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Minisatellite Alterations in *ZRT1* Mutants Occur via *RAD52*-dependent and *RAD52*-independent Mechanisms in Quiescent Stationary Phase Yeast Cells

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Abstract

Alterations in minisatellite DNA repeat tracts are associated with a variety of human diseases including type 1 diabetes, progressive myoclonus epilepsy, and some types of cancer. However, in spite of their role in human health, the factors required for minisatellite alterations are not well understood. We previously identified a stationary phase specific increase in minisatellite instability caused by mutations in the high affinity zinc transporter *ZRT1*, using a minisatellite inserted into the *ADE2* locus in *Saccharomyces cerevisiae*. Here, we examined *ZRT1*-mediated minisatellite instability in yeast strains lacking key recombination genes to determine the mechanisms by which these alterations occur. Our analysis revealed that minisatellite alterations in a $\Delta zrt1$ mutant occur by a combination of *RAD52*-dependent and *RAD52*-independent mechanisms. In this study, plasmid-based experiments demonstrate that *ZRT1*-mediated minisatellite alterations occur independently of chromosomal context or adenine auxotrophy, and confirmed the stationary phase timing of the events. To further examine the stationary phase specificity of *ZRT1*-mediated minisatellite alterations, we deleted *ETR1* and *POR1*, genes that were previously shown to differentially affect the viability of quiescent or nonquiescent cells in stationary phase populations. These experiments revealed that minisatellite alterations in $\Delta zrt1$ mutants occur exclusively in quiescent stationary phase cells. Finally, we show that loss of *ZRT1* stimulates alterations in a derivative of the human *HRAS1* minisatellite. We propose that the mechanism of *ZRT1*-mediated minisatellite instability during quiescence is relevant to human cells, and thus, human disease.

Keywords

Minisatellites; Recombination; Genome Stability; Stationary Phase

1. Introduction

Minisatellite DNA repeat tracts are found throughout eukaryotic genomes [1, 2]. They consist of repeat units ranging from 16 to 100bp in length. The highly polymorphic nature of

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minisatellites makes them ideal for use as genetic markers, but they also have important biological functions. Minisatellites have been shown to act as enhancer elements for adjacent genes [2, 3]. The human minisatellite associated with the *HRAS1* oncogene has been shown to bind the $\text{rel/NF-}\kappa\text{B}$ transcription factor and can enhance expression of a reporter gene [4, 5]. Minisatellites also frequently act as fragile sites, possibly due to stalling of DNA polymerases at non-B DNA structures formed by the repeats (reviewed in [1, 6]). It has also been suggested that minisatellites may have a role in chromosome pairing, specifically during male meiosis [2].

In addition to their important biological functions, these repetitive sequence elements also have a profound influence on human health. Rare altered alleles of human minisatellites have been correlated with increased risk of type 1 diabetes, progressive myoclonus epilepsy, and various cancer subtypes [3, 7–9]. Recently minisatellite alleles have also been associated with disorders as varied as asthma [10], ulcerative colitis [11], and attention-deficit hyperactivity disorder [12, 13].

Little is known about how minisatellites alter to give rise to disease-associated minisatellite alleles. Human minisatellites have been shown to change in tract length and repeat composition during meiosis, while remaining relatively stable during mitotic cell cycles [1]. We previously demonstrated that these phenotypes are recapitulated in the budding yeast *Saccharomyces cerevisiae* [14, 15]. Since the patterns of minisatellite alteration are similar in yeast and human cells, we used the more genetically tractable yeast to identify factors that control minisatellite stability during meiosis. Our work demonstrated that meiotic minisatellite alterations require the meiosis-specific endonuclease Spo11p and the DNA loop repair protein Rad1p, while others have shown the recombination protein Rad50p is also critical [14, 16]. Additional studies have demonstrated that mutation of the key replication genes *RAD27*, *DNA2*, *POL3*, or the PCNA complex can destabilize minisatellite tracts during mitotic cell cycles [17–20]. In addition, the loss of the helicase *PIF1* can destabilize minisatellite tracts during mitotic growth [21].

We previously conducted a screen for mutants that destabilize a minisatellite reporter tract in the *ADE2* gene [22]. The reporter, designated *ade2-min3*, employed three 20 bp direct repeats plus a 5 bp linker inserted into the *ADE2* gene (Fig. 1a). This insertion causes a frameshift mutation in *ADE2*, resulting in a red colony color. Deletion of one repeat unit or addition of two repeat units to the array restores white colony color. If such a repeat tract expansion or contraction occurs during log phase growth of the *ade2-min3* colony, then a red-white sectoring phenotype will be observed.

We isolated mutants of the zinc-homeostasis genes *ZRT1* and *ZAP1* with a novel color segregation phenotype, called blebbing, in the *ade2-min3* background ([22], Fig. 2). In *ZRT1* or *ZAP1* mutants, red colonies form, then white microcolonies ('blebs') develop on the surface of the colony. We confirmed by PCR that cells in the white blebs have minisatellite tracts in which the repeat number has been altered. As we observe no sectoring in these colonies, we concluded that the blebs arise from cells in which the minisatellite tract has altered after colony growth has ceased, and the cells within the colony have entered a post-mitotic, stationary phase state. In support of our interpretation of this colony morphology phenotype, time course experiments and fluctuation analysis demonstrated that blebbing is the result of minisatellite alterations that occur after cells have entered stationary phase. Our work was the first to address the stability of minisatellites during stationary phase – potentially a significant advance since most human somatic cells spend the majority of their lifespan in a quiescent state.

Preliminary genetic analysis revealed a partial decrease in the intensity of blebbing in $\Delta zrt1 \Delta rad50$ mutants [22]. These data, combined with the fact that blebbing is caused by alteration in minisatellite repeat copy number, implied a role for recombination in the minisatellite alterations of *ZRT1* mutants. In *S. cerevisiae*, the majority of mitotic recombination requires *RAD52* [23], but some rare recombination events are *RAD52*-independent - these require *RAD50* or *RAD51*. It was clear that the mechanism responsible for these events would be of significant interest given the limited data on DNA repair in quiescent cells.

In this study, we examine the requirements for stationary phase minisatellite tract alterations in the $\Delta zrt1$ zinc homeostasis mutant. We show that stationary phase minisatellite alterations occur by both *RAD52*-dependent and *RAD52*-independent mechanisms. In addition, we demonstrate that *ZRT1*-dependent minisatellite alterations likely have broad genomic relevance, as they occur independently of chromosomal context, adenine auxotrophy, or marker assay gene. We confirm the stationary phase timing of minisatellite alterations in the *ZRT1* mutant and demonstrate that these events are limited to the quiescent population of stationary phase cells. Finally, we establish that loss of *ZRT1* can destabilize a tract derived from the human *HRAS1*-associated minisatellite. Our findings indicate that the mechanism of *ZRT1*-dependent minisatellite alteration is likely to be relevant to minisatellites found in a broad range of biological contexts.

2. Materials and Methods

2.1. Media, plasmids and strains

Media was made as previously described [24]. YPD + G418 media was composed of standard YPD solid media + 200 mg/l of G418 sulfate (geneticin). Sporulation and dissection protocols were used as described [14].

