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Friedreich's Ataxia $(GAA)_n$•$(TTC)_n$ Repeats Strongly Stimulate Mitotic Crossovers in *Saccharomyces cerevisae*

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Abstract

Expansions of trinucleotide GAA•TTC tracts are associated with the human disease Friedreich's ataxia, and long GAA•TTC tracts elevate genome instability in yeast. We show that tracts of $(GAA)_{230}$•$(TTC)_{230}$ stimulate mitotic crossovers in yeast about 10,000-fold relative to a "normal" DNA sequence; $(GAA)_n$•$(TTC)_n$ tracts, however, do not significantly elevate meiotic recombination. Most of the mitotic crossovers are associated with a region of non-reciprocal transfer of information (gene conversion). The major class of recombination events stimulated by $(GAA)_n$•$(TTC)_n$ tracts is a tract-associated double-strand break (DSB) that occurs in unreplicated chromosomes, likely in G1 of the cell cycle. These findings indicate that $(GAA)_n$•$(TTC)_n$ tracts can be a potent source of loss of heterozygosity in yeast.

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Introduction

Several inherited human diseases are a consequence of the expansion of trinucleotide tracts [1,2]. Although the mechanism by which tract expansions are generated is not yet understood, most of the trinucleotide tracts prone to expansion can form secondary structures such as "hairpin-like" DNA (intrastrand pairing) or triplexes (intramolecular pairing events involving complexes with three paired strands). Friedreich's ataxia is caused by expansion of tracts of the trinucleotide GAA•TTC, a sequence that is associated with triplex formation [3].

In the yeast *Saccharomyces cerevisiae*, $(GAA)_n$•$(TTC)_n$ tracts greater than 40 repeats in length result in an orientation-dependent stall of the replication fork [4,5]. The stall of the replication fork is observed when the $(GAA)_n$ sequence is located on the lagging strand template. Long $(GAA)_n$•$(TTC)_n$ tracts have high frequencies of contractions and expansions (primarily contractions) in both orientations, although these alterations are somewhat more frequent when the $(GAA)_n$ sequences are on the lagging strand template; in our subsequent discussion, we will refer to tracts in this orientation as $(GAA)_n$ tracts and the same sequence in the opposite orientation as $(TTC)_n$ tracts. The poly(GAA) tracts are also associated with a high rate of double-stranded DNA breaks (DSBs) and a high rate of terminal chromosome deletions [5]. In addition, $(GAA)_{230}$ tracts stimulate ectopic recombination between *lys2* heteroalles 200-fold more than $(TTC)_{230}$ tracts [5]. In contrast to the strong orientation-dependence observed in studies of replication fork stalling, DSB formation, and ectopic recombination, the frequency of large-scale expansions of the long $(GAA)_n$•$(TTC)_n$ tracts is affected only slightly by tract orientation [6].

In addition to studies done in yeast, the properties of $(GAA)_n$•$(TTC)_n$ repeats were also examined in bacterial and mammalian systems. In *E. coli*, $(GAA)_n$•$(TTC)_n$ tracts stimulate plasmid-plasmid recombination by a mechanism that is dependent on both the orientation and length of the repetitive tract [7]. In mammalian cells, length-dependent expansions of $(GAA)_n$•$(TTC)_n$ and $(CTG)_n$•$(CAG)_n$ tracts are observed; these expansions are stimulated by transcription, and are observed in non-dividing cells, indicating that they are not initiated by stalled replication forks [8–10].

The yeast studies of $(GAA)_n$•$(TTC)_n$ tracts described above were done in haploid strains. In the analysis described below, we examined the properties of long (230 repeats) and short (20 repeats) tracts on reciprocal mitotic crossovers (RCOs) between homologous chromosomes in diploids. The diploid strains described in the Results section allow the selection and mapping of mitotic crossovers. In addition, crossovers are often associated with gene conversion events, the local non-reciprocal transfer of information near the site of the crossover [11,12]. Most meiotic gene conversion events reflect heteroduplex formation between allelic sequences, followed by repair of the resulting mismatch [11,13]. During meiotic recombination in yeast, the length of a gene conversion tract is usually about 1–2 kb [14], although mitotic conversion tracts are often much longer with a median length of 7 kb [15]. In our study, both crossovers and conversion events were mapped.

Author Summary

Although meiotic recombination has been much more studied than mitotic recombination, mitotic recombination is a universal property. Meiotic recombination rates are quite variable within the genome, with some chromosomal regions (hotspots) having much higher levels of exchange than other regions (coldspots). For mitotic recombination, although some types of DNA sequences are known to be associated with elevated recombination rates (highly-transcribed genes, inverted repeated sequences), relatively few hotspots have been described. In this report, we show that a 690 base pair region consisting of 230 copies of the $(GAA)_n$•$(TTC)_n$ trinucleotide repeat stimulates mitotic crossovers in yeast 10,000-fold more strongly than an "average" yeast sequence. This sequence is a preferred site for chromosome breakage in stationary phase yeast cells. Our findings may be relevant to understanding the expansions of the $(GAA)_n$•$(TTC)_n$ trinucleotide repeat tracts that are associated with the human disease Friedreich's ataxia.

We find a strong stimulation of RCOs for long (230-repeat), but not short (20-repeat) tracts. This hotspot activity is observed in strains heterozygous, as well as homozygous, for the long tracts, and this stimulation is not substantially affected by the orientation of the tract relative to the replication origin. Analysis of the recombination events suggests that the recombinogenic property of the long tracts is a consequence of a double-strand DNA break (DSB) formed within an unreplicated chromosome.

Results

Experimental system to detect reciprocal crossovers and associated gene conversion events

The method allowing the selection and mapping of crossovers and associated gene conversion events is shown in Figure 1 [15–17]. A G2-associated RCO can generate two daughter cells that are homozygous for markers that were heterozygous in the starting diploid strain. On one copy of chromosome V, the diploid has the *can1-100* allele, an ochre-suppressible mutation in a gene regulating sensitivity to canavanine; yeast strains with the wild-type *CAN1* allele are killed by this drug. On the other copy of chromosome V, the *CAN1* gene has been deleted and replaced by *SUP4-o*, a tRNA gene encoding an ochre suppressor. In addition, the diploid is homozygous for *ade2-1*, also an ochre mutation. In the absence of an ochre suppressor, *ade2-1* strains are adenine auxotrophs and form red colonies as a consequence of accumulation of a pigmented precursor to adenine [18]. The starting diploid strain is canavanine-sensitive (Can^S), and forms white colonies. A RCO can be selected as a red/white sectored canavine-resistant colony.

In Figure 1, we show only one of the two possible segregation patterns, the one in which the recombined chromosomes segregate with the unrecombined chromosomes. If the two recombined chromosomes segregate into one daughter cell and the two unrecombined chromosomes segregate into the other, no canavanine-resistant sectored colony will be observed. In *S. cerevisiae*, these two segregation patterns are equally frequent [19]. Thus, the rate of RCOs is equivalent to twice the frequency of Can^R sectored colonies in the 120 kb *CEN5-can1-100/SUP4-o* interval [16].

By constructing diploid strains from haploids with diverged sequences, Lee *et al.* [15] used single-nucleotide polymorphisms (SNPs) located on chromosome V to map recombination events. Thirty-four polymorphisms that altered restriction enzyme recognition sites were used. Genomic DNA from each sector of a red/white Can^R colony was purified and used as a template to generate PCR products containing the SNPs. By treating these fragments with diagnostic restriction enzymes, followed by gel electrophoresis, Lee *et al.* [15,17] could determine whether the sector was homozygous or heterozygous for the polymorphism.

As described in the Introduction, crossovers are frequently associated with gene conversion events. For example, in Figure 2A, we show conversion of one of the polymorphic sites adjacent to the RCO, resulting in the converted allele being found in three of the four chromosomes involved in the initial exchange; this type of event is termed a "3:1" conversion. These events can be detected by examining the markers in both sectors of a sectored colony. In addition to 3:1 conversion tracts (Figure 2A), in analyzing spontaneous mitotic crossovers, Lee *et al.* [15] also found two other types of conversion tracts: 4:0 tracts (Figure 2B) and 3:1/4:0 hybrid tracts (Figure 2C). These events are likely to reflect a DSB in one homologue in G1 of the cell cycle, followed by replication of the broken chromosome, and repair of two broken chromatids in G2. Replication of a chromosome broken in G1 is an expected outcome, since single DSBs formed in G1 do not activate the DNA damage checkpoint machinery [20] and are inefficiently processed to recombination intermediates [21,22]. If the conversion tracts associated with repair of both DSBs include the same markers, a 4:0 event is generated. If one conversion tract is more extensive than the other, a hybrid 3:1/4:0 event would be observed. This explanation of the spontaneous mitotic RCOs and associated conversions is supported by the observation that the RCOs resulting from gamma-radiation of G1-synchronized yeast cells have 4:0 and 3:1/4:0 hybrid tracts, whereas cells irradiated in G2 do not [17].

