Low Levels of DNA polymerase Alpha Induce Mitotic and Meiotic Instability in the Ribosomal DNA Gene Cluster of *Saccharomyces cerevisiae*

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Abstract

The ribosomal DNA (rDNA) genes of *Saccharomyces cerevisiae* are located in a tandem array of about 150 repeats. Using a diploid with markers flanking and within the rDNA array, we showed that low levels of DNA polymerase alpha elevate recombination between both homologues and sister chromatids, about five-fold in mitotic cells and 30-fold in meiotic cells. This stimulation is independent of Fob1p, a protein required for the programmed replication fork block (RFB) in the rDNA. We observed that the fob1 mutation alone significantly increased meiotic, but not mitotic, rDNA recombination, suggesting a meiosis-specific role for this protein. We found that meiotic cells with low polymerase alpha had decreased Sir2p binding and increased Spo11-catalyzed double-strand DNA breaks in the rDNA. Furthermore, meiotic crossover interference in the rDNA is absent. These results suggest that the hyper-Rec phenotypes resulting from low levels of DNA polymerase alpha in mitosis and meiosis reflect two fundamentally different mechanisms: the increased mitotic recombination is likely due to increased double-strand DNA breaks (DSBs) resulting from Fob1p-independent stalled replication forks, whereas the hyper-Rec meiotic phenotype results from increased levels of Spo11-catalyzed DSBs in the rDNA.

Introduction

The maintenance of genetic stability during DNA replication is of critical importance. DNA polymerases can stall at DNA lesions such as crosslinks, strand breaks, natural pause sites, and regions that form secondary structures [1,2]. Stalled replication forks are a potential source of genetic instability, because they can be processed to a double-strand break (DSB) [3,4]. Recombination proteins form foci at stalled forks, and homologous recombination (HR) is thought to be one mechanism by which collapsed forks are re-initiated [5,6].

Almost 10% of the *S. cerevisiae* genome is within the rDNA array, a cluster of 150–200 tandemly repeated 9 kb units on the right arm of chromosome XII [7,8]. Each 9 kb unit has a natural replication fork barrier (RFB) site. The RFB prevents replication fork progression in the direction opposite 3'5' transcription, presumably to prevent collisions between DNA and RNA polymerases [9–11]. The Fob1p binds directly to the RFB sequence and is required for replication fork blocking [12]. Double-strand breaks (DSBs) are observed near the RFB site in logarithmically growing cells [13,14] and are a source of genetic instability within the array, leading to high levels of unequal sister-chromatid exchange, unequal gene conversion, and intra-chromatid recombination [15–18]. Cells that lack the Fob1p do not experience fork stalling at the RFB and have reduced mitotic rDNA recombination [14,17–19]. In these studies, the effect of the fob1 mutation on rDNA recombination between homologues was not examined.

In contrast to the relatively high levels of mitotic recombination in the rDNA, meiotic recombination between rDNA arrays on homologous chromosomes is suppressed 70- to 100-fold [8,20]. The mechanism preventing meiotic rDNA recombination between homologs is not yet fully understood. Meiosis-specific DSBs are undetectable in the array [21], and Spo11p, which catalyzes meiotic DSBs, is at low levels within the array [22]. Strains that lack Sir2p have increased Spo11p-associated DSBs in the rDNA [22] and significantly elevated meiotic and mitotic unequal sister-chromatid rDNA recombination [22–24].

In this study, we designed a system that allows us to measure rDNA recombination both between homologues and between sister chromatids. Using this system, we examined the relationship between DNA replication and recombination by investigating mitotic and meiotic rDNA recombination in cells with low levels of Pol1p, the catalytic subunit of the lagging strand DNA polymerase alpha [23]. Reduced levels of Pol1p were previously shown to elevate the rates of translocations, chromosome loss events, microsatellite alterations, deletions and point mutations in non-rDNA regions [26,27]. Below, we show that low levels of Pol1p significantly increase recombination in the rDNA array, both between homologues and between sister chromatids. This increase...
**Author Summary**

In many organisms, the genes that encode the ribosomal RNAs are present in multiple copies arranged in tandem. This unique section of the genome is under strict cellular control to minimize changes in the number of ribosomal DNA (rDNA) genes as a consequence of unequal crossover between repeats. In addition, the rate of unequal crossovers and gene conversion in the rDNA is elevated in mitosis and strongly influence the level of sequence divergence between repeats. Crossovers can result from repair processes initiated at stalled replication forks, and in this study we investigated the effect of a low level of DNA polymerase on rDNA stability. We found that low levels of DNA polymerase modestly increase rDNA recombination in mitosis and strongly elevate rDNA recombination in meiosis.