Plasmid pKK055 was used to integrate the *ade2-hras7.5* allele's 28bp minisatellite repeats into the *ADE2* gene (Fig. 1). To construct this plasmid, a PCR product containing the *HRAS1* minisatellite repeats from DTK759a [15] and *XbaI*-cleavable ends was amplified using primers 35138391 and 42180832. This cassette was digested with *XbaI* and ligated into the *XbaI* site of the *ADE2* fragment contained in the plasmid pEAS8 [25]. Insertion was verified by sequencing. The insert includes 28bp of unique sequence from the human genome on the 5' end, one 14bp *HRAS1* minisatellite repeat fragment, seven 28bp *HRAS1* minisatellite repeats, and 57bp of unique sequence from the human genome on the 3' end. With the 6bp duplication of the *XbaI* restriction site, the insert is 301bp in length. The sequence of the insert (5' to 3' and excluding *XbaI* sites) is:

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5'GAAGCTGGTCACTCGGAGGCTGCTGGGGAGAAGGGGGAGTGTGGCATTCC
CTGGACAGAAGGGCAAGTGTGGCGTCCCCTGGAGAGAAGGGCGAGTGTGG
CGTCCCCTGGAGAGAAGGGGGAGTGTGGCGTCCCCTGGACAGAAGGGGGA
GTGTGGCGTCCCCTGGAGAGAAGGGGGAGTGTGGCGTCCCCTGGAGAGAA
GGGCGAGTGTGGCGTCCCCTGGACAGAAGGGGGAGTGTCCCTGCAGCCCT
GGCCAGCCAGCGGCATGGCCTACTAGCTCTCCCCAACCTCAGGG 3'.
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S. cerevisiae strains used in this study (Table 1) were derived from EAS28 (MATa *his7-2 trp1-289 ura3-52*) from Sia *et al.* 2001. Those strains whose construction is not mentioned here are described in [22]. Strain DTK1188, containing the *ade2-h7.5* allele, was made by transforming DTK260 [22] with *BglII* digested pKK055. Ura⁻ derivatives were then selected on 5-fluoroorotic acid (5-FOA) plates. Integration of the *ade2-h7.5* allele was verified by sequencing.

The following strains were constructed by amplifying genomic DNA as template from the Yeast Deletion Consortium Strains. The PCR products are composed of the G418 resistance gene flanked by 3' and 5' homology to the target sequence. DTK944 and DTK945, bearing deletions of *RAD1*, were constructed by amplifying a $\Delta rad1::KAN$ cassette using oligos 6934207 and 6934208. Strains DTK1074 and DTK1076, bearing deletions of *RAD51*, and strains DTK1309 and DTK1316, bearing deletions of *DNL4*, were constructed in a similar manner using oligos 26876476 and 26876477 for $\Delta rad51::KAN$ and oligos 39724850 and 39724851 for $\Delta dnl4::KAN$. Strains DTK1244 and DTK1360, bearing a deletion of *ETR1*, were constructed by amplifying an $\Delta etr1::KAN$ cassette using oligos 37616572 and 37616573. DTK1245 and DTK1361, bearing a deletion of *POR1*, were constructed using $\Delta por1::KAN$ cassette DNA with oligos 37616574 and 37616575. Strain DTK1666, bearing a deletion of *DNL4*, was constructed using genomic DNA from strain DTK1316 using oligos 39724850 and 39724851 for $\Delta dnl4::KAN$. DTK1690, bearing a deletion of *RAD52*, was constructed using genomic DNA amplified from DTK1253 using oligos 34756508 and 34756507 for $\Delta rad52::URA3$. Strain DTK1200, *ade2-h7.5* with a deletion of *ZRT1*, was also constructed using a PCR construct generated using oligos 12966370 and 12966371 for $\Delta zrt1::KAN$ with genomic DNA from strain DTK878 as a template. Parental strains were transformed with PCR product, grown for 4 hours in liquid YPD and then plated on YPD + G418 or SD-ura solid media to select for integration events. Transformants were checked by PCR.

To construct the *RAD52* null mutant DTK1191, the $\Delta rad52::URA3$ strain DNY101 [26] was crossed to DTK904, sporulated, and dissected as previously described [14]. A spore bearing the *ade2-min3* allele, the $\Delta zrt1::LEU2$ allele, and the $\Delta rad52::URA3$ allele was isolated by color and survival on SD-leu, SD-ura, and YPD+G418 sulfate media. This isolate was backcrossed twice to DTK904 and dissected as above to generate DTK1191. The *RAD59* null strain DTK1229 was constructed by crossing DTK1195, a haploid spore isolate from sporulation and dissection of the $\Delta rad59::KAN$ Yeast Deletion Consortium strain, to DTK904 and sporulating and dissecting. A spore bearing the *ade2-min3* allele, the $\Delta zrt1::LEU2$ allele, and the $\Delta rad59::KAN$ allele was isolated by red color, survival on SD-leu, and G418 sulfate resistance. This isolate was backcrossed twice to DTK904 and dissected to generate DTK1229. DTK1230 was constructed in a similar fashion, by crossing DTK1196, a haploid spore isolate of the $\Delta rad59::KAN$ Yeast Deletion Consortium strain, to DTK271 and sporulating and dissecting as above. A spore bearing *ade2-min3* and $\Delta rad59::KAN$ was isolated by color and G418 sulfate resistance, then backcrossed twice to DTK271. The following strains were constructed by crossing, sporulating, and dissecting: DTK1253 (DTK1191 $\times$ DTK284), DTK1254 (DTK1057 $\times$ DTK1076), DTK1268 (DTK1253 $\times$ DTK1057), DTK1269 (DTK1191 $\times$ DTK1076), DTK1289 (DTK1268 $\times$ DTK284), DTK1290 (DTK1269 $\times$ DTK284), DTK1299 (DTK1254 $\times$ DTK284), DTK1320 (DTK1191 $\times$ DTK1299), DTK1323 (DTK1191 $\times$ DTK1299), DTK1400 (DTK1188 $\times$ DTK1056), DTK1401 (DTK1188 $\times$ DTK1074), DTK1403 (DTK1200 $\times$ DTK1191), DTK1406 (DTK1200 $\times$ DTK1056), DTK1407 (DTK1400 $\times$ DTK1191), DTK1426 (DTK1400 $\times$ DTK1191), DTK1427 (DTK1426 $\times$ DTK1188), DTK1428 (DTK1401 $\times$ DTK1200), DTK1438 (DTK1401 $\times$ DTK1407), DTK1677 (DTK1316 $\times$ 1229) and DTK1678 (DTK1316 $\times$ DTK1229).

2.2 PCR primers

The following primers were used in this study:

Primer 6934207 (*Rad1F*): TCTGTTTGCCTTTATTTTGC

Primer 6934208 (*Rad1R*): GAAGATGAATTGCGGATG

Primer 12966370 (*Zrt1F*): TACGCACGGCATTAGCTC

Primer 12966371 (*Zrt1R*): ACTCGTAGATGGCACGGTC
 Primer 26876476 (*Rad51F*): GTGTAGCGACAAAGAGCAGACGTA
 Primer 26876477 (*Rad51R*): GCAGTAGGGTTGCGAGGTATATGA
 Primer 34756508 (*Rad52F*): AAGAGTCTGCTCTTCCCGTTAG
 Primer 34756507 (*Rad52R*): ACGACACATGGAGGAAAGAA
 Primer 37616572 (*Etr1R*): TTGAAGGGTCGACGTCCCCTTTTA
 Primer 37616573 (*Etr1F*): TGTACCCAGGGGTGGTTTCCAT
 Primer 37616574 (*Por1R*): TTCTCACTGCCAAGCAACCA
 Primer 37616575 (*Por1F*): CCAATCAAACACCGCCATTTCG
 Primer 39724850 (*Dnl4F*): GAGTTAAGATCGTTTTTCGATCCCT
 Primer 39724851 (*Dnl4R*): GCGCATCTTCCACTCTTATTG
 Primer 35138391 (*Hras1* minisatellite F):
 AAATCTAGACCCTGAGGTTGGGGGAGAGC
 Primer 42180832 (*Hras1* minisatellite R):
 AAATCTAGAGAAGCTGGTCACTCGGAGGCTG

2.3 Fluctuation analysis

Fluctuation analysis was performed as described [27], with the following modifications: Plasmid pEAS20 [25], containing the *ura3-min3* construct (Fig. 1), was transformed into DTK271 and DTK904 and plated on SD-ura to select for plasmid uptake. Ten Ade⁺ and ten Ade⁻ transformants for each strain were picked and used to inoculate liquid cultures. SD-trp media, rather than YPD, was used for growing liquid cultures in order to maintain selection for the plasmid, without selecting for a particular conformation of the *ura3-min3* repeats. SD-trp liquid cultures of strains bearing pEAS20 were grown at 30°C. Cultures were diluted and plated on YPD and SD-trp 5-FOA plates at 0, 4, 8, and 24 hours after inoculation. The OD₆₀₀ of the cultures was measured at each timepoint to monitor culture growth. Three independent trials were conducted for each strain and the average frequency of minisatellite alteration and standard error for each strain was calculated. In contrast to previous applications of this technique, we did not extend our calculations to determine rate of minisatellite alteration over generations; as described in Results, we determined that minisatellite alterations occur specifically in quiescent cells in stationary phase, thus making it impossible to calculate a rate measurement.