An alternative explanation of the 4:0 and 3:1/4:0 hybrid tracts is that they represent two independent repair events of DSBs generated in G2. The rate of RCOs in WXT46 is 8.5×10^{-5}/division (Table 1). Of the 29 conversion events associated with the RCOs, 8 were 3:1 events and 21 were 4:0 or 3:1/4:0 hybrid tracts. If the 3:1 events are interpreted as the frequency of single repair events in G2, we calculate that the frequency of single events is about 2.3×10^{-5} ([8/29] × [8.5×10^{-5}]). The expected frequency of independent double events would be $(2.3\times10^{-5})^2$ or about 5.3×10^{-10}. The observed frequency of "double events" (conversion events of the 4:0 or 3:1/4:0 classes) was 5.3×10^{-5}. We conclude, therefore, that the 4:0 and 3:1/4:0 hybrid tracts do not reflect two independent cycles of DSB formation and DSB repair.

Orientation-dependent blockage of replication forks for poly GAA•TTC insertions on chromosome V

Previously, we used the system shown in Figure 1 and Figure 2 to measure the frequency and location of spontaneous or gamma-ray-induced recombination events in the 120 kb interval between *CEN5* and the *can1-100/SUP4-o* markers on chromosome V. In the current study, we constructed yeast strains with insertions of $(GAA)_n$•$(TTC)_n$ tracts of two different sizes (230 and 20 repeats) in two different orientations near the *URA3* gene on chromosome V (details of the constructions in Text S1). In the strains used in our study, the $(GAA)_n$•$(TTC)_n$ tracts are embedded within *lys2* sequences inserted in the intergenic region between *GEA2* and *URA3*. This position is about 22 kb centromere-proximal to *ARS508* and about 31 kb centromere-distal to *ARS510*; both of these *ARS* elements are active origins [23].

In previous studies [4,5], it was shown that long (>100-repeat) $(GAA)_n$ tracts on the lagging strand template result in a replication

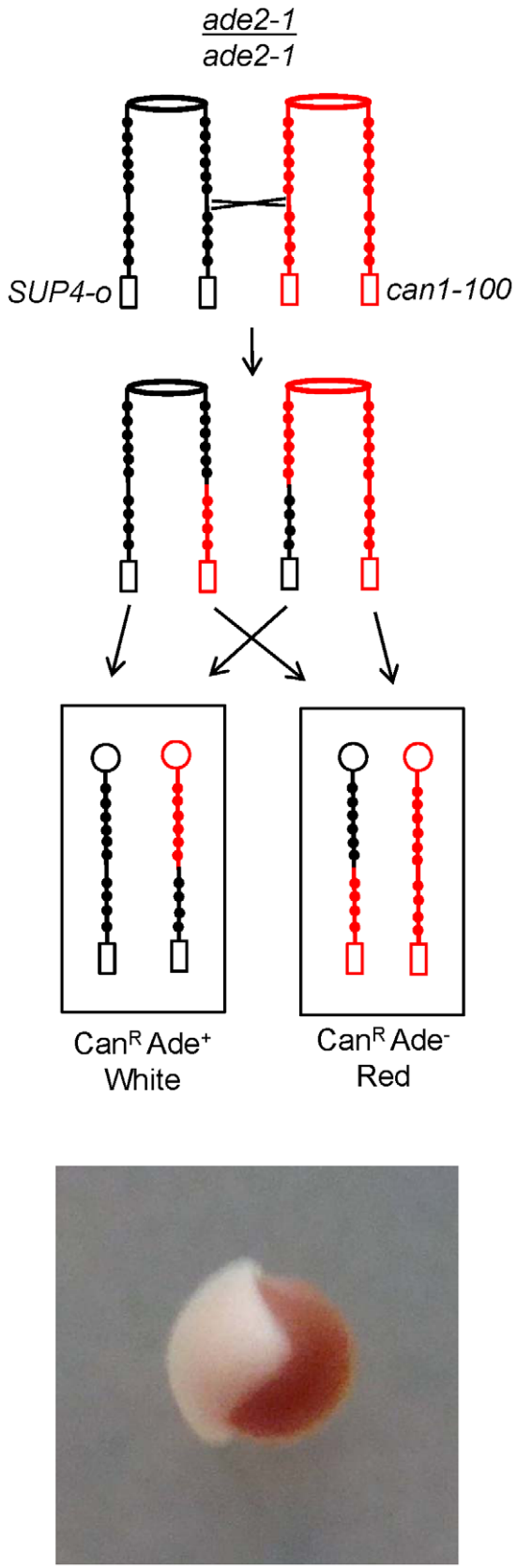

Figure 1. Detection of reciprocal crossovers. A reciprocal crossover (RCO) in G2 is shown, with chromatids indicated by vertical lines, the centromeres as ovals, and heterozygous polymorphisms as circles. The diploid strains used in this study were heterozygous for the *can1-100* allele (an ochre-suppressible mutation) located about 120 kb from the centromere of chromosome V. On the other homologue at the same position as *can1-100*, the strain had an insertion of *SUP4-o*, encoding an ochre-suppressing tRNA. The strain was homozygous for the *ade2-1* mutation, also an ochre-suppressible mutation. Strains with the unsuppressed *ade2-1* mutation form red colonies. The starting diploid was Can^S and formed white colonies. An RCO results in two Can^R cells that can divide to produce a red/white sectored Can^R colony. Polymorphisms distal to the crossover become homozygous in the two sectors. A photograph of a sectored colony is shown below the depiction of the RCO.
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fork block whereas long TTC tracts on the lagging strand template do not. To determine how replication forks were blocked for strains with the $(GAA)_n$•$(TTC)_n$ tracts inserted on chromosome V, we constructed two isogenic haploid strains in which a $(GAA)_{230}$•$(TTC)_{230}$ tract was inserted in two orientations. In the haploid MD512, the tract was oriented such that the GAA sequence was on the "Watson" strand as designated in *Saccharomyces* Genome Database, and the haploid MD510 had the tract in the opposite orientation. By two-dimensional gel electrophoresis, we found a blocked replication fork in MD510 but not in MD512 (Figure 3). Since a replication fork initiated at *ARS510* would encounter the GAA tract on the lagging strand in MD510, this result suggests that tracts are replicated primarily by a replication fork initiated at *ARS510* rather than *ARS508*, although we have not directly examined fork movement. In our subsequent discussion of yeast strains, tracts oriented in the same direction as MD510 will be termed "$(GAA)_n$" tracts and those with the opposite orientation will be termed "$(TTC)_n$" tracts; this nomenclature is consistent with previous studies [5]. It should be noted that, in other genetic backgrounds, the chromosomal region in which we inserted the $(GAA)_n$•$(TTC)_n$ tracts is replicated using forks that move in the opposite direction from the one observed in our genetic background [24].

$(GAA)_{230}$•$(TTC)_{230}$ tracts stimulate the frequency of mitotic RCOs

We first performed a pilot experiment to examine the recombinogenic effects of $(GAA)_{230}$ and $(TTC)_{230}$ tracts in diploids heterozygous for insertion near *URA3*. As described above, the rate of RCOs in the *CEN5-can1-100/SUP4-o* interval can be calculated from the frequency of Can^R red/white sectored colonies. The rates of RCOs in MD506 (heterozygous for the $[GAA]_{230}$ tract) and MD508 (heterozygous for the $[TTC]_{230}$ tract) were 13×10^{-5}/division ($\pm3\times10^{-5}$) and 6.2×10^{-5}/division ($\pm2\times10^{-5}$), respectively; 95% confidence limits are shown in parentheses. The rate of RCOs in an isogenic diploid without the tract insertion is 5.8×10^{-6}/division [15]. Thus, the heterozygous tract insertions stimulated RCOs in the *CEN5* to *can1-100/SUP4-o* interval by about 10- to 20-fold and tracts in both orientations were recombinogenic.

The diploids MD506 and MD508 did not have the polymorphisms required to map the recombination events (details of their genotypes in Text S1 and Table S1). Consequently, we constructed six other diploids that were heterozygous for polymorphisms that allowed mapping of RCOs and associated conversions. The strain names, and their tract sizes and orientations are: WXTMD42, $(GAA)_{20}$/$(GAA)_{20}$; WXTMD46, $(GAA)_{230}$/$(GAA)_{20}$; WXTMD43, $(GAA)_{230}$/$(GAA)_{230}$; WXTMD40, $(TTC)_{20}$/$(TTC)_{20}$; WXTMD45, $(TTC)_{20}$/$(TTC)_{230}$; WXTMD41, $(TTC)_{230}$/$(TTC)_{230}$. In all strains, the $(GAA)_n$•$(TTC)_n$ tracts were inserted at the same position on chromosome V near the *URA3* gene (green rectangles in Figure 4).

