered throughout the genome (Fig. 2a, c and Supplementary Fig. 7a, b) and at the mating type locus (Supplementary Fig. 8), where Sap1 and Abp1 have been described to regulate mating type switching[14], [15]. Both Sap1 and Abp1/Cbh1 also localized to genomic regions independently of each other, suggesting that they do not form a stable complex or mediate their mutual recruitment. In particular, Abp1 exhibited binding to tRNA genes (Fig. 2b and Supplementary Fig.7b), known to be potent replication pause sites[13], [16]. Abp1 and Cbh1 co-localize to a highly A/T rich region located in positions 100–150 of the LTR (Fig. 2c and Supplementary Fig. 7a, b). The localization of Sap1 within the LTR was concentrated in the first 50bp of sequence (Fig. 2c), coinciding with a predicted Sap1 binding site[17] (Supplementary Fig. 7a, c). We tested this sequence by EMSA and detected specific binding in WT extracts (Fig. 2d) as well as decreased binding and altered mobility in extracts from Δ*abp1*Δ*cbh1sap1-c* mutants (Supplementary Fig. 7d). Interestingly, solo LTR and full length Tf2 insertions were associated with a prominent peak of Sap1 binding located outside the 3′ end of the transposon sequence (Fig. 2c). These observations indicate that Sap1 binding precedes and possibly guides Tf element integration. To test this prediction, we plotted the average enrichment of Sap1, Abp1 and Cbh1 around more than 70,000 *de novo* Tf1 integration sites recently reported[18] and observed a dramatic association of these integration sites with a peak of Sap1 binding immediately downstream of the insertion site (Fig. 2e and Supplementary Fig. 8), with no appreciable CENP-B enrichment. These results strongly suggest that Sap1 binding sequences determine the targeting and orientation of Tf retroelement transposition.

To evaluate the mutual influence of Sap1 and Abp1/Cbh1 on LTR binding we performed ChIP analysis of Sap1 in Δ*abp1*Δ*cbh1* mutants and of Abp1 in ts *sap1* mutants that affect DNA binding activity[12]. Sap1 binding to the LTR was unaffected inΔ*abp1* andΔ*cbh1* mutants, and was slightly increased in Δ*abp1*Δ*cbh1* double mutants (Fig. 2f), but consistently reduced (2 fold) in Δ*abp1*Δ*cbh1sap1-c*. Conversely, Abp1 binding to the LTR was increased between 2 and 3 times at the permissive temperature in *sap1-1* and *sap1–48* mutants (Fig. 2g). These results indicate that Sap1 and Abp1/Cbh1 bind to the LTR independently of each other and mutually counteract their recruitment, and that the *sap1-c* mutation impairs its binding to the LTR *in vivo* as well as *in vitro* (Fig. S4).

A failure of replication fork stability at LTR, which are distributed throughout the genome, would explain the widespread DNA damage in Δ*abp1*Δ *cbh1* mutants. We assessed the behavior of the replication fork as it traversed the LTR using 2D agarose gel electrophoresis. Sap1 dependent programmed fork blocks are directional and only hinder fork progression in one orientation[10], [11]. We cloned a full length LTR and its first 50 base pairs (containing the Sap1 binding site) in a plasmid in both orientations with respect to the replication origin *ars1*. 2D gel electrophoresis in a WT strain transformed with this episomal system showed a modest accumulation of fork signal at the location of the cloned LTR (Fig. 3a) but only when the Sap1 binding site was proximal to the origin, and not in the opposite orientation (Supplementary Fig. 9). The Sap1 binding site was sufficient for this blocking activity, with the same orientation requirement (Supplementary Fig. 9). We next assayed the LTR for pausing activity in Δ*abp1*, Δ*cbh1* and *sap1-c* mutants (Fig. 3a). Strikingly, the paused fork signal was consistently enhanced and always at the same location in Δ*abp1* and Δ*cbh1* mutants, while theΔ*abp1*Δ*cbh1* double mutant exhibited additional signals outside the replication arc suggestive of recombination intermediates[19]. The fork blocking activity of the LTR disappeared in Δ*abp1*Δ*cbh1sap1-c* mutants. Unresolved fork blocks can collapse and undergo HR for fork recovery. We confirmed the presence of HR in the Δ*abp1*Δ*cbh1* double mutants by measuring the increase in the formation of Rad22 (homologous to Rad52 in *S cerevisiae*) foci in a Rad22-YFP strain[20] (Fig. 3b), and we observed that Δ*abp1*Δ*cbh1* double mutant cells accumulated the HR protein Rad22 at the LTR (Fig. 3c). Consistently, the recombination factor Rhp51 (Rad51 homolog) was essential for viability of Δ*abp1*Δ*cbh1* double mutants (Supplementary Fig. 10), indicating that HR is necessary for recovery from fork stalling at LTR. These results indicate that Abp1/Cbh1 counteract Sap1 barrier activity and stabilize the replication fork at LTR. This results in loss of genome integrity and HR at the LTR in Δ*abp1*Δ*cbh1* mutants.