2.4 Bleb quantitation

To quantify the amount of blebbing in our strains we counted the number of blebs on the surface of each colony after 7 days (30°C for 3 days and then room temperature for 4 days). For each strain we surveyed at least 100 colonies over 3 independent experiments, calculating the mean number of blebs per colony and the 95% confidence interval for the mean. The size of the 95% confidence intervals was examined to determine the repeatability of the assay. In order to ensure accurate comparisons, we measured the diameter of each colony and calculated the mean colony diameter and the 95% confidence interval for the mean for each strain. Mean colony diameters for all strains in this analysis ranged from 1.24–1.29mm and 95% confidence intervals for all strains overlapped, indicating that there was not a significant difference in colony size between strains. The timepoint at which we counted blebs was chosen because at that time blebs are readily detected but still small enough to preclude overlap between blebs, which could potentially result in an undercount.

We note that the extremely small 95% confidence intervals observed in our measurements demonstrate that our bleb counts in this assay are highly reproducible and are therefore likely to be a true indication of the alteration frequency of the minisatellite tract in the strain.

3. Results

3.1. Minisatellite alterations in stationary phase $\Delta zrt1$ mutants occur in quiescent cells

The *ade2-min3* allele allows us to monitor minisatellite stability in *S. cerevisiae* (Fig. 1a, [22]). Three minisatellite repeats inserted into the *ADE2* gene disrupt the reading frame; cells carrying this allele are Ade⁻ and form red colonies. Loss of a repeat unit from the minisatellite tract restores the correct reading frame; cells carrying these altered alleles are Ade⁺ and form white colonies.

Using the *ade2-min3* reporter, we previously demonstrated that *ZRT1* mutants exhibit a novel color segregation phenotype that we called blebbing (Fig. 2). The blebbing phenotype consists of white microcolonies growing on the surface of the red colony. Colony morphology examination, PCR analysis, and timing experiments determined that blebbing in an *ade2-min3* strain results from the loss of one minisatellite repeat specifically during stationary phase [22]. Previous studies have shown that stationary phase cultures are composed of quiescent cells that have exited the cell cycle and nonquiescent cells which may still be undergoing some cell division [28]. These studies also demonstrated that loss of *ETR1* specifically reduces the capacity of the quiescent population of stationary phase cells to reenter the cell cycle when plated on rich media. The loss of *POR1* differentially affects the reproductive capacity of nonquiescent cells in a similar manner. *ETR1* encodes a 2-enoyl thioester reductase, which is involved in fatty acid synthesis [29], while *POR1* is a mitochondrial porin, necessary for maintenance of mitochondrial osmotic stability and aerobic respiration [30].

These distinct genetic requirements for cell cycle reentry in quiescent and nonquiescent stationary phase cells provide a means to distinguish which cell population is responsible for *ZRT1*-mediated minisatellite alteration. If the reproductive capacity of the cell fraction in which the minisatellite alterations are occurring is reduced, then the cells will be unable to return to growth after minisatellite tract changes restore adenine biosynthesis, preventing blebs from forming. In order to determine whether minisatellite alterations in a $\Delta zrt1$ mutant occur in quiescent or nonquiescent stationary phase cells, we deleted *ETR1* or *POR1* in a $\Delta zrt1$ strain background.

The parental $\Delta zrt1$ strain has 20.6 blebs/colony (Table 2). The extremely small 95% confidence interval for this strain (20.6 $\pm$ 0.8), as well as all other strains examined, demonstrated that our bleb counts for this assay are highly repeatable. Loss of *ETR1* in a $\Delta zrt1$ strain reduced blebbing to 0.09 blebs/colony (Table 2, Fig. 2). This indicates that most, if not all, minisatellite alterations in a $\Delta zrt1$ mutant occur in quiescent cells. In contrast, loss of *POR1* had little effect on blebbing, with 17.9 blebs/colony in a $\Delta zrt1$ *por1* strain, indicating that minisatellite alterations in a $\Delta zrt1$ mutant likely do not occur in nonquiescent cells.

We also examined the effect of *ETR1* or *POR1* deletion on minisatellite alteration in wild-type *ZRT1* cells. A wild-type *ade2-min3* strain has 3.7 blebs/colony. Loss of *ETR1* completely suppressed bleb formation in this strain background, reducing it to 0.06 blebs/colony (Table 2). Loss of *POR1* reduced blebbing to 1.8 blebs/colony. Therefore, while it is likely that background minisatellite alterations occur primarily in quiescent cells, some events may be occurring in nonquiescent stationary phase cells.

3.2. *ZRT1*-dependent minisatellite instability is independent of chromosomal context and adenine auxotrophy

Minisatellite stability can be affected by *cis*-acting sequence elements [1]. In addition, loss of *ADE2* function can be highly mutagenic [31, 32]. To determine if local DNA context or the use of the *ADE2* gene as the marker in our minisatellite stability assay influences the stability of the minisatellite tract, we used another minisatellite assay system in which the *min3* minisatellite tract is incorporated into the *URA3* gene on a plasmid, pEAS20 [25]. This *TRP1* CEN plasmid contains three tandem repeats, identical to those in the *ade2-min3* allele, in-frame with the *URA3* coding region, forming the *ura3-min3* allele (Fig. 1b). Alterations in the minisatellite repeats will throw the *URA3* gene out of frame, making cells carrying the altered plasmid Ura⁻ and resistant to 5-fluoroorotic acid (5-FOA).

We employed a modified fluctuation analysis assay [27] to determine the stability of the minisatellite tract in pEAS20. In our fluctuation assay, we calculated the frequency of 5-FOA resistant colony formation (and thus minisatellite alteration) rather than the rate over generations, as the events being measured do not occur until after cells have ceased dividing and entered stationary phase. Cells bearing pEAS20 were grown in SD-trp liquid media in order to maintain selection for the plasmid without selection for a particular minisatellite conformation. We determined the frequency of minisatellite alteration in cells at 0, 4, 8, and 24 hours after inoculation, while monitoring the growth of the cells in the cultures to determine entry into stationary phase. An increase in minisatellite alteration, as indicated by a dramatic increase in the frequency of 5-FOA-resistant colonies in the $\Delta zrt1$ strain, was not detected until the 24 hour timepoint (Table 3), which corresponded with the onset of stationary phase (data not shown). Entry into stationary phase and the increase in minisatellite alteration frequency in *ura3-min3* $\Delta zrt1$ cells was consistent with prior data from *ade2-min3* $\Delta zrt1$ cells [22], although stationary phase onset, as determined by OD₆₀₀ observation of the liquid cultures, was earlier in the *ura3-min3* experiments, presumably due to the use of synthetic drop-out media (required to maintain selection for the *ura3-min3* pEAS20 plasmid) rather than rich media. Wild-type pEAS20 cells maintained a level of minisatellite alteration at least 86-fold lower than the corresponding $\Delta zrt1$ cells at every time point, demonstrating that loss of *ZRT1* significantly increases the frequency of minisatellite alterations independent of chromosomal context (Table 3). Control experiments indicated that the low level of white colonies present at early timepoints in both the wild-type DTK271 and $\Delta zrt1$ DTK904 strains were likely derived from cells that entered stationary phase in the colony that was chosen for the initial liquid media inoculation, as their frequency could be dramatically reduced by using colonies that were grown for a much shorter period of time prior to use. Finally, there was no significant difference in the frequency of minisatellite alteration between Ade⁺ and Ade⁻ $\Delta zrt1$ transformants (2.87×10^{-1} events/cell vs. 2.47×10^{-1} events/cell at 24hrs after inoculation), indicating that *ZRT1*-dependent minisatellite instability is also independent of adenine auxotrophy.