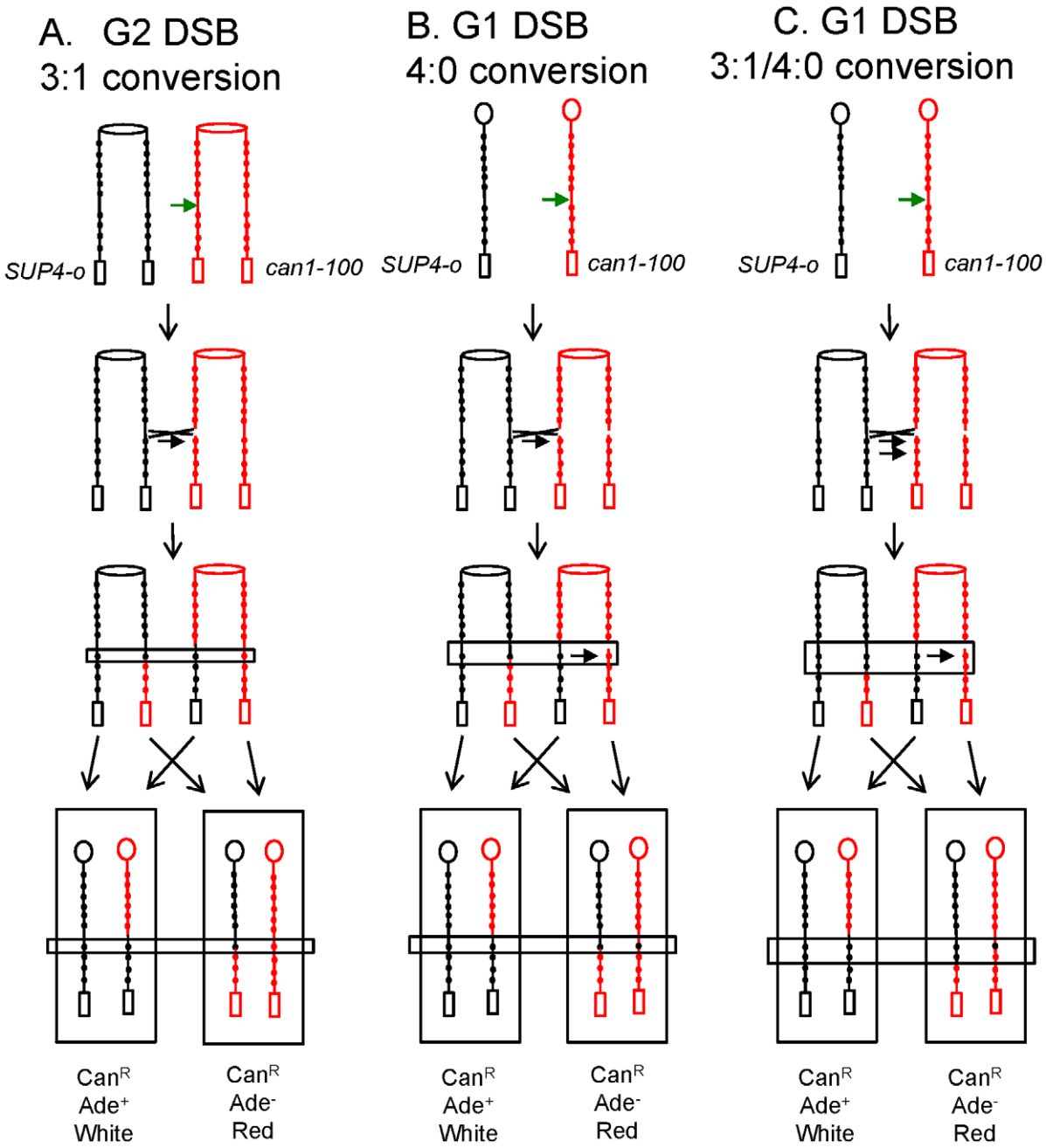

Figure 2. Gene conversion events associated with crossovers in G2 and G1. The depictions of chromosomes, centromeres, and polymorphic markers are the same as in Figure 1. The regions involved in the conversion event are enclosed within horizontal rectangles. A. 3:1 conversion associated with an RCO initiated by a double-strand DNA break (DSB) in G2. Following a DSB on the *can1-100*-containing chromosome, a conversion event in which information is transferred from the *SUP4-o*-containing chromosome occurs; this conversion event is associated with the RCO. For 3:1 conversion events, one sector is homozygous for the polymorphic marker and the other sector retains heterozygosity. B. 4:0 conversion associated with a DSB in G1. One of the unreplicated chromosomes is the target of a DSB, and the broken chromosome is replicated to yield two broken chromatids. The repair of the first chromatid is associated with an RCO and a conversion event. The repair of the second chromatid is unassociated with an RCO but underwent conversion of the same marker. In the resulting colony, both sectors are homozygous for the same polymorphic marker. C. 3:1/4:0 hybrid conversion associated with a DSB in G1. As in Figure 2B, the recombination-initiating lesion occurs in unreplicated chromosomes. In this event, however, repair of the first DSB converts two markers, whereas repair of the second converts only one marker. The net result is a 3:1/4:0 hybrid tract.
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These diploid strains were constructed from two haploid parents (PSL2 and PSL5) with numerous sequence polymorphisms allowing mapping of the positions of the crossovers as described further below.

The rates of RCOs with 95% confidence limits, based on an average of the number of sectored colonies in at least 20 cultures, are shown in Table 1. Strains homozygous for $(GAA)_{20}$ or $(TTC)_{20}$ tracts (WXTMD42 and WXTMD40) had rates of RCOs of about

Table 1. Rates of reciprocal mitotic crossover in diploid strains homozygous and heterozygous for $(GAA)_n$•$(TTC)_n$ tracts.

Strain name	Orientation and length of GAA/TTC tracts[1]	Rates of RCO (95% confidence limits)[2]
WXTMD42	$(GAA)_{20}/(GAA)_{20}$	4.2×10^{-6} ($3.8-5\times10^{-6}$)
WXTMD46	$(GAA)_{230}/(GAA)_{20}$	8×10^{-5} ($7.6-8.4\times10^{-5}$)
WXTMD43-2-1	$(GAA)_{230}/(GAA)_{230}$	2.2×10^{-4} ($2-2.6\times10^{-4}$)
WXTMD40-1-1	$(TTC)_{20}/(TTC)_{20}$	3.2×10^{-6} ($2.2-4.4\times10^{-6}$)
WXTMD45-2-1	$(TTC)_{20}/(TTC)_{230}$	8.2×10^{-5} ($6.6-10.2\times10^{-5}$)
WXTMD41-1-1	$(TTC)_{230}/(TTC)_{230}$	1.5×10^{-4} ($1.3-1.8\times10^{-4}$)
PSL101[3]	No tract insertions	6.6×10^{-6} ($6.2-7\times10^{-6}$)

[1]As elsewhere in the text, GAA indicates that the $(GAA)_n$ tract is the lagging strand template, and TTC is the opposite orientation relative to the replication origin. In this column, the upper number represents the tract located in the PSL2–derived homologue and the bottom number represents the tract in the PSL5–derived homologue.
[2]The average frequency of sectored colonies in 20 to 40 independent cultures was determined. This value was multiplied by two to calculate the rate of RCOs.
[3]This strain was analyzed previously [15].
doi:10.1371/journal.pgen.1001270.t001

4×10^{-6}/division. These rates are very similar to that observed in the isogenic PSL101 strain (6×10^{-6}) that had no GAA•TTC tracts [15]. The strains homozygous for either the $(GAA)_{230}$ or $(TTC)_{230}$ tracts (WXTMD43 and WXTMD41, respectively) had RCO rates of about 2×10^{-4}/division. Thus, the addition of a GAA•TTC tract that is only 690 base pairs in length elevated the rate of RCOs in a 120 kb interval by more than 30-fold. The strains heterozygous for the long tracts (WXTMD46 and WXTMD45) also had substantially (20-fold) elevated rates of RCOs; the rates of RCOs in the heterozygous strain were about half those observed in the homozygous strains, indicating the GAA•TTC sequences on the two homologues functioned independently. As found previously for the MD506 and MD508 strains, the orientation of the GAA•TTC tract has no strong effect on its recombinogenic properties. It should be noted that Break-Induced Replication (BIR) [12] and local gene conversion events can generate unsectored canavanine-resistant colonies; however, these colonies cannot be unambiguously distinguished from RCOs that occur prior to plating cells on canavanine-containing medium [16].