We suggest that in mitotic cells the increased recombination is likely due to increased double-strand DNA breaks (DSBs) resulting from stalled replication forks. However, in meiotic cells, we found evidence that high levels of recombination result from increased double-strand DNA breaks (DSBs) resulting from stalled replication forks but not within the rDNA (Figure 1).

**Results**

**Low Levels of Pol1p Increase Mitotic rDNA Recombination**

In order to investigate the effect of low Pol1p levels on rDNA instability, we used a diploid strain homozygous for the GAL-POL1 allele, in which the POL1 gene is fused to the GAL1/10 promoter [26]. Low galactose levels (0.005%) induce Pol1p expression at ~10% of the wild-type level, whereas high galactose (0.05%) induces ~900% expression of Pol1p compared to wild-type. For our analysis, we constructed strains containing heterozygous markers surrounding and within the rDNA array. On one homologue, we inserted the URA3 gene (encoding uracil phosphoribosyltransferase) at the centromere-proximal junction of the rDNA. On the other homologue, we inserted the HIP1 gene (encoding hygromycin resistance) centromere-proximal to the rDNA. To determine whether forks blocked at the RFB are the primary source of rDNA instability in cells with low Pol1p, we analyzed mitotic recombination rates in fob1 mutant derivatives of our strains. Previous studies reported decreased extrachromosomal rDNA circle (ERC) formation and an approximately three-fold reduction in internal rDNA marker loss in fob1 mutants [14,17--

Since these two patterns of segregation are equally frequent [28], we calculate that the rate of RCO is twice the frequency of sectoring colonies. Non-reciprocal recombination events (Break-Induced Replication [BIR] [29]) can result in a loss of one marker (for example, URA3 as shown in Figure 2D) and duplication of another (TRP1). Loss of the TRP1 marker by intrachromatid “pop-out” exchange (Figure 2E), single-strand annealing (not depicted), or unequal sister-chromatid exchange (Figure 2F) can also be detected.

To analyze further this type of recombination event responsible for sectoring, we did several types of analysis. First, we purified all sector colonies and, in colonies with Ura⁺/Ura⁻ sectors, we determined whether the Ura⁺ cells had a high rate of 3-fluoroorotate-resistant (3-FOAR) derivatives (indicating that the cells were hyper-recombinogenic for the URA3 insertion) or a had a very low rate of 3-FOAR derivatives (indicating that the cells were hyper-recombinogenic for the insertion). In strains heterozygous for the insertion, a 5-FOAR derivative could arise by loss of the wild-type URA3 allele by a subsequent mitotic crossover or by chromosomal loss. We also subjected each sector side of the colony to tetrad analysis. Some Trp⁺/Trp⁻ sectorial colonies were further analyzed by Southern analysis to determine whether sectoring arose by unequal crossover, intrachromatid recombination, or other events; this analysis is discussed in detail in Text S1. Based on this analysis (summarized in Table 1), we grouped these mitotic events into saw categories: rDNA recombination between homologues and between sister chromatids. In cells with low levels of Pol1p (Figure 3A), we observed a four- to five-fold increase relative to wild-type cells or cells with high levels of Pol1p in both of these categories (p<0.0001 and p = 0.0031, respectively).

**Increased Mitotic rDNA Recombination in Cells with Reduced Pol1p Is Not Fob1p-Dependent**

To determine whether forks blocked at the RFB are the primary source of rDNA instability in cells with low Pol1p, we analyzed mitotic recombination rates in fob1 mutant derivatives of our strains. Previous studies reported decreased extrachromosomal rDNA circle (ERC) formation and an approximately three-fold reduction in internal rDNA marker loss in fob1 mutants [14,17--

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**Figure 1. Positions of heterozygous markers used to monitor rDNA recombination in diploids.** Grey boxes indicate the rDNA array and the black circles show the centromeres. Chromosome XII sequences surrounding the array are not drawn to scale. Positions of BamHI (B) and NgoMIV (N) restriction sites are indicated. BamHI has no sites within the rDNA or within the TRP1 insertion, whereas NgoMIV has no sites within the rDNA but a site within the TRP1 gene. Using CHEF gel analysis of BamHI- or NgoMIV-treated DNA samples, we determined the sizes of the rDNA clusters and the position of insertion of TRP1 within the clusters. We depict the location of the markers in the wild-type diploid AMC45. The sizes of the HPH-containing cluster, the positions of the TRP1 marker within the cluster (from the centromere-proximal junction), and the sizes of the URA3-containing clusters in kb for the other strains are, respectively: 1080, 880, 1110 (GAL-POL1 strain, AMC20); 1170, 940, 920 (fob1 strain, AMC156); 960, 580, 1110 (GAL-POL1 fob1 strain, AMC160).