3.3. Multiple recombination pathways facilitate minisatellite alterations in $\Delta zrt1$ mutants

Blebbing in the $\Delta zrt1$ mutant is partially suppressed by loss of *RAD50* [22], which encodes a recombination protein [33]. In *S. cerevisiae*, mitotic recombination can occur by the canonical *RAD52*-dependent pathway, or one of two *RAD52*-independent pathways requiring either *RAD50* or *RAD51* [23]. To determine which recombination pathways are required for minisatellite alteration in the $\Delta zrt1$ mutant, we constructed *ade2-min3* $\Delta zrt1$ strains containing deletions for key recombination genes. We then quantified the average number of blebs per colony for each of our strains (Table 2).

We examined $\Delta zrt1$ strains bearing deletions of *RAD50*, *RAD51*, and *RAD52*, along with double and triple mutant combinations of these genes. The parental $\Delta zrt1$ strain has 20.6

blebs/colony (Table 2). Minisatellite alterations in the $\Delta zrt1 \Delta rad50$ double mutant are reduced over 50% to 9.0 blebs/colony. Similarly, minisatellite alterations in the $\Delta zrt1 \Delta rad51$ and $\Delta zrt1 \Delta rad52$ double mutants are reduced to 10.0 blebs/colony and 9.4 blebs/colony, respectively. Therefore, minisatellite alterations in a $\Delta zrt1$ strain are partially dependent on *RAD51* and *RAD52*, as well as *RAD50*.

To distinguish between the relative contributions of the *RAD52*-dependent and the *RAD52*-independent pathways, we examined mutants with combinations of recombination gene deletions in the $\Delta zrt1$ background. The extent of blebbing in a $\Delta zrt1 \Delta rad50 \Delta rad52$ triple mutant was reduced to 3.9 blebs/colony (Table 2), lower than any of the double mutants, suggesting that *ZRT1*-dependent minisatellite alterations can occur by a *RAD52*-dependent mechanism or a *RAD52*-independent mechanism that requires *RAD50*. In contrast, a $\Delta zrt1 \Delta rad51 \Delta rad52$ strain was phenotypically identical to the $\Delta zrt1 \Delta rad51$ and $\Delta zrt1 \Delta rad52$ parents (9.8 blebs/colony vs 10.0 and 9.4 blebs/colony). This result indicates that the *RAD52*-independent pathway that requires *RAD51* does not significantly contribute to minisatellite alterations in a $\Delta zrt1$ mutant. A $\Delta zrt1 \Delta rad50 \Delta rad51$ strain was also phenotypically identical to the corresponding parent strains (9.1 blebs/colony vs. 9.0 and 10.0 blebs/colony). These data indicate that *RAD51* does not contribute to *ZRT1*-dependent minisatellite alterations as long as wild-type *RAD50* or *RAD52* is present.

In order to explain the residual minisatellite alterations in the $\Delta zrt1 \Delta rad50 \Delta rad52$ triple mutant, we examined a $\Delta zrt1 \Delta rad50 \Delta rad51 \Delta rad52$ quadruple mutant. Blebbing in this strain was reduced to 0.5 blebs per colony (Table 2). Thus, any remaining events in a $\Delta zrt1 \Delta rad50 \Delta rad52$ mutant are dependent on a *RAD52*-independent mechanism that requires *RAD51*, although this pathway does not contribute to minisatellite alterations in the presence of wild-type *RAD50* or *RAD52*.

A significant loss in cell viability could be one possible explanation for the observed decrease in blebbing in the recombination mutants, rather than loss of recombination activity. To address this possibility we examined cell viability, by comparing cell number via hemocytometer counts with viable colony forming units, in colonies that were beginning to exhibit blebbing. We observed no significant difference in cell viability in wild type (DTK271), $\Delta zrt1$ (DTK904), $\Delta rad50 \Delta rad51 \Delta rad52$ (DTK1320), and $\Delta zrt1 \Delta rad50 \Delta rad51 \Delta rad52$ (DTK1323) strains – all exhibited viability between 25% and 32%. These data also indicate that it is unlikely that the loss of *ZRT1* is causing a mutator phenotype, as significantly increased mutation in these haploid strains would likely lead to increased cell death.

Single-strand annealing (SSA) is a *RAD52*-dependent recombination pathway that can result in repeat deletions like those seen in *ZRT1*-dependent minisatellite alteration [34]. SSA is partially dependent on *RAD59* and *RAD1* [35, 36]. When *RAD59* or *RAD1* is deleted in a $\Delta zrt1$ background, blebbing is reduced to 16.0 blebs/colony and 17.2 blebs/colony, respectively. While this is a smaller reduction than is seen in the $\Delta zrt1 \Delta rad52$ double mutant (9.4 blebs/colony, Table 2), it indicates that at least some of the *RAD52*-dependent minisatellite alterations in the $\Delta zrt1$ mutant may occur by SSA.

As *RAD50* is also involved in non-homologous end joining, we wanted to determine if non-homologous end-joining (NHEJ) has a role in *ZRT1*-dependent minisatellite alterations. We introduced a deletion of *DNL4*, a critical NHEJ ligase [37], into the $\Delta zrt1$ background. The $\Delta zrt1 \Delta dnl4$ double mutant had an average of 18.4 blebs/colony, which was not substantially different from the $\Delta zrt1$ single mutant (Table 2). A $\Delta zrt1 \Delta dnl4 \Delta rad59$ triple mutant exhibited a similar blebbing frequency. However, a $\Delta zrt1 \Delta dnl4 \Delta rad52$ mutant had a significantly reduced frequency (2.4 blebs/colony), which is well below the $\Delta zrt1 \Delta rad52$

mutant (9.4 blebs/colony). Thus, while NHEJ does not play a major role in the minisatellite alterations occurring in $\Delta zrt1$ strains, nor does it affect events occurring by SSA, it does influence some of the events remaining after the *RAD52*-dependent pathway has been eliminated.

We also determined if recombination plays a role in the infrequent minisatellite alterations occurring in a strain with a wild-type *ZRT1* gene. The parental *ade2-min3* strain has 3.7 blebs per colony (Table 2). Loss of *RAD50*, *RAD51*, *RAD52* or *DNL4* in a wild-type background reduced minisatellite alterations to 0.9, 2.1, 1.4, and 1.8 blebs/colony, respectively. These results indicate that the small number of minisatellite tract alterations seen in the wild-type *ade2-min3* strain involve DNA double-strand break repair, but require both homologous recombination and non-homologous end joining (NHEJ).

3.4. A tract derived from the human *HRAS1* minisatellite is destabilized in $\Delta zrt1$ mutants

Both the *ade2-min3* and the *ura3-min3* minisatellite constructs utilize three identical tandem repeats with roughly 50% GC content (Fig. 1a, b). However, human minisatellites are often composed of variable repeats that are enriched for CG content [2], and therefore may be altered by different mechanisms than our reporter minisatellite constructs. To determine whether the stability of a human minisatellite is affected by loss of *ZRT1*, we constructed the *ade2-h7.5* allele, which is composed of one partial and seven complete 28bp repeats of the human *HRAS1* minisatellite plus *HRAS1* locus flanking DNA inserted into the *ADE2* gene (Fig. 1c). This minisatellite has a CG content of 68%, and variable nucleotides in each repeat at the 14th and 22nd positions.