Mapping of crossovers and associated gene conversion events associated with $(GAA)_{230}$•$(TTC)_{230}$ tracts

From the results described above, one obvious possibility is that $(GAA)_{230}$•$(TTC)_{230}$ tracts are preferred sites for formation of a DSB or some other type of recombinogenic DNA lesion. By this model, one would expect most of the tract-stimulated recombination events to map at or near the position of the tract. In addition, in meiotic and mitotic recombination events in yeast analyzed previously, if a diploid is heterozygous for a preferred site of DSB formation, the chromosome with the preferred site is the recipient of genetic information in a gene conversion event [12]. We examined the positions of crossovers and associated gene conversion events in two strains: WXTMD46 (a diploid

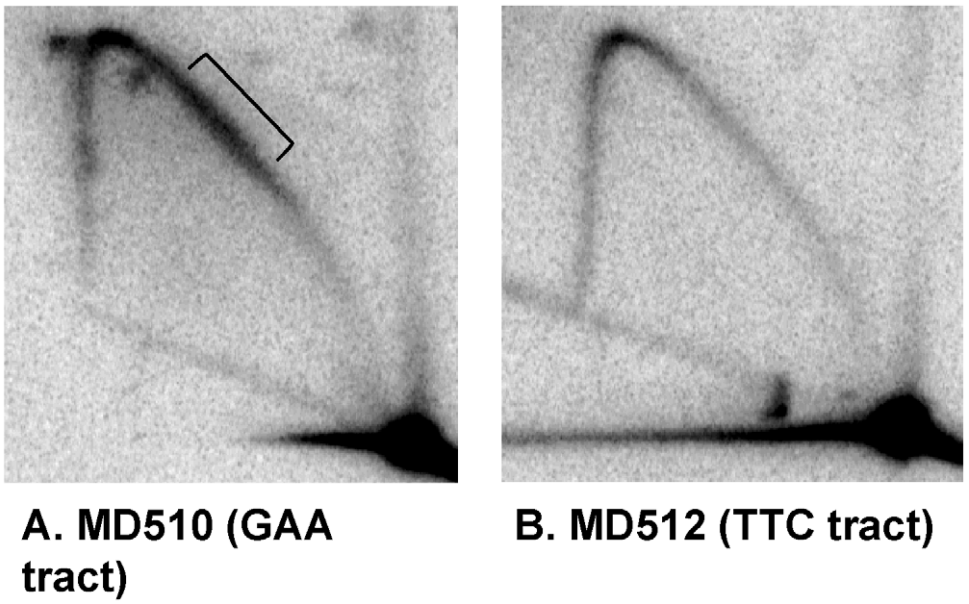

Figure 3. Two-dimensional gel analysis of replication forks stalled by the $(GAA)_{230}$ tracts. We examined replication forks in two strains, MD510 and MD512 by two-dimensional gel electrophoresis (details in Materials and Methods). These strains were identical except for the orientation of the $(GAA)_n$•$(TTC)_n$ tracts. Accumulation of replication intermediates leads to a "bulge" on the Y-arc (indicated by brackets) in MD510 but not in MD512. Thus, we define the orientation in MD510 as the GAA orientation and the opposite orientation as TTC.
doi:10.1371/journal.pgen.1001270.g003

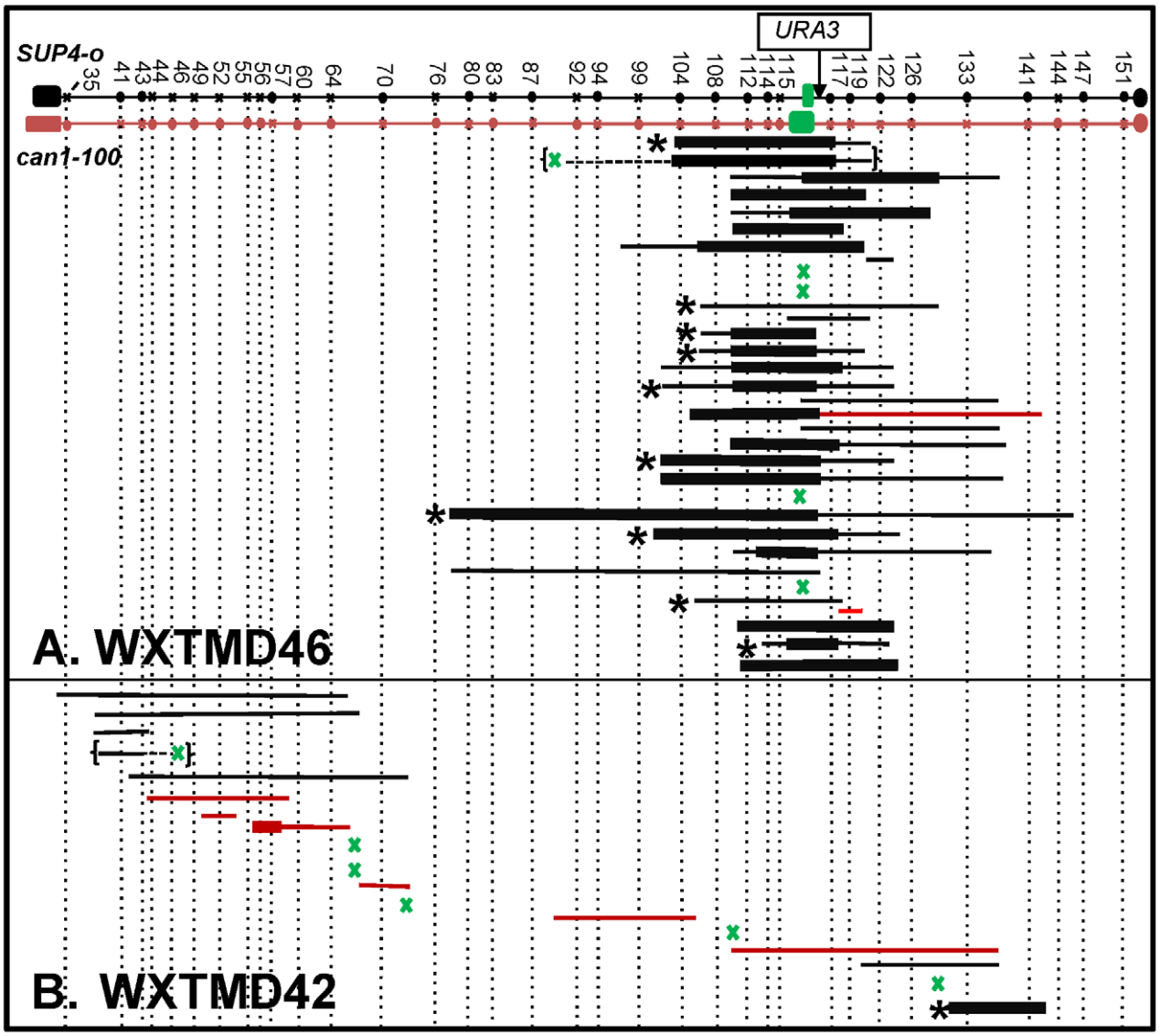

Figure 4. Mapping of RCOs and associated gene conversion events in WXTMD46 (GAA_{230}/GAA_{20}) and in WXTMD42 (GAA_{20}/GAA_{20}). The two horizontal lines at the top of the figure represent the two copies of chromosome V of the diploids with black showing the chromosome derived from the haploid PSL5/YJM789 background and red indicating the chromosome derived from PSL2/W303a. The green boxes show the position and approximate sizes of the $(GAA)_n$ tracts in WXTMD46. In WXTMD46, the "black" chromosome contains the GAA_{20} tract and the "red" chromosome has the long GAA_{230} tract; both tracts are short in WXTMD42. By PCR and restriction analysis [15], we determined whether the markers (shown as X's and circles) were heterozygous or homozygous in each sector. At the top of the figure, an X indicates that the PCR fragment did not have the diagnostic restriction site, and a circle indicates that it had the site. The numbers show the approximate SGD coordinates of the marker in kb. The data from each colony are shown on a separate line. Conversion events of the 3:1 and 4:0 types are indicated by thin and thick lines, respectively. The color of the lines indicates whether the donated marker was from the black or red chromosomes. Green X's show RCOs that are not associated with an observable gene conversion. A. Analysis of 33 sectored colonies of WXTMD46. In this strain, many of the conversion tracts were hybrid 3:1/4:0 events. In some of these events (marked with an asterisk), there was a crossover within the conversion tract. An example of one of these events is shown in Figure S1. Although most of the conversion events involve donating information from the black chromosome to the red chromosome, there were two conversions in which the transfer of information occurred in the opposite direction. There was also one example of a conversion event that was separated from the crossover by markers not involved in the conversion (shown as a green X connected to the conversion event by a dotted line). B. Analysis of 18 sectored colonies of WXTMD42. In this strain, as expected, the conversion and crossover events are distributed throughout the 120 kb interval between *CEN5* and the *can1-100/SUP4-o* markers.
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heterozygous for a $(GAA)_{230}$ tract) and WXTMD42 (a diploid homozygous for $[GAA]_{20}$ tracts).

The positions of the crossovers and gene conversion events were mapped by the methods described previously [15]. In brief, using PCR and restriction analysis, for both sectors of a Can^R red/white sectored colony, we determined whether polymorphic sites on chromosome V were homozygous for the PSL2 form of the polymorphism (shown in red in Figure 4), the PSL5 form of the polymorphism (shown in black in Figure 4), or were heterozygous. In the previous studies of spontaneous or gamma-ray-induced mitotic crossovers, four types of sectored colonies were commonly observed: 1) RCOs unassociated with an adjacent gene conversion

tract, 2) RCOs associated with an adjacent 3:1 tract (as defined in Figure 2), 3) RCOs associated with an adjacent 4:0 tract, and 4) RCOs associated with a hybrid 3:1/4:0 tract. Spontaneous recombination events are distributed throughout the 120 kb interval with a minor "hotspot" located near the *can1-100/SUP4-o* marker and a minor "coldspot" near *CEN5* [15].

A summary of the mapping of crossovers and associated conversions in WXTMD46 is shown in Figure 4A. All markers proximal to the crossover are heterozygous in both red and white sectors, and homozygous distal to the crossover in both sectors (as illustrated in Figure 2). As observed for spontaneous events previously, most of the crossovers (29 of 33) were associated with conversion tracts of various sizes. 3:1 and 4:0 conversion tracts (as defined in the Introduction) are indicated by thin and thick vertical lines in Figure 4, respectively. 3:1/4:0 hybrid tracts are shown by adjacent thick and thin lines. Conversion tracts shown in black indicate that genetic information was transferred from the PSL5-related homologue and red tracts show transfer of information from the PSL2-related homologue.