The Sap1 binding sequence is conserved in Tf1 and Tf2 retrotransposon LTR (Supplementary Fig. 6c) suggesting that it plays a role in the retrotransposon life cycle. We assayed the effect of *sap1* and *abp/cbh1* on Tf2 stability by measuring the frequencies of loss of a *ura4* reporter transgene inserted in the Tf2-6 transposon[21]. Mutation of *abp1* resulted in a dramatic decrease of Tf2 ectopic recombination, which returned to normal levels when *sap1* was also mutated (Fig. 4). In the presence of *sap1+* there is a preference for gene conversion, which normally constitutes the majority of ectopic recombination events[22], but in Δ*abp1sap1-c* and Δ*cbh1sap1-c* mutants the proportion of eviction and conversion events is similar (Fig. 4). Therefore, we propose that the LTR recruits Sap1 in order to control the direction of transposon replication and increase transposon persistence in the genome, perhaps by coordinating lagging strand synthesis, which prevents single strand annealing from complementary direct repeats (Supplementary Fig. 11a,b). CENP-B counteracts this activity, possibly by promoting replication fork progression through the Sap1 dependent barrier. Thus, CENP-B and Sap1 promote genome and transposon integrity, respectively, in a “tug-of-war” between transposon and host. Abp1 stimulates fork progression by recruiting the fork restart protein MCM10[4] which has primase activity, and the histone deacetylase Mst1, which has roles in replication fork stability, also interacts directly with Cbh1[23] (Supplementary Fig. 11a). In *S cerevisiae* the histone deacetylase (HDAC) Sir2 silences and inhibits recombination in repetitive DNA[24]. CENP-B factors

recruit the HDACs Clr3 and Clr6, which carry out LTR silencing[2]. The result of these functions would be to preserve genome integrity at LTRs by preventing DNA damage and recombination. This novel role of CENP-B may not be limited to LTR and tDNA, as mutation of the replication fork blocking factor *reb1*, which is specific to rDNA repeats, also suppresses the slow growth of the *abp1* mutant[25]. Similarly, our ChIP-seq data indicates that Sap1 may also be implicated in the functionality of the replication terminator RTS1 (Supplementary Fig 8) in collaboration with Rtf1. In this manner, the function and regulation of the Sap1 bound regions is determined by the binding in their vicinity of different factors affecting replication fork progression.

Because of their repetitive nature, transposons have a close relationship with replication and recombination. For example, the IS608 transposon of *E coli* is targeted to the lagging strand and always replicated in the same direction[26]. This might prevent recombination between tandemly arranged copies. We have shown that retrotransposons influence DNA replication via recruitment of directional fork blocking factor Sap1 and that activity of CENP-B is required for replication fork management Additionally, retrotransposition is targeted to the genomic localization of Sap1. These mechanisms influence the replicative dynamics of the host genome. The genomes of eukaryotes show widespread colonization by retrotransposons, and pericentromeric satellite repeats are often of transposon origin[27]. When such sequences are arranged as tandem repeats, control of replication direction by CENP-B would prevent chromosome breaks and preserve genome integrity. This mechanism accounts for the role of other regulators of fork progression in inter-LTR recombination[28], [29]. In contrast, when flanked by LTR in opposite orientations, fragile sites fail to replicate and result in chromosome breaks[13], [30].