The insertion of the *HRAS1* minisatellite-derived DNA throws the *ADE2* gene out of frame, making the cells carrying this allele red and Ade⁻. Alterations in the minisatellite tract can restore *ADE2* to the correct reading frame, making cells in which alterations have taken place white and Ade⁺. Thus, colony color segregation serves as a reporter for minisatellite alteration in strains with the *ade2-h7.5* allele, as with the *ade2-min3* allele. The *ade2-h7.5* minisatellite allele is very stable, with a blebbing frequency of 0.7 blebs/colony in a WT strain background (Table 2).

To examine the effect of *ZRT1* on the stability of the *ade2-h7.5* minisatellite repeats, we deleted *ZRT1* in the *ade2-h7.5* background. The *ade2-h7.5* $\Delta zrt1$ strain displayed a blebbing phenotype similar to the *ade2-min3* $\Delta zrt1$ strain, but the frequency of minisatellite alterations in the *ade2-h7.5* $\Delta zrt1$ strain (4.8 blebs/colony) was lower than the *ade2-min3* $\Delta zrt1$ strain (20.6 blebs/colony) (Table 2). However, the seven-fold increase seen in the $\Delta zrt1$ *ade2-h7.5* strain relative to the wild-type parental *ade2-h7.5* strain is comparable to the six-fold increase seen in *ade2-min3* strains (Table 2). Thus, the *ZRT1*-dependent pathway of minisatellite alteration in yeast may also be relevant to minisatellite stability in human cells. The lower frequency of minisatellite alterations in the *ade2-h7.5* $\Delta zrt1$ strain may be due to the fact that the *HRAS1* minisatellite repeats are variable at two nucleotides in their sequence (Fig. 1c), which could influence homologous recombination events occurring between repeats. Prior work in our lab demonstrated that single nucleotide heterozygosities in *HRAS1* minisatellite repeats act to stabilize the DNA tracts [15].

We examined the nature of the tract alterations that led to bleb formation in the $\Delta zrt1$ *ade2-h7.5* strain. Whole-cell PCR was used to examine the *ade2-h7.5* allele in 104 independent white blebs. While almost all alleles (99/104 – 95%) were contractions of the repetitive tract, we did observe tract expansions in 5% (5/104) of the blebs examined. All expansion alleles gained two repeat units. Of the 99 tracts exhibiting loss, 91 had lost a single repeat while 8 lost four repeats.

We sequenced the tracts in 27 of the 104 *ade2-h7.5* alleles examined by PCR. As shown in Figure 3, both expansions sequenced added repeat units to the middle of the tract. The majority of single repeat losses occurred at the fourth or fifth repeat. The first allele with a four-repeat contraction lost the second through fifth repeats, while the second four-repeat contraction allele lost DNA from the middle of the first repeat (between the variable 14th and 22nd nucleotides) to the middle of the fifth repeat (between the variable nucleotides) to generate a hybrid Type 3 repeat.

Finally, we asked if the *ZRT1*-dependent alterations in the *ade2-h7.5* minisatellite, like alterations in the *ade2-min3* minisatellite, occur via *RAD52*-dependent and – independent pathways. As with *ade2-min3* strains, we deleted key recombination factors in the *ade2-h7.5* parent and *ade2-h7.5 Δzrt1* mutant strains. The *ade2-h7.5 Δzrt1* mutant displays an average of 4.8 blebs/colony. Loss of *RAD50*, *RAD51*, or *RAD52* in the *ade2-h7.5 Δzrt1* background reduced blebbing to 1.1 blebs/colony, 1.8 blebs/colony, and 2.5 blebs/colony, respectively (Table 2). An *ade2-h7.5 Δzrt1 Δrad50 Δrad52* strain showed a further reduction in blebbing – to 0.9 blebs/colony. Finally, loss of *RAD50*, *RAD51*, and *RAD52* reduced blebbing in the *ade2-h7.5 Δzrt1* background to 0.3 blebs/colony, a level not significantly higher than the *ZRT1* wild-type strain. This indicates that *HRAS1* minisatellite alterations in the *Δzrt1* mutant likely occur via a combination of *RAD52*-dependent and *RAD52*-independent mechanisms, as is the case in for *ade2-min3* minisatellite alterations.

4. Discussion

In this work we examined minisatellite alterations during stationary phase in *S. cerevisiae*. First, we demonstrated that *ZRT1*-dependent stationary phase minisatellite alterations occur specifically in quiescent cells, and not in nonquiescent cells within the stationary phase population. We showed that minisatellite alterations in *Δzrt1* mutants are not dependent on chromosomal context or adenine auxotrophy. We determined that recombination factors are required for alterations in minisatellite tracts during quiescence. The majority of minisatellite alterations in a *Δzrt1* strain occurs by *RAD52*-dependent homologous recombination or a *RAD52*-independent pathway that requires *RAD50*. Finally, we find that mutation of *ZRT1* can destabilize a human minisatellite, implying that zinc homeostasis may play a role in minisatellite stability in human cells.

Stationary phase cultures of *S. cerevisiae* consist of a quiescent cell fraction, in which cells are uniformly arrested and bulk DNA synthesis does not occur, and a nonquiescent cell fraction, in which some budded cells are present and DNA replication may occur [28]. Therefore, we considered that *ZRT1*-dependent minisatellite alterations during stationary phase might occur as a result of polymerase slippage during whole-genome DNA synthesis in nonquiescent cells. However, we found that loss of *ETR1*, which specifically reduces the ability of quiescent cells to reenter the cell cycle, completely eliminated blebbing in a *Δzrt1* mutant. In contrast, loss of *POR1*, which specifically reduces the reproductive capacity of nonquiescent cells, has little effect on blebbing in a *Δzrt1* mutant. Therefore, our data argue that *ZRT1*-dependent minisatellite alterations occur as a result of events in quiescent cells that do not require bulk DNA synthesis. Recent work [38] demonstrates limited DNA synthesis at specific locations in the genome of stationary phase yeast cells; the events that lead to minisatellite tract alterations may utilize this type of limited repair synthesis.

Using the chromosomal *ade2-min3* allele (Fig. 1), we determined that loss of *ZRT1* triggers an increase in minisatellite alterations exclusively in quiescent cells. We utilized the *ura3-min3* allele to show that minisatellite alterations in a *Δzrt1* mutant strain occur independently of chromosomal context and adenine auxotrophy. In stationary phase *Δzrt1* cells, we see an increase in plasmid-borne *ura3-min3* minisatellite alterations, eliminating

the possibility that the $\Delta zrt1$ minisatellite instability phenotype is an artifact of *cis*-acting sequences surrounding *ADE2* and implying that chromosomal context is not likely to have an influence on these events. Alterations occurred with equal frequency in Ade⁺ or Ade⁻ strains, indicating that the alterations are not due to the disruption of the adenine biosynthetic pathway. Taken together, these results argue that loss of *ZRT1* could potentially destabilize minisatellites at many loci and in many genetic backgrounds.