Almost all of the conversion events in WXTMD46 included one or both of the markers flanking the GAA•TTC tract, as expected if the recombination event initiated within the tract. All four of the crossovers unassociated with conversion (shown as green Xs) occurred in the region containing the tract. In Figure 5, we compare the distribution of conversion events in WXTMD46 and PSL101 (an isogenic diploid without a GAA•TTC tract; data from Lee *et al.* [15]). The difference in the distributions of conversion events in the two strains is evident. In addition, in WXTMD46, the conversion tracts were strongly biased in the direction that represents transfer of information from the PSL5-related homologue. This result is consistent with the recombinogenic lesion occurring on the PSL2-related homologue that contains the $(GAA)_{230}$ tract rather than the chromosome with the $(GAA)_{20}$ tract.

Several other features of the conversion events are important. First, most of the conversion tracts were either 4:0 tracts or hybrid 3:1/4:0 tracts. As discussed previously, such tracts are most simply interpreted as representing repair in G2 of a DSB formed in G1 (Figure 2B and 2C). This issue will be discussed in more detail below. Second, although some of the observed conversion events extended symmetrically to both sides of the tract, others were asymmetric. Thus, conversion events can extend either unidirectionally or bidirectionally from the initiating DNA lesion. Third, as observed with spontaneous recombination events and events induced by gamma rays in G1 [15,17], the conversion tracts were long compared to those observed in meiosis. We estimated tract length by averaging the minimal tract length (the distance between the markers included in the tract) and the maximal tract length (the distance between the closest flanking markers not included in the tract). The median length of the tracts was 20.3 kb (95% confidence limits of 12.5–23.4 kb), somewhat larger than the length observed in spontaneous events without the $(GAA)_n$•$(TTC)_n$

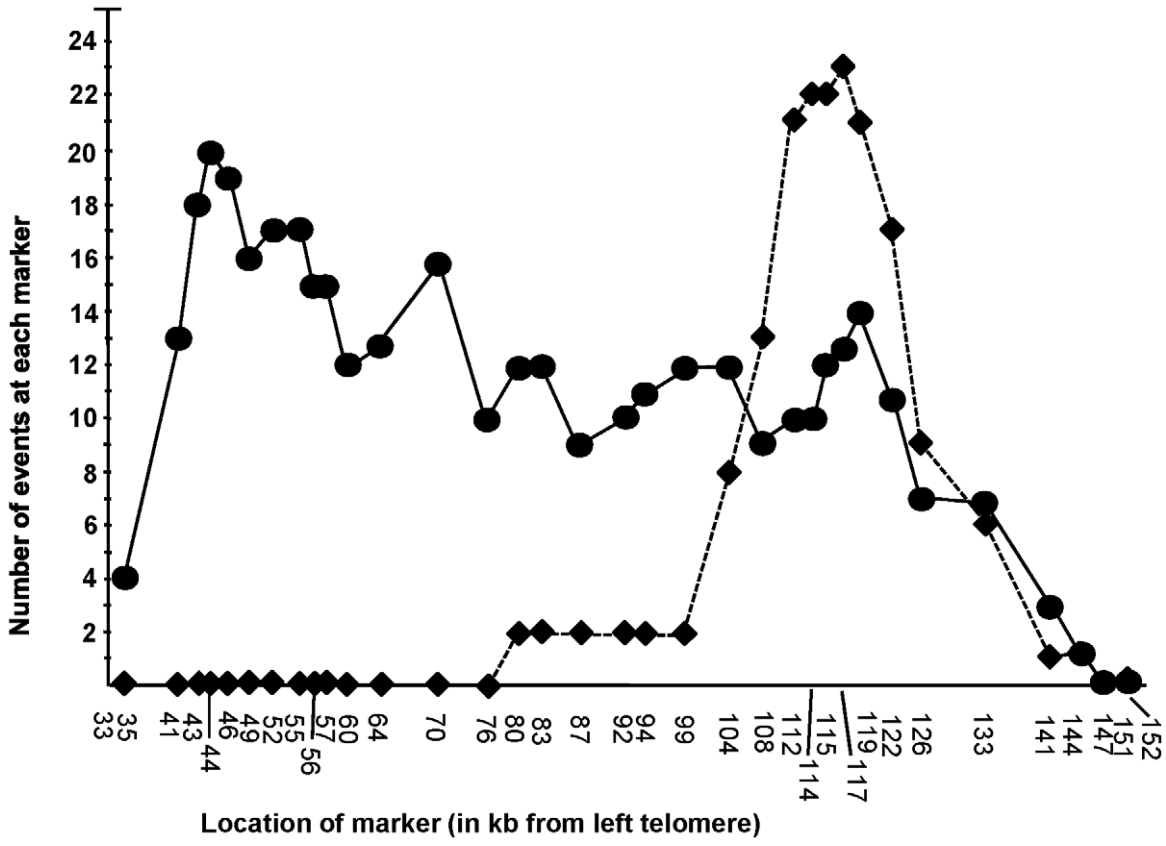

Figure 5. Comparison of the distribution of mitotic gene conversion events in PSL100/PSL101 (lacking the $(GAA)_n$ tract) and WXTMD46 (containing the $(GAA)_{230}$ tract). For each heterozygous marker, we show the number of events that include that marker in PSL100/PSL101 (circles connected by undotted lines) and WXTMD46 (diamonds connected by dotted lines). The conversion events in PSL100/PSL101 occurred throughout the 120 kb interval with an elevation of events near markers 43 and 44, and a reduction in conversion near the centromere. In WXTMD46, the events are strongly biased toward the position of the GAA tract between markers 115 and 117.
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tracts (6.5 kb; [15]). The median size of meiotic conversion tract lengths is about 2 kb [14]. Fourth, as in previous studies, we found a number of examples of crossovers within a conversion tract; these events are indicated by asterisks in Figure 4. As discussed in Lee *et al.* [15], most of these events are explicable as representing the independent repair of two broken chromatids. An example of this class of conversion event is shown in Figure S1.

We also mapped a small number of RCOs in WXTMD42, the strain homozygous for the $(GAA)_{20}$ tracts (Figure 4B). As expected, these events were distributed throughout the *CEN5* to *can1-100/SUP4-o* interval. In addition, the conversion events involved transfer of information from both homologues with approximately the same frequency. The median conversion tract length in WXTMD42 is 11.6 kb (95% confidence limits of 3.7–22.3 kb).

Physical evidence for DSBs associated with $(GAA)_{230}$•$(TTC)_{230}$ tracts in stationary phase cells

The genetic evidence predicts the existence of a tract-associated DSB in G1 diploid cells. To look for such DSBs directly, we prepared DNA samples from stationary phase cells (>95% unbudded cells) of two isogenic haploid strains, WXT10 with a $(TTC)_{20}$ tract and WXT11 with a $(TTC)_{230}$ tract. Intact chromosomal DNA was isolated from cells suspended in agarose plugs to prevent shearing and the resulting samples were analyzed by contour-clamped homogeneous electric field gel electrophoresis (CHEF gels; [25]). The separated chromosomal DNA molecules were transferred to nylon membranes and hybridized to *URA3*-specific probe. We observed a chromosomal fragment at the position expected for a DSB within the tract (Figure 6A) in WXT11, but not in WXT10 (Figure 6B). The fraction of broken chromosomal molecules observed in three independent experiments was about 0.013 (average of 0.013, 0.017, and 0.01). Although this frequency of DSBs is considerably higher than the observed frequency of RCOs (about 10^{-4}), it is likely that many of the DSBs are repaired by pathways, such as BIR and gene conversion unassociated with RCOs, that do not generate RCOs [12].

$(GAA)_{230}$•$(TTC)_{230}$ tracts are not strong hotspots for meiotic recombination

In yeast, long $(CTG)_n$•$(CAG)_n$ tracts are preferred sites for DSB formation in mitosis [26]. In meiosis, long (greater than 75 repeats) $(CTG)_n$•$(CAG)_n$ tracts were hotspots of recombination in one study [27], but were not in another [28]. Short (10-repeat) $(CTG)_n$•$(CAG)_n$ tracts were not meiotic recombination hotspots [29].

As shown above long $(GAA)_n$•$(TTC)_n$ promote DSB formation in mitosis. It was reasonable to ask, therefore, whether long GAA•TTC tracts stimulate meiotic recombination, as well. To address this question, we performed tetrad analysis, measuring meiotic recombination distances in three intervals on chromosome V: *CEN5-ura3*; *ura3-can1-100/SUP4-o* (the interval containing the tracts), and *can1-100/SUP4-o* to *V9229::HYG*. The heterozygous *HYG* gene (encoding a protein that results in resistance to hygromycin) was inserted approximately 20 kb centromere distal to the *can1-100* gene. This analysis was done in WXTMD46 (which contains $(GAA)_{230}$ on one homologue and $(GAA)_{20}$ on the other) and PSL101 (which lacks $(GAA)_n$•$(TTC)_n$ tract insertions). No significant differences were observed in map distances for any of the intervals (details of the analysis in Table S4). The map distance for the interval containing the insertion was 36 cM in WXTMD46 and 37 cM in PSL101 (total of about 100 tetrads examined in each strain).