Methods summary

ChIP was performed using tagged TAP-Abp1 and TAP-Cbh1 strains with an Anti-Calmodulin Binding Protein antibody (Millipore) and a polyclonal serum against the native Sap1 protein[9]. High throughput sequencing was performed on the illumina G2 genome analyzer, and analyzed for polymorphism detection or statistical analysis of enrichment. 2D gel electrophoresis was performed as described[11], see supplementary information for construction of the episomal system. EMSA was performed as described previously[17].

Full methods and any associated references are available in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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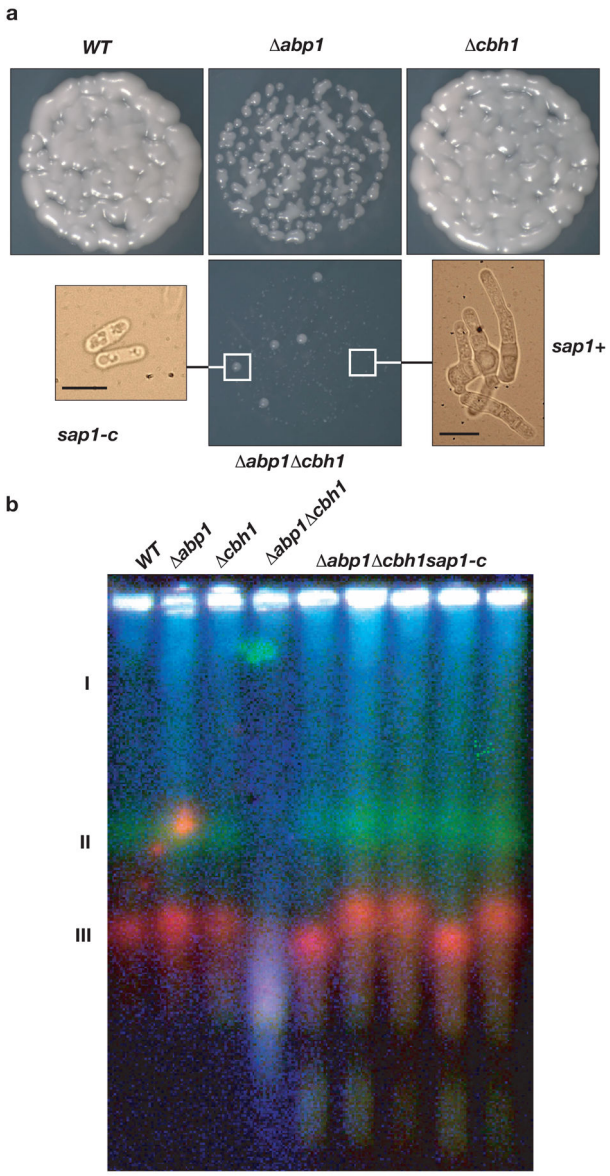

Figure 1.

DNA damage in CENP-B mutants is suppressed by *sap1* mutation. a, Images of 10^3 plated cells of WT, Δ*abp1* and Δ*abp1*Δ*cbh1* with Δ*abp1*Δ*cbh1sap1-c* colonies. Microscopy image inserts: Images showing branched phenotype in Δ*abp1*Δ*cbh1* background (right) and Δ*abp1*Δ*cbh1sap1-c* mutant (left). Scale bar is 10 μm. b, Pulsed Field Gel blot analysis of wild type (WT), CENP-B mutants (Δ*abp1*, Δ*cbh1*, Δ*abp1*Δ*cbh1*) and 5 CENP-B/*sap1-c* mutant isolates (Δ*abp1*Δ*cbh1sap1-c*). The position of the three chromosomes is indicated on the right. The image is a false-colored composite of hybridizations for all three chromosomes.