Our data demonstrate that most minisatellite alterations in a $\Delta zrt1$ mutant require homologous recombination. Loss of *RAD50*, *RAD51*, or *RAD52* in a $\Delta zrt1$ mutant strain reduces the minisatellite alterations by approximately 50% compared to the parental $\Delta zrt1$ single mutant (Table 2). Similarly, loss of both *RAD50* and *RAD51* or both *RAD51* and *RAD52* in a $\Delta zrt1$ mutant also reduce minisatellite alterations by ~50%. However, loss of both *RAD50* and *RAD52* in a $\Delta zrt1$ strain reduces minisatellite alterations by ~81%, indicating that both *RAD52*-dependent recombination and a *RAD50*-dependent, *RAD52*-independent mechanism are required for minisatellite alterations in the $\Delta zrt1$ mutant. Finally, loss of *RAD50*, *RAD51*, and *RAD52* in a $\Delta zrt1$ mutant reduces minisatellite alterations to a level not significantly different from the parental $\Delta rad50 \Delta rad51 \Delta rad52$ strain, indicating *RAD51* plays a role in *ZRT1*-dependent minisatellite alterations in the absence of *RAD50* and *RAD52*. Finally, deletion of the non-homologous end-joining (NHEJ) ligase *DNL4* in a $\Delta zrt1$ mutant strain does not reduce the frequency of minisatellite alterations, but a significant reduction is seen when *RAD52* is also deleted, indicating that NHEJ can play a role in stability maintenance if the homologous recombination pathway has been compromised. These results are consistent with a model in which two homologous recombination pathways, one *RAD52*-dependent and the other *RAD52*-independent and requiring *RAD50*, are required for most alterations occurring in the $\Delta zrt1$ mutant cells, with other pathways becoming active when homologous recombination has been compromised.

While recombinational mechanisms have previously been implicated in minisatellite instability during yeast mitosis [1, 22], minisatellite alterations in $\Delta zrt1$ mutants occur during stationary phase, a stage at which most yeast cells are quiescent [39]. Recombination modulation previously has been linked to genome alterations during stationary phase in prokaryotic *E. coli* cells (see, for example [40–42]); our data extend these findings to eukaryotic yeast cells.

Stationary phase in our $\Delta zrt1$ strain consists of a relatively uniform G1 arrest; visual inspection of the *ade2-min3 $\Delta zrt1$* strain shows that by the time the culture enters stationary phase, which occurs at ~48hrs after inoculation [22], approximately 98% of the cells are unbudded (G1) (data not shown). G1 cells have only one copy of each chromosome while G2 cells, which have undergone DNA replication, have two sister chromatids. Thus, minisatellite alterations in a $\Delta zrt1$ mutant are not likely to be a result of recombination between sister chromatids. This is significant because all the strains we have used to examine minisatellite stability in this study are haploid and therefore cannot undergo recombination between homologous chromosomes.

What is the molecular mechanism underlying stationary phase minisatellite tract alteration? Since we have shown that *ZRT1*-dependent minisatellite alterations occur in quiescent, G1-arrested stationary phase haploid cells, the mechanism for these events must not rely on bulk DNA synthesis or involve exchange between sister chromatids or homologous chromosomes. Therefore, we propose that minisatellite alterations in $\Delta zrt1$ mutants during stationary phase occur by two mechanisms: single strand annealing (SSA) and intramolecular repair events. Simple misalignment of repeats during SSA could easily result in minisatellite repeat deletion, and the role of SSA in repeat array contraction has been well established [34]. An intramolecular repair event could be initiated by a single-strand gap

(Fig. 4). During the limited DNA synthesis needed to repair the gap, polymerase slippage and misalignment could result in repeat units forming a single-stranded loop. If the loop forms on the template DNA strand and is removed, a deletion of repeat units will result. If the loop forms on the newly synthesized DNA strand and is repaired by nicking the template DNA strand opposite the loop, an expansion of the repeat tract will result (Fig. 4). The frequency of this type of event is dependent on the frequency of polymerase slippage and the formation of single-stranded nicks or gaps. It is quite possible that the polymerase slippage frequency is significantly increased at repetitive tracts in stationary phase cells. Nucleotide reserves in quiescent cells are likely to be small relative to the nucleotide pools available in actively-dividing cells, leading to increased polymerase pausing (especially at repetitive DNA tracts) thereby increasing the likelihood of polymerase dissociation and re-association events. Finally, defects in zinc transport have been shown to elevate single-stranded DNA damage in mammalian cells [43, 44].

Our data on the types of minisatellite alterations observed in $\Delta zrt1$ mutants are consistent with these models. We previously showed that minisatellite alterations in $\Delta zrt1$ mutants consist of deletions of repeat units from the *ade2-min3* tract [22]. In this study we observed both repeat deletions and tract expansion in the *ade2-h7.5* minisatellite (Fig. 3). Intramolecular repair events and SSA can both lead to deletions in direct repeat tracts when repeats are misaligned [34]. We find that minisatellite alterations in a $\Delta zrt1$ mutant during stationary phase occur by both *RAD52*-dependent and *RAD52*-independent mechanisms. SSA is a *RAD52*-dependent process for shorter repeat arrays [45, 46]. *RAD52*-independent mechanisms of SSA have been demonstrated only for large *CUP1* and rDNA repeat tracts [47]. Since our *ade2-min3* and *ade2-h7.5* reporters are significantly smaller than those tracts both in terms of repeat unit length and number of repeats, it is unlikely that *RAD52*-independent SSA contributes to our events. *RAD59* and *RAD1* are involved in SSA [36, 48, 49]. Deletion of either *RAD1* or *RAD59* in a $\Delta zrt1$ strain leads to a ~20% reduction in minisatellite alterations (Table 2, data not shown), clearly implicating SSA in these events. Since some SSA has been demonstrated to occur even without *RAD59* [49], it is difficult to estimate what proportion of *ZRT1*-dependent minisatellite alterations result from SSA. It may be that all *RAD52*-dependent minisatellite alterations in the $\Delta zrt1$ mutant occur by SSA. However, as we have argued above, *RAD52*-independent SSA is not likely to contribute to minisatellite alterations in the *ade2-min3* minisatellite tract. Therefore, all *RAD52*-independent minisatellite alterations in the *ZRT1* mutant may occur via some form of intramolecular repair. Consistent with this, we find that most *RAD52*-independent minisatellite alterations require *RAD50*; prior studies demonstrated a requirement for *RAD50* in *RAD52*-independent intramolecular recombination [34]. Rad50p possesses zinc hooks that are employed in linking DNA ends [50, 51], which could play a role in forming the single stranded DNA loops in our model of intramolecular minisatellite repair. Previous work has demonstrated that single stranded DNA loops over 16 nt can be repaired by loop removal, but the genetic requirements for this process remain elusive, likely because redundant pathways facilitate it [52]. Therefore, it is difficult to provide evidence for this aspect of our model. Finally, our *DNL4* data show that NHEJ can be used as a repair mechanism when homologous recombination has been compromised.

Importantly, we demonstrate here that a human *HRAS1* minisatellite tract alters in quiescent cells, and that loss of *ZRT1* further destabilizes the minisatellite (Table 2). The yeast *ZRT1* protein is a member of the ZIP (Zrt-, Irt-like Protein) family of zinc transporters, which are found throughout bacteria and eukaryotes, including humans [53]. Mammals have three orthologs of the yeast *ZRT1* gene: ZIP1 (also known as ZIRT1), ZIP2 and ZIP3 [54–59]. The ZIP1 protein in humans (hZip1) is likely to be the major zinc transporter for most of the cells in the body, as it is expressed in most cell types [59]. The human Zip2 protein is expressed in prostate and uterine epithelial cells. Mutations in ZIP family zinc transporters

lead to zinc deficiency and associated health problems in humans [60]. For example, mutation of Zip2 or Zip3 has been linked to prostate cancers [54].

Our data provide a mechanism to link zinc deficiencies with human disease generation. DNA strand breaks, triggered by zinc deficiency, could be the initiating lesions for minisatellite alterations in post-mitotic cells. In agreement with this model, zinc deficiency has been linked to increased DNA strand breakage in mammals [43, 44]. Variations in the *HRAS1* minisatellite tract are known to alter *HRAS1* transcription [4, 61, 62], modifying the activity of this important oncogene and influencing the onset of particular cancers. These alteration events may be elevated in quiescent, post-mitotic cells, as our data demonstrate that they are particularly sensitive to loss of zinc transporters.