Strong meiotic recombination hotspots are associated with high rates of gene conversion and crossovers [30]. The $(GAA)_{230}$ tract in WXTMD46 is located about 1 kb from the mutant *ura3* allele and the $(GAA)_{20}$ tract is located the same distance from the wild-type *URA3* allele. If the $(GAA)_{230}$ tract is a preferred site for meiotic DSB formation, we would expect an elevation in gene conversion events of the 3 Ura^+:1 Ura^- class, since the chromosome that receives the DSB acts as a recipient for information derived from the uncut chromosome [12]. This effect should be detectable since the strong meiotic recombination *HIS4* hotspot stimulates meiotic conversion events at sites located 2.7 kb from the hotspot [31], a distance longer than that between the $(GAA)_{230}$ tract and *URA3*. In PSL101, we observed two conversion events, both 1 Ura^+: 3 Ura^- tetrads, in a total of 118 tetrads. In 105 tetrads derived from WXTMD46, we found no gene conversions of the 3^+:1^- or 1^+:3^- classes, but four tetrads that had 4 Ura^+: 0 Ura^- spores. This 4:0 type of conversion is consistent with a mitotic gene conversion occurring within a sub-population of the WXTMD46 cells prior to sporulation [11]. Consistent with this hypothesis, in two of the tetrads with 4 Ura^+ spores, all four spores had the *SUP4-o* marker and were Hyg^S. These segregation patterns are consistent with a mitotic gene conversion at the *ura3* locus associated with a mitotic crossover.

We also examined the meiotic stability of the $(GAA)_n$•$(TTC)_n$ tracts by PCR analysis of spore DNA in 20 tetrads. Three patterns were observed. In 10 tetrads, two of the tracts were about 20 repeats in length and two were about 230 repeats in length. In 5 tetrads, two of the tracts were 20 repeats in length and two were of equal size but shorter than 230 repeats; this class is consistent with a sub-population of WXTMD46 cells in which the 230-repeat tract had undergone a mitotic deletion. In the third class (5 tetrads), two spores had 20-repeat tracts, one had a 230-repeat tract, and one had a tract of intermediate size; this class is consistent with a meiotic deletion event in one of the two 230-repeat tracts. Taken together with the mapping and gene conversion data, these results argue that the long $(GAA)_n$•$(TTC)_n$ tracts are somewhat meiotically unstable, but the DSBs formed within the tract do not strongly stimulate meiotic recombination between the homologous chromosomes. This issue will be discussed further below.

Discussion

The main conclusions from our study are: 1) $(GAA)_{230}$•$(TTC)_{230}$ tracts in both orientations strongly stimulate recombination between homologous chromosomes in mitosis, but not in meiosis, 2) the recombinogenic properties of the $(GAA)_n$•$(TTC)_n$ tracts suggest that most of the events are initiated by a DSB formed in G1 of the cell cycle (a conclusion supported by a physical analysis of tract-associated DSBs), 3) the gene conversion events associated with the $(GAA)_n$•$(TTC)_n$ repeats resemble those associated with spontaneous mitotic crossovers, and 4) single conversion events can be propagated from the location of the $(GAA)_n$•$(TTC)_n$ insertion either unidirectionally (in either direction) or bidirectionally. Each of these conclusions will be discussed in detail below.

Mitosis-specific recombinogenic effects of $(GAA)_n$•$(TTC)_n$ tracts

Although the tendency of certain trinucleotide tracts to expand in size was first demonstrated in humans, much of the experimental research concerning the effects of genome-destabilizing effects of these sequences has been done in bacteria and the yeast *Saccharomyces cerevisiae* [1,2,32]. In yeast, three types of repetitive trinucleotide tracts, $(CTG)_n$•$(CAG)_n$, $(CGG)_n$•$(CCG)_n$,

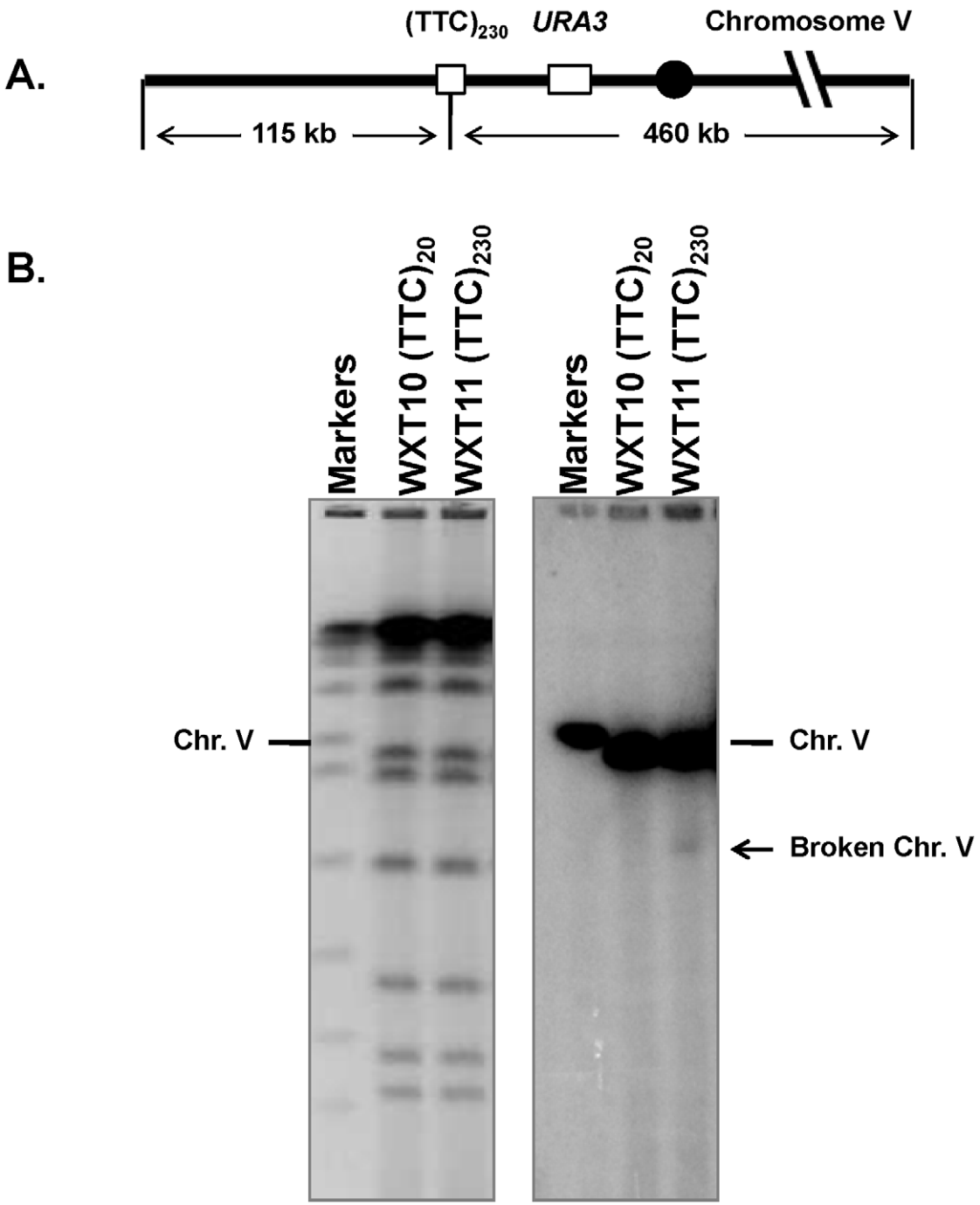

Figure 6. Physical detection of tract-associated DSBs in stationary-phase (G1/G0) haploid cells. Haploid strains with short (WXT10, 20 repeats) or long (WXT11, 230 repeats) were grown to stationary phase in rich medium. Chromosomal DNA molecules were separated by contour-clamped homogeneous electric field gel electrophoresis (CHEF gels) and Southern analysis was performed using a *URA3*-specific hybridization probe. A. Location of the $(GAA)_n$•$(TTC)_n$ tracts on chromosome V. Based on the location, a DSB located within or near the tract should produce two chromosomal fragments, one of about 460 kb that should hybridize to a *URA3*-specific probe and one of about 115 kb. B. Detection of a DSB associated with the long, but not the short, tract. The left panel shows the ethidium bromide-stained gel following electrophoresis, and the right panel shows the Southern hybridization pattern of the same gel to a *URA3*-specific probe. Since we chose electrophoresis conditions to maximize separation of the smaller chromosomes, all 16 yeast chromosomes are not visualized as bands on the ethidium bromide-stained gels. The leftmost lane has marker DNA molecules (Biorad) isolated from a yeast strain that has chromosomes of somewhat different sizes than our strains.
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and $(GAA)_n$•$(TTC)_n$, have been examined in detail. All three types of tracts undergo frequent size alterations with the frequencies of alterations increasing as a function of the number of repeats [32]. The frequency of these alterations is also affected by the orientation of the repetitive tract with respect to the replication origin. All three tracts are capable of forming secondary structures *in vitro* with one strand forming a more stable secondary structure than the other [1]. The orientation in which the strand with the

most stable secondary structure is on the lagging strand for replication has the highest frequency of tract alterations. This orientation is also associated with replication fork pausing [1]. For the $(GAA)_n$•$(TTC)_n$ repeats, as discussed above, replication fork pausing is observed when the $(GAA)_n$ repeats are on the lagging strand [4]. Somewhat unexpectedly, large-scale expansions of $(GAA)_n$•$(TTC)_n$ tracts occur with approximately the same frequency regardless of the orientation of the tract [6].