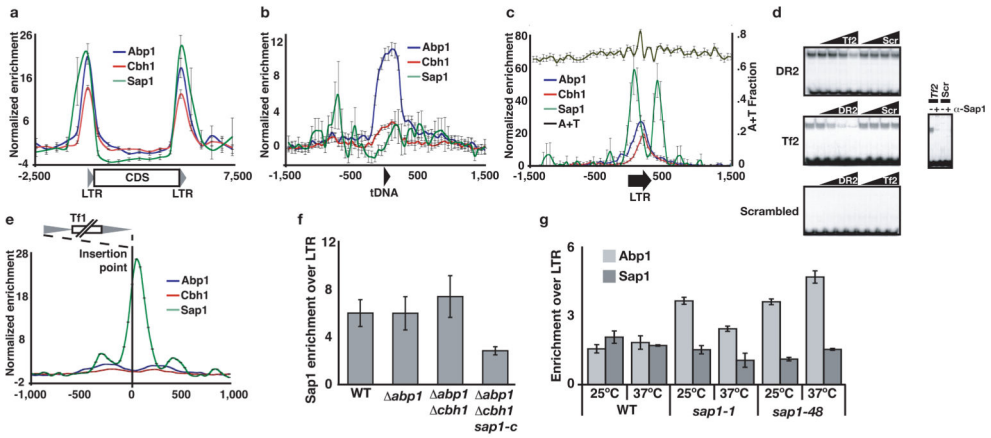

Figure 2.

Sap1 and CENP-B colocalize at the LTR of retrotransposons *in vivo*. Average genome-wide enrichment by ChIP-seq of Sap1, Abp1 and Cbh1 on a, all *Tf2* elements, b, euchromatic tRNA and c, solo LTR. Error bars represent Standard Error. d, Left panel: Competition EMSA. Right panel: Inactivation by incubation with anti-Sap1 serum[9]. e, Average Sap1, Abp1 and Cbh1 enrichment around Tf1 *de novo* insertion points[18]. f, ChIP of Sap1 with LTR of *Tf2* in CENP-B and *sap1-c* mutants and g, of Abp1 with LTR of *Tf2* in and *sap1* ts mutants. Error bars represent standard deviation for triplicates.

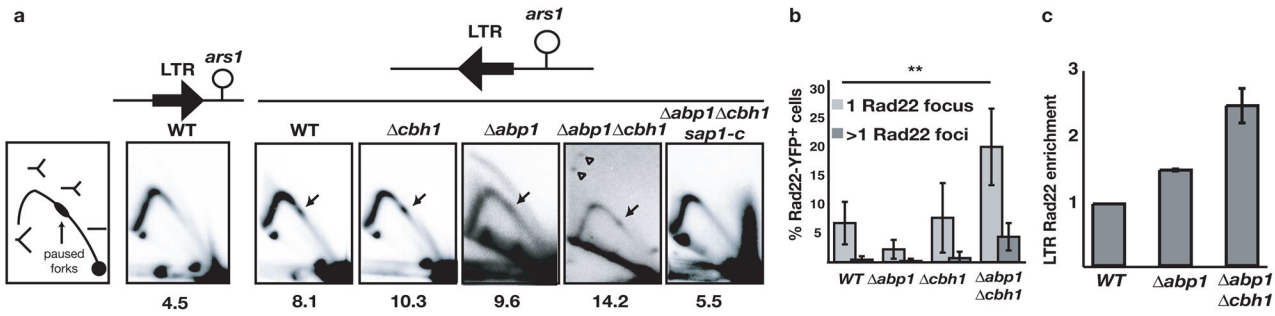

Figure 3.

CENP-B promotes replication fork progression through the Sap1 dependent barrier present at the LTR and prevents HR. a, 2D gel electrophoresis of a plasmid fragment containing the *Tf2* LTR oriented towards (left) and away (right) from the *ars1* origin. Arrows indicate paused replication intermediates and open arrows recombination intermediates. The percentage of signal over the LTR is indicated below each panel. b, Quantification of Rad22 -GFP foci (N >400 nuclei for all mutants). Error bars depict standard errors. c, Rad22 -YFP ChIP with LTR in WT, Δ*abp1* and Δ*abp1*Δ*cbh1* mutants. Error bars represent standard deviation for triplicates.