Links that support this hypothesis have been detected between zinc homeostasis and minisatellite stability in human disease. Prostate cancer has been linked to both zinc deficiency and rare *HRAS1* minisatellite alleles [8, 43]. Type 1 diabetes has long been associated with rare alleles of the *IDDM1* minisatellite, and has recently been correlated with SNPs in the ZIP family zinc transporter SLC30A8 [3, 63]. Thus, the proposal that zinc homeostasis may directly affect minisatellite stability and influence minisatellite-correlated disease in humans clearly merits further investigation.

5. CONCLUSIONS

We previously discovered that a reporter minisatellite DNA tract is destabilized in stationary phase yeast cells with defects in zinc homeostasis [22]. Here we demonstrate that minisatellites are destabilized specifically in quiescent cells. Tract alterations occur regardless of minisatellite context, and can also take place in a human minisatellite inserted into the yeast genome. We have determined that both *RAD52*-dependent and *RAD52*-independent recombination pathways are necessary to alter minisatellite tracts in quiescent $\Delta zrt1$ cells. Our findings indicate that analogous zinc-mediated minisatellite rearrangements may be occurring in human cells, leading to minisatellite-associated pathologies such as cancer.

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Figure 1.

Minisatellite reporter constructs used in this study.

A) The *ade2-min3* allele. Three tandem 20bp repeats of the sequence shown above, plus one additional base, were inserted into the *ADE2* gene at the *XbaI* site, duplicating the 4bp overhang of the site. This insertion throws the *ADE2* gene out of frame. Deletion of one 20bp repeat unit restores the correct reading frame.

B) The *ura3-min3* allele on plasmid pEAS20. Three tandem 20bp repeats (identical to those in *ade2-min3*) are located between fragments of *LEU2* and *HIS4* fused to the amino terminus of the *URA3* gene, such that the *URA3* gene is in the correct reading frame [25]. Deletion of one 20bp repeat unit throws the *URA3* gene out of frame.

C) The *ade2-h7.5* allele. Seven and a half 28bp *HRAS1* human minisatellite repeats, plus flanking unique sequences from the *HRAS1* locus as indicated, were inserted into the *ADE2* gene at the *XbaI* site, duplicating the restriction site. This insert throws the *ADE2* gene out of frame. Deletion of one 28bp repeat restores the correct reading frame. The sequence of the *HRAS1* minisatellite can vary at two bases (+14 and +22), with either a C or a G as indicated. Repeats with a G at nt 14 and a C at nt 22 are Type 1 repeats, while repeats with two Gs are Type 2, repeats with a C at 14 and a G at 22 are Type 3, and repeats with two Cs are Type 4. The actual sequence at +14 and +22 is given within each repeat unit, while the repeat type is indicated below the repeat unit.

Figure 2.

Colony morphology for *ade2-min3* strains. Strains were incubated at 30°C for three days, then at room temperature for four days.

The wild-type *ade2-min3* parent is DTK271. The following strains were constructed from DTK271 as described in Materials and Methods: $\Delta zrt1$ (DTK904), $\Delta zrt1 \Delta etr1$ (DTK1244), and $\Delta zrt1 \Delta por1$ (DTK1245).

Class	# of alleles identified	ade2-h7.5 allele structure
Gain:	1	412123213
2 Repeats	1	412323213
Parental		4123213
	1	423213
Loss:	11	412213
1 Repeat	10	412313
	1	412323
Loss:	1	413
4 Repeats	1	313

Figure 3. Analysis of tract alterations in the *ade2-h7.5* minisatellite allele in $\Delta zrt1$ strains. The *ade2-h7.5* allele was sequenced in 27 independent white bleb isolates from DTK1200 (*ade2-h7.5* $\Delta zrt1$). The parental repeat structure, using the repeat type designations from Figure 1, is given for reference. Added or modified repeats are indicated in grey, while the location of deleted repeats is depicted as a break in the repeat tract. Added repeats are indicated to the right of the repeats they duplicate but, as it is not possible to distinguish which repeats are original and which have been added, the designation is done in this manner only for consistency.

Figure 4.

A model for intramolecular minisatellite alterations.

One strand of a minisatellite DNA tract (with repeats shown as a series of vertical lines) is damaged, generating a single-stranded gap. Repair synthesis initiates (red arrow), but the polymerase dissociates during replication. In (A) the polymerase re-associates further along the template strand, forming a single-stranded loop in the template. This loop is cleaved and the resulting nick is repaired, generating a minisatellite tract that has lost repeat units. In (B) the polymerase re-associates with repeat units that have already been replicated, forming a loop on the nascent strand. The template strand subsequently is nicked, and the single-stranded loop undergoes repair synthesis, generating a minisatellite tract with additional repeat units.

Table 1

Yeast Strains

Strain	Relevant genotype	Construction details
EAS28	Wild-type	<i>MATa his7-2 trp1-289 ura3-52</i> (Sia <i>et al.</i> 2001)
DNY101	<i>rad52::URA3</i>	(Nag and Petes, 1993)
DTK260	<i>lev2::HisG</i>	EAS28 with pNKY85 (Kelly <i>et al.</i> 2007)
DTK264	<i>ade2-min3</i>	DTK260 with pDTK123 (Kelly <i>et al.</i> 2007)
DTK271	<i>ade2-min3, MATa</i>	DTK264 mating-type switch (Kelly <i>et al.</i> 2007)
DTK284	<i>ade2-min3, arg8::HisG</i>	DTK264 with pDS27 (Kelly <i>et al.</i> 2007)
DTK878	<i>ade2-min3, zrl1::KAN</i>	DTK271 with <i>zrl1::KAN</i> (Kelly <i>et al.</i> 2007)
DTK904	<i>ade2-min3, zrl1::LEU2</i>	DTK284 with <i>zrl1::LEU2</i> (Kelly <i>et al.</i> 2007)
DTK944	<i>ade2-min3, rad1::KAN</i>	DTK271 with <i>rad1::KAN</i> ^a
DTK945	<i>ade2-min3, zrl1::LEU2, rad1::KAN</i>	DTK904 with <i>rad1::KAN</i> ^a
DTK1056	<i>ade2-min3, rad50::KAN</i>	DTK271 with <i>rad50::KAN</i> (Kelly <i>et al.</i> 2007)
DTK1057	<i>ade2-min3, zrl1::LEU2, rad50::KAN</i>	DTK904 with <i>rad50::KAN</i> (Kelly <i>et al.</i> 2007)
DTK1074	<i>ade2-min3, rad51::KAN</i>	DTK271 with <i>rad51::KAN</i> ^a
DTK1076	<i>ade2-min3, zrl1::LEU2, rad51::KAN</i>	DTK904 with <i>rad51::KAN</i> ^a
DTK1188	<i>ade2-h7.5</i>	DTK260 with pKK055, FOA ^R isolate
DTK1191	<i>ade2-min3, zrl1::LEU2, rad52::URA3</i>	DNY101 × DTK904, isolated spore
DTK1195	<i>rad59::KAN</i>	Spore isolated from Yeast Deletion Consortium strain dissection.
DTK1196	<i>rad59::KAN</i>	Spore isolated from Yeast Deletion Consortium strain dissection.
DTK1200	<i>ade2-h7.5, zrl1::KAN</i>	DTK1188 with <i>zrl1::KAN</i> ^a
DTK1229	<i>ade2-min3, zrl1::LEU2, rad59::KAN</i>	DTK904 × DTK1195, isolated spore
DTK1230	<i>ade2-min3, rad59::KAN</i>	DTK271 × DTK1196, isolated spore
DTK1244	<i>ade2-min3, zrl1::LEU2, Δetr1::KAN</i>	DTK904 with <i>etr1::KAN</i> ^a
DTK1245	<i>ade2-min3, zrl1::LEU2, Δpor1::KAN</i>	DTK904 with <i>por1::KAN</i> ^a
DTK1253	<i>ade2-min3, rad52::URA3</i>	DTK1191 × DTK284, isolated spore
DTK1254	<i>ade2-min3, zrl1::LEU2, rad50::KAN, rad51::KAN</i>	DTK1057 × DTK1076, isolated spore
DTK1268	<i>ade2-min3, zrl1::LEU2, rad50::KAN, rad52::URA3</i>	DTK1253 × DTK1057, isolated spore
DTK1269	<i>ade2-min3, zrl1::LEU2, rad51::KAN, rad52::URA3</i>	DTK1191 × DTK1076, isolated spore