Since DSBs are recombinogenic [12] and since DSBs are observed at the sites of long $(CTG)_n$•$(CAG)_n$ and $(GAA)_n$•$(TTC)_n$ tracts [5,26], one would expect that such tracts would be hotspots for recombination. Long $(CTG)_n$•$(CAG)_n$ tracts stimulate intrachromosomal recombination between repeats and sister-chromatid exchanges [26,33]; long $(GAA)_n$•$(TTC)_n$ tracts elevate the frequency of recombination between repeats on non-homologous chromosomes in yeast [5] and plasmid-plasmid recombination in *E. coli* [7]. In these assays, it was unclear whether the recombination events were reciprocal (producing two recombined DNA molecules) or non-reciprocal. The assay used in our current study selects for reciprocal events.

We found that the 230-repeat tract elevates the rate of RCOs in 120 kb interval from about 5×10^{-6}/division (strains with no tract or a 20-repeat tract) to about 2×10^{-4}/division. We calculate that the rate of RCOs/kb in the strains without the tract is about 4×10^{-8}/kb/division. The 690 bp tract has a rate of RCOs of about 3×10^{-4}/kb/division. Consequently, the $(GAA)_{230}$•$(TTC)_{230}$ tract is about 10^4-fold more recombinogenic than an average yeast sequence.

In contrast to the strong recombinogenic effects of the tract on mitotic recombination, no strong stimulation was observed for meiotic exchange. Since we observed meiosis-specific alterations in tract length in about 25% of the tetrads that were analyzed, it is likely that the long $(GAA)_n$•$(TTC)_n$ tracts are substrates for DSB formation in meiosis. The lack of a detectable effect of the tracts on meiotic recombination can be explained in two ways. First, it is possible that tract-associated DSBs are repaired by intrachromosomal interactions (Synthesis-Dependent Strand Annealing, SDSA) or sister-chromatid exchanges [12]; neither of these events would be detected by standard tetrad analysis. Meiosis-specific intra-allelic changes in the lengths of minisatellites consistent with SDSA events have been observed previously in humans [34] and yeast [35,36]. Second, it is possible that the effects of a weak tract-associated hotspot would be obscured by the very high frequency of meiosis-specific DSBs catalyzed by Spo11p. We note, however, that a strong tract-associated hotspot would have been detected by our analysis. The strong *HIS4* recombination hotspot, for example, increases the map length in the *LEU2-HIS4* interval from 20 cM to 36 cM [37].

Evidence that the tract-associated RCOs reflect a DSB formed in unreplicated (G1) chromosomes

Previously, we showed that about 40% of spontaneous RCOs were associated with 4:0 or 3:1/4:0 hybrid conversion tracts [15]. We suggested that such events were a consequence of DSB formation on an unreplicated chromosome, followed by replication of the broken chromosome, and repair of the two resulting broken chromatids (Figure 2B and 2C). Since most of the RCOs stimulated by the $(GAA)_n$•$(TTC)_n$ repeats are associated with 3:1/4:0 tracts, it is likely that the recombinogenic DSBs are formed in G1. This conclusion, based on genetic analysis, is also supported by the physical analysis demonstrating tract-associated DSBs in stationary phase cells (Figure 6). Since the DSBs occur in G1/G0, the observation that the tract-associated stimulation of RCOs is independent of the orientation of the tract is expected.

It should be emphasized that our results do not show that tract-associated DSBs occur only in G1/G0. We observed previously that $(GAA)_n$•$(TTC)_n$ tracts stimulate ectopic recombination between repeats on non-homologous chromosomes in an orientation-dependent mechanism [5]. We suggest that these events are likely to be non-reciprocal and, therefore, regulated differently than the RCOs that are the subject of the present study.

In summary, our studies of the properties of $(GAA)_n$•$(TTC)_n$ tracts indicate that they promote genetic instability by several different mechanisms. One mechanism is dependent on the orientation of the repeats and is likely to reflect breakage of replication forks [5]; this mechanism is also associated with small tract contractions/expansion and ectopic recombination events [4,5]. A second mechanism is the orientation-independent large expansion of $(GAA)_n$•$(TTC)_n$ tracts that may involve strand-switching events in which the leading strand copies an Okazaki fragment [6]. The third mechanism is also independent of the orientation and likely reflects DSB formation in G1 to yield RCOs.

Although we have not determined the source of the G1-induced DSBs, they may reflect the action of DNA repair enzymes and/or topoisomerases interacting with secondary structures formed by the tracts. Replication-independent instability has been observed in mammalian cells for both $(GAA)_n$•$(TTC)_n$ and $(CTG)_n$•$(CAG)_n$ tracts [8,10]. This instability appears to be related to DNA repair events associated with transcription [9,10]. In most of the strains examined in our study, the $(GAA)_n$•$(TTC)_n$ tracts were embedded in a promoter-less fragment of the *LYS2* gene.

Other properties of repeat-stimulated gene conversions

The most obvious difference in the patterns of spontaneous RCOs observed previously [15] and those seen in the current study is the location of the events. All of the events observed in the current study are at or near the site of the $(GAA)_n$•$(TTC)_n$ tracts, presumably because all events are initiated at or near the tracts. Although the distribution of spontaneous events observed by Lee *et al.* is not completely random, it is clear that the events can be initiated at many sites within the 120 kb interval (Figure 5). Although the properties of DNA sequences that regulate the probability of initiating a mitotic recombination events have not yet been completely established, mitotic recombination is promoted by closely-spaced inverted repeats [38] and by high rates of transcription [39,40].

The median length of the tract-stimulated conversion events in WXTMD46 (20.3 kb) is longer than those observed for spontaneous events in the absence of the repetitive sequence (6.5 kb) and conversion events generated in G1-arrested cells by gamma radiation (7.3 kb; [17]). The median tract length is much longer than the median length observed associated with RCOs induced by gamma radiation in G2-arrested cells (2.7 kb; [17]).

Most of the conversion tracts are 3:1/4:0 hybrid tracts (Figure 4A). As discussed in the Introduction, such tracts can be explained by independent repair of two DSBs. If the DSBs occur within the GAA•TTC insertions, we expect that the 4:0 region of the hybrid tract should include one or both of the markers flanking the tract, and this expectation is met (Figure 4A). If processing of the broken DNA ends is bidirectional and symmetric from the site of the DSB, most tracts should have a 4:0 region flanked by 3:1 regions. Although we observe this pattern for some of the conversion events, for other events, the 4:0 region is at one end of the hybrid tract. Thus, we infer that the mechanism that generates the gene conversion in mitosis can be asymmetric. In addition, single conversion events can be propagated from the initiation site either toward the centromere or toward the

telomere. Meiotic gene conversion tracts share these properties [41,42].

Two different mechanisms can result in a gene conversion event. During meiotic recombination, most conversion events reflect heteroduplex formation followed by repair of any resulting mismatches. One key early intermediate in this process is a broken end that has been "processed" by 5′ to 3′ degradation on one of the two strands [13]. It is possible that mitotic conversion events involve much more extensive processing than meiotic events or extensive branch migration of the Holliday junction(s) associated with the strand invasion. An alternative possibility is that the conversion events involve the repair of a double-stranded gap [43]. Although there is strong evidence that mitotic events that generate relatively short conversion tracts are a consequence of heteroduplex formation followed by mismatch repair [44,45], it is currently unclear whether the very long tracts are a consequence of mismatch repair or gap repair [15].

In summary, we have demonstrated that $(GAA)_{230}$•$(TTC)_{230}$ tracts strongly stimulate RCOs and our analysis indicates that these events are initiated by a DSB in unreplicated DNA. These results have several implications relevant to the genetic instability observed in patients with Friedreich's ataxia. First, a G1-associated DSB may be an intermediate in the expansion process in at least a sub-set of the expansion events. Second, since we find that the $(GAA)_{230}$•$(TTC)_{230}$ tracts are highly recombinogenic by a mechanism that is independent of DNA replication, our findings may be relevant to the observation that the FRDA-associated tracts are unstable in post-mitotic (non-dividing) cells and these expansions contribute to pathogenesis. For example, the highest rate of somatic instability is observed in dorsal root ganglia, which is the most damaged tissue in FRDA patients [46]. In addition, expanded $(GAA)_n$•$(TTC)_n$ tracts may elevate the frequency of loss of heterozygosity (LOH) on the chromosome containing the expanded tract, allowing heterozygous mutations to become homozygous. Since there are other $(GAA)_n$•$(TTC)_n$ runs within mammalian genomes that are prone to expansions [47], such tracts may also promote LOH on other chromosomes. It would be of interest to examine tissues of FRDA patients or cell lines derived from patients for tract-associated DSBs (using ligation-mediated PCR) or LOH of single-nucleotide polymorphisms located centromere-distal to the expanded tracts.