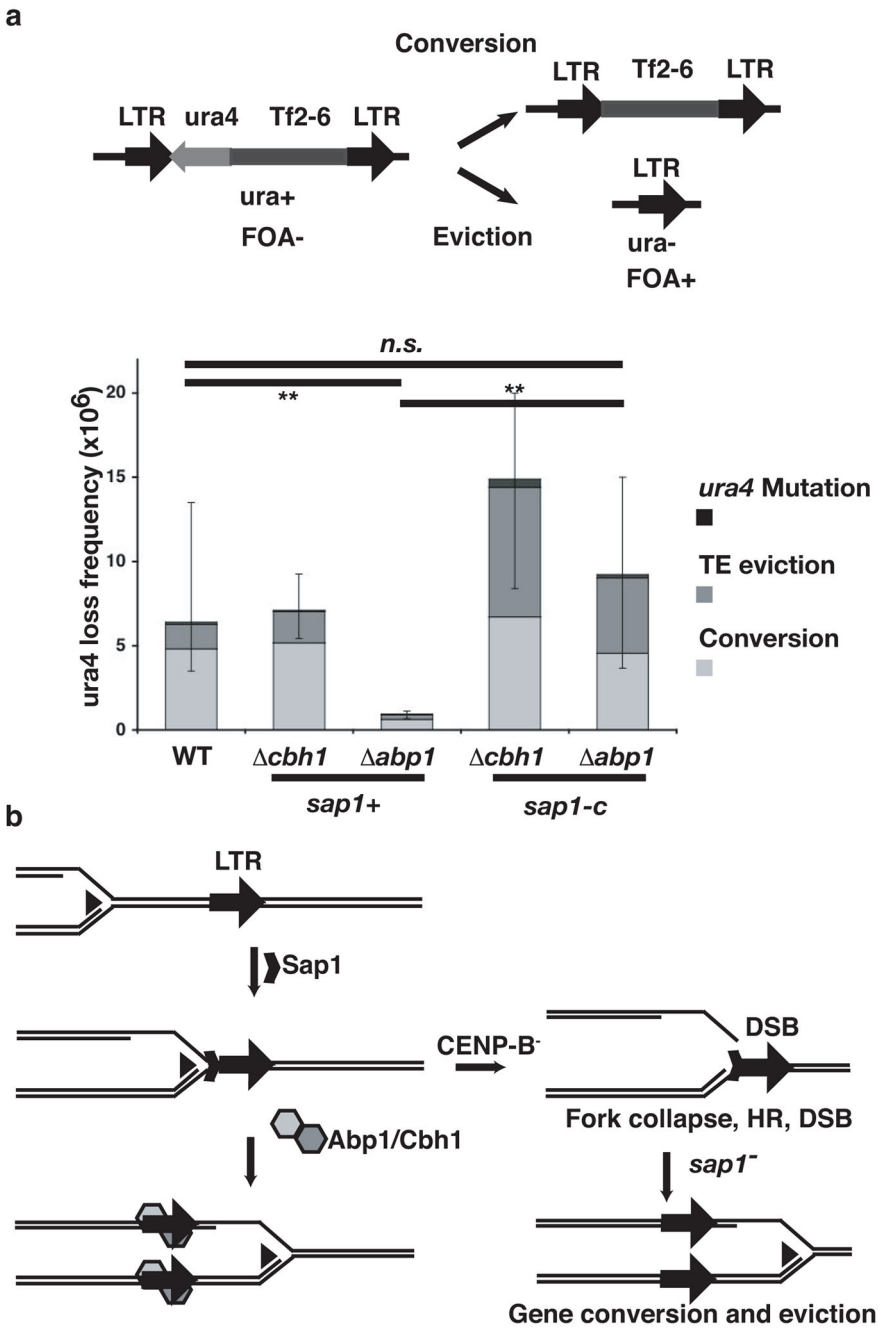

Figure 4.

CENP-B and Sap1 have opposite effects on Tf2 stability. a, ectopic recombination fluctuation assay. Two potential mechanisms of *ura4* loss from the marked *Tf2-6::ura4* are indicated, gene conversion and eviction by LTR recombination. Columns represent total median *ura4* loss frequency in WT, Δ*abp1*, Δ*cbh1*, Δ*abp1sap1-c* and Δ*cbh1sap1-c* mutants, error bars represent 95% confidence intervals. Colors indicate distribution of mode of ectopic recombination events in the *ura4*- colonies obtained from WT (n=93), Δ*abp1*

(n=88), Δ*cbh1* (n=94), Δ*abp1sap1-c* (n=91), and Δ*cbh1sap1-c* (n=89) mutants. b, Model for the interactions between Abp1, Cbh1, Sap1 and the replication fork at the LTR.