Strain	Relevant genotype	Construction details
DTK1289	<i>ade2-min3, rad50::KAN, rad52::URA3</i>	DTK1268 × DTK284, isolated spore
DTK1290	<i>ade2-min3, rad51::KAN, rad52::URA3</i>	DTK1269 × DTK284, isolated spore
DTK1299	<i>ade2-min3, rad50::KAN, rad51::KAN</i>	DTK1254 × DTK284, isolated spore
DTK1309	<i>ade2-min3, zrl1::LEU2, dnl4::KAN</i>	DTK904 with <i>dnl4::KAN</i> ^a
DTK1316	<i>ade2-min3, dnl4::KAN</i>	DTK271 with <i>dnl4::KAN</i> ^a
DTK1320	<i>ade2-min3, rad50::KAN, rad51::KAN, rad52::URA3</i>	DTK1191 × DTK1299, isolated spore
DTK1323	<i>ade2-min3, zrl1::LEU2, rad50::KAN, rad51::KAN, rad52::URA3</i>	DTK1191 × DTK1299, isolated spore
DTK1360	<i>ade2-min3, Δetr1::KAN</i>	DTK271 with <i>etr1::KAN</i> ^a
DTK1361	<i>ade2-min3, Δpor1::KAN</i>	DTK271 with <i>por1::KAN</i> ^a
DTK1400	<i>ade2-h7.5, Δrad50::KAN</i>	DTK1188 × DTK1056, isolated spore
DTK1401	<i>ade2-h7.5, Δrad51::KAN</i>	DTK1188 × DTK1074, isolated spore
DTK1403	<i>ade2-h7.5, Δzrl1::LEU2, Δrad52::URA3</i>	DTK1200 × DTK1191, isolated spore
DTK1406	<i>ade2-h7.5, Δzrl1::KAN, Δrad50::KAN</i>	DTK1200 × DTK1056, isolated spore
DTK1407	<i>ade2-h7.5, Δzrl1::LEU2, Δrad50::KAN, Δrad52::URA3</i>	DTK1400 × DTK1191, isolated spore
DTK1426	<i>ade2-h7.5, Δrad50::KAN, Δrad52::URA3</i>	DTK1400 × DTK1191, isolated spore
DTK1427	<i>ade2-h7.5, Δrad52::URA3</i>	DTK1426 × DTK1188, isolated spore
DTK1428	<i>ade2-h7.5, Δzrl1::KAN, Δrad51::KAN</i>	DTK1401 × DTK1200, isolated spore
DTK1438	<i>ade2-h7.5, Δzrl1::LEU2, Δrad50::KAN, Δrad51::KAN, rad52::URA3</i>	DTK1401 × DTK1407, isolated spore
DTK1612	<i>ade2-min3 with pEAS20 (ura3-min3)</i>	DTK271 transformed with pEAS20
DTK1613	<i>ade2-min3, zrl1::LEU2 with pEAS20 (ura3-min3)</i>	DTK904 transformed with pEAS20
DTK1666	<i>ade2-min3, zrl1::LEU2, rad52::URA3, dnl4::KAN</i>	DTK1191 with <i>dnl4::KAN</i> ^a
DTK1677	<i>ade2-min3, zrl1::LEU2, rad59::KAN, dnl4::KAN</i>	DTK1316 × DTK1229, isolated spore
DTK1678	<i>ade2-min3, dnl4::KAN, rad59::KAN</i>	DTK1316 × DTK1229, isolated spore
DTK1690	<i>ade2-min3, dnl4::KAN, rad52::URA3</i>	DTK1316 with <i>rad52::URA3</i> ^a

^a Indicates that the strain was made using a PCR-generated construct.

Table 2

		Mean blebs/colony +/- 95% Confidence Interval	
Strain ^I	Genotype	WT	$\Delta zrtI$
<i>ade2-min3</i> strains:			
DTK271/DTK904	Parental	3.7 +/-0.4	20.6 +/-0.8
DTK1360/DTK1244	$\Delta etr1$	0.06 +/-0.05	0.09 +/-0.05
DTK1361/DTK1245	$\Delta por1$	1.8 +/-0.06	17.9 +/-0.7
DTK1056/DTK1057	$\Delta rad50$	0.9 +/-0.2	9.0 +/-0.5
DTK1074/DTK1076	$\Delta rad51$	2.1 +/-0.3	10.0 +/-0.7
DTK1253/DTK1191	$\Delta rad52$	1.4 +/-0.2	9.4 +/-0.6
DTK1289/DTK1268	$\Delta rad50 \Delta rad52$	0.6 +/-0.2	3.9 +/-0.3
DTK1290/DTK1269	$\Delta rad51 \Delta rad52$	1.7 +/-0.2	9.8 +/-0.6
DTK1299/DTK1254	$\Delta rad50 \Delta rad51$	1.2 +/-0.3	9.1 +/-0.5
DTK1320/DTK1323	$\Delta rad50 \Delta rad51 \Delta rad52$	0.3 +/-0.1	0.5 +/-0.1
DTK1230/DTK1229	$\Delta rad59$	2.0 +/-0.3	16.0 +/-0.6
DTK944/DTK945	$\Delta rad1$	4.5 +/-0.5	17.2 +/-0.6
DTK1316/DTK1309	$\Delta dnl4$	1.8 +/-0.3	18.4 +/-0.9
DTK1678/DTK1677	$\Delta dnl4 \Delta rad59$	3.8 +/-0.4	18.9 +/-0.5
DTK1690/DTK1666	$\Delta dnl4 \Delta rad52$	0.9 +/-0.2	2.4 +/-0.3
<i>ade2-h7.5</i> strains:			
DTK1188/DTK1200	Parental	0.7 +/-0.2	4.8 +/-0.4
DTK1400/DTK1406	$\Delta rad50$	0.3 +/- 0.08	1.1 +/-0.2
DTK1401/DTK1428	$\Delta rad51$	0.4 +/- 0.1	1.8 +/- 0.2
DTK1427/DTK1403	$\Delta rad52$	0.2 +/- 0.09	2.5 +/-0.3
DTK1426/DTK1407	$\Delta rad50 \Delta rad52$	0.1 +/-0.06	0.9 +/-0.2
NA/DTK1438	$\Delta rad50 \Delta rad51 \Delta rad52$	NA	0.3 +/-0.09

^I WT strain number/ $\Delta zrt1$ strain number

Table 3

Frequency of 5-FOA Resistance (events/cells) ± Standard Error

Time after Inoculation (hrs)	WT strain	$\Delta zrl1$ strain	Fold Difference WT vs. $\Delta zrl1$
0	$1.26 \times 10^{-4} \pm 1.19 \times 10^{-4}$	$1.25 \times 10^{-2} \pm 2.96 \times 10^{-3}$	99x
4	$3.16 \times 10^{-4} \pm 2.39 \times 10^{-4}$	$2.71 \times 10^{-2} \pm 1.57 \times 10^{-2}$	86x
8	$4.04 \times 10^{-4} \pm 2.25 \times 10^{-4}$	$4.05 \times 10^{-2} \pm 2.60 \times 10^{-2}$	100x
24	$4.06 \times 10^{-4} \pm 6.38 \times 10^{-5}$	$2.47 \times 10^{-1} \pm 1.50 \times 10^{-1}$	608x