Materials and Methods

Yeast strains

Most of the experiments involve diploids generated by crosses of haploids with diverged DNA sequences. The haploid strain PSL5 [15] is derived from the YJM789 genetic background whereas PSL2 [15] is derived from W303a [39]. The details of the constructions and genotypes of the haploid and diploid strains are given in Text S1 and Tables S1, S2, S3. The diploids strains used to measure the effect of GAA•TTC tracts on RCOs were homozygous for the *ade2-1* mutation, and heterozygous on chromosome V for *can1-100* and an allelically-placed copy of *SUP4-o*. As described in the text, this system allows the selection of RCOs as Can^R red/white sectored colonies.

Genetic analysis and Southern analyses of replication fork intermediates and tract-associated DSBs

Standard yeast procedures were used for transformations, mating, sporulation, and tetrad dissection [48]. Media were prepared as described previously [15,16]. The two-dimensional gel analysis of replication forks was done as described previously [5]. DNA samples for the gel analysis were treated with the *Afl*II restriction enzyme, and the Southern blot was hybridized to the 3.9 kb *LYS2*-specific *Afl*II fragment isolated from pFL39*LYS2* (described in Text S1). To analyze tract-associated DSBs, we grew haploid strains to stationary phase (three days of growth in rich growth medium [YPD] at 30°C), and then prepared DNA by methods described previously [25]; in the stationary-phase cultures, >95% of the cells were unbudded as expected for cells in G1/G0. Chromosomal DNA molecules were separated using the Bio-Rad CHEF Mapper XA. The Southern analysis was done using a *URA3*-specific probe that was prepared by PCR amplification of genomic DNA with the primers: URA3-f (5′ GGTTCTGGCGAGGTATTGGATAGTTCC) and URA3-r (5′ GCCCAGTATTCTTAACCCAACTGCAC). The hybridization signals were detected and quantitated using a PhosphorImager.

Measurements of frequencies of RCOs

The methods used to quantitate RCOs in various strains were identical to those described previously [15]. In brief, individual colonies formed on rich growth medium were suspended in water, and plated on non-selective medium (omission medium lacking arginine [SD-arg]) or on medium containing canavanine (SD-arg with 120 micrograms/ml canavanine). Plates were incubated at room temperature for four days, followed by storage for one day at 4°C (which accentuates the red color of sectors). The rate of RCOs for each strain was determined by averaging the frequency of crossovers observed in at least 20 independent cultures (colonies).

Mapping of mitotic crossovers and gene conversion events

Red and white Can^R strains were purified from each half of the sectored colonies. DNA was isolated by standard procedures [48]. As we have done previously, we mapped crossovers by examining 34 single-nucleotide polymorphisms (SNPs) located in the 120 kb interval between *CEN5* and the *can1-100/SUP4-o* markers. For each SNP, the DNA from one of the haploid parents contained a diagnostic restriction enzyme recognition that was altered for the other parent. For each SNP, we amplified genomic DNA using primers flanking the heterozygous marker, treated the fragment with the diagnostic restriction enzyme, and examined the products by gel electrophoresis. From this analysis, we could determine whether the sectored colony was homozygous for the YJM789 form of the SNP, homozygous for the W303a form of the SNP, or heterozygous for the polymorphism. The sequence of the primers and restriction enzymes used in the analysis are given in Lee *et al.* [15].

Statistical analysis

Statistical analyses were done using the VassarStats Website (http://faculty.vassar.edu/lowry/VassarStats.html). Most of the comparisons involved the Fisher exact test. 95% confidence limits on the rates of RCOs were calculated by determining the 95% confidence limits on the proportions (number of sectored colonies/number of colonies on non-selective plates) using the Wilson procedure with a correction for continuity. Calculations of median conversion tract lengths and 95% confidence limits on the median were done as described previously [17].

Supporting Information

Figure S1 Patterns of crossovers and conversions resulting in hybrid 3:1/4:0 conversions with apparent crossovers within the tract. As discussed in the text, some of the conversion events associated with RCOs in the WXTMD46 strain (indicated by asterisks in Figure 4) appeared to have a crossover within the

conversion tract. One such example is shown schematically in this figure. In the white sector, the strain was homozygous for the "black" markers A, B, C, and D, and heterozygous for the other markers. The red sector was homozygous for "red" markers A and B, heterozygous for C, homozygous for the "black" markers D and E, and heterozygous for markers F and G. Thus, this sectored colony has a 3:1/4:0 hybrid tract. It appears, however, that there was a crossover between the two 3:1 segments of the tract, since the C marker is homozygous in the white sector, but the E marker is opposite in the opposite sector. This pattern can be explained by the following series of events. Following replication of a chromosome broken in G1, one of the broken chromatids is repaired by an event that converts markers D and E (interaction of chromatids 2 and 3), and is unassociated with a RCO. In the second repair event (involving chromatids 2 and 4), there is a conversion involving markers C and D, associated with an RCO between markers B and C. Segregation of chromatids 1 and 4 into one daughter cell and 2 and 3 into the other would produce the observed segregation pattern of the markers.
Found at: doi:10.1371/journal.pgen.1001270.s001 (0.64 MB TIF)

Table S1 Genotypes of haploid strains. **1** The haploid strains were derived from two different genetic backgrounds: W303a (*MAT*a *ade2-1 leu2-3,112 can1-100 ura3-1 trp1-1 his3-11,15 rad5-535*; [2]) and YJM789 [3]. The genotypes have a complex nomenclature because of the complex manipulations. For example, the term "*V9229::HYG*" indicates that the gene encoding resistance to hygromycin was inserted at *Saccharomyces* Genome Database (SGD) coordinate 9229 on yeast chromosome V. The GAA•TTC tracts were inserted into *lys2* sequences either 4.5 kb or 1.2 kb in size between bases 115902 and 115903 on chromosome V; examples of the nomenclature for these two types of insertion are *V115902::4.5 lys2::*$(GAA)_{230}$ and *V115902::4.5 lys2::*$(GAA)_{230}$, respectively. **2** Additional details concerning the constructions are described in Text S1.
Found at: doi:10.1371/journal.pgen.1001270.s002 (0.09 MB DOC)

Table S2 Primers used in strain constructions or analysis of $(GAA)_{230}$•$(TTC)_{230}$ tract lengths[1]. **1** The use of the primers in construction of yeast strains is described in Text S1. Primers used to diagnose markers in the *CEN5* to *can1-100/SUP4-o* intervals are described in Lee *et al.* (1).
Found at: doi:10.1371/journal.pgen.1001270.s003 (0.06 MB DOC)

Table S3 Genotype of diploid strains. **1** For the diploid genotypes in the strains WXTMD40-WXTMD43, WXTMD44, and WXTMD45, the genes derived from the W303a background are on the top line and those derived from YJM789 are on the bottom line. An SGD coordinate without an insertion (for example, V9229) indicates that one chromosome has no insertion at this position. Other elements of the nomenclature are explained in Table S1.
Found at: doi:10.1371/journal.pgen.1001270.s004 (0.07 MB DOC)

Table S4 Meiotic crossovers on chromosome V in WXTMD46 and PSL101[1]. **1** This table shows the numbers of tetrads in each class of event (first-division segregation [FDS], second-division segregation [SDS], parental ditype [PD], non-parental ditype [NPD], and tetratype [T]) and the calculated map distance in centiMorgans. The strain WXTMD46 has a $(GAA)_{230}$ tract on one copy of V and an $(GAA)_{20}$ tract on the other; these tracts are located in the *URA3* to *can1-100/SUP4-o* interval. The PSL101 is an isogenic derivative lacking the AAG tracts. 2 For markers that are reasonably close to the centromere, gene-centromere distances can be calculated from the segregation patterns of the marker of interest with a tightly-centromere linked on a different chromosome (8). The strains WXTMD46 and PSL101 were heterozygous at the *TRP1* locus, a very tightly-centromere-linked marker. The distance in centiMorgans is approximately half of the percentage of second-division segregation events (8). **3** The numbers of tetrads in each class were used to calculate the map distance by the Perkin's equation (8).
Found at: doi:10.1371/journal.pgen.1001270.s005 (0.05 MB DOC)

Text S1 Friedreich's Ataxia (GAA)n•(TTC)n Repeats Strongly Stimulate Mitotic Crossovers in *Saccharomyces cerevisae*. The details of strain constructions and relevant literature references are included in this section.
Found at: doi:10.1371/journal.pgen.1001270.s006 (0.09 MB DOC)

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Author Contributions

Conceived and designed the experiments: WT ZH KSL HMK VN SMM TDP. Performed the experiments: WT MD PWG ZH HMK VN SMM. Analyzed the data: WT MD PWG ZH KSL SMM TDP. Contributed reagents/materials/analysis tools: WT KSL HMK. Wrote the paper: WT KSL SMM TDP.

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