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We found that the \textit{fob1} mutation alone resulted in a 10-fold reduction, relative to wild-type, in the rate of mitotic rDNA recombination between homologs ($p = 0.011$), but a statistically insignificant ($p = .671$) reduction in sister chromatid recombination (Figure 3B). Surprisingly, we showed that the \textit{fob1} mutation resulted in a significant \textit{increase} in recombination between homologues ($p = 0.027$) in the \textit{GAL-POL1} strain grown on low galactose compared to the level observed in the \textit{FOB1 GAL-POL1} strain grown under the same conditions. The classes of mitotic recombination events in strains with the \textit{fob1} mutation (Table S1) were similar to those shown in Table 1. Thus, the hyper-Rec.
Table 1. Frequencies of sectored colonies of various phenotypes reflecting mitotic recombination in a wild-type strain and in strains with high and low levels of DNA polymerase alpha.

| Phenotype          | High Galα, n = 5156 colonies | Low Pol1p, n = 2303 colonies | Control, n = 5156 colonies |
|--------------------|-------------------------------|------------------------------|-----------------------------|
| CEN12-HPH          | Total                         | BIR initiated on HPH chromatid | BIR initiated on HPH chromatid |
|                    | Number of colonies            | 0                            | 3.6 \times 10^{-1}           |
|                    | CEN12-HPH chromatid Total     | 2                             | 2.13 \times 10^{-1}          |
|                    | Total                         | 1.9 \times 10^{-3}            | 1.42 \times 10^{-3}          |

Following growth of cells in medium containing glucose (AMC45), or raffinose with 0.05% or 0.005% galactose (AMC20), cells were plated on glucose-containing medium (AMC45) or raffinose with 0.05% galactose (AMC20) and allowed to form colonies. These colonies were then replica-plated to medium lacking uracil or tryptophan, or containing hygromycin. The first number in each column is the number of colonies of each sectoring class. The number in parentheses represent the rate of the event. Since we can detect only one half of the reciprocal crossover (RCO) events, the frequency of sectored colonies was doubled to estimate the rate of RCOs (see text for details). The "Total" column shows the total rate of all recombination events occurring between homologues within the rDNA. Some patterns of sectoring are unambiguous in defining the type and position of the exchange. Other patterns yield two Ura+ sectors (Figure 2B); tetrad dissection of the Ura+ sectors (Figure 2A). A RCO between the TRP1 marker and the other sector is is HygS Trp+ (Figure 2C). An RCO between the TRP1 markers results in Ura+ Trp+ spores. A break-induced replication (BIR) event initiated on the HPH-containing chromatid results in a Trp+ spore. The conversion event could be local (deleting only the inserted DNA) or represent a BIR event that occurs distal to the insertion. A BIR event initiated by a break on the HPH-containing chromatid results in the formation of extrachromosomal circles (Figure 3C).

Alteration in the Size of the rDNA Gene Cluster Induced in Cells with Low Pol1p

Unequal sister-chromatid recombination will result in loss of the TRP1 marker only if the crossover occurs between the misaligned insertions (Figure 2F). In contrast, all unequal crossovers or intrachromatid crossovers that alter the size of the rDNA array by 50 kb or more can be detected by CHEF gel electrophoresis of BamHI-treated DNA samples. To determine whether low DNA polymerase alpha resulted in increased size variability of the rDNA, we examined the sizes of rDNA clusters in sub-cultured isolates of the GAL-POL1 diploid grown on plates containing high or low levels of galactose (Figure 4). The rDNA clusters in the initial GAL-POL1 diploid colony were about 1100 kb and 855 kb. We observed slight size variation following two cycles of high galactose subculturing (lanes 3–7). The size variation in cultures subcultured in low galactose (lanes 9–13) was considerably greater, and two of the five colonies had three rDNA clusters, reflecting either chromosome XII trisomy or the presence of sub-populations within the culture with varying array sizes.

The rDNA bands derived from strains subcultured on low galactose were blurry in comparison to those subcultured in high galactose. It is likely that this blurring reflects a very high rate of recombination resulting in small changes in cluster size. We also observed that, in DNA samples isolated from the GAL-POL1 diploids in exponential phase, most of the chromosome XII DNA molecules were retained in the well of the gel rather than migrating in the normal position (Figure S1). Since branched DNA molecules remain in the well of CHEF gels, it is likely that the observation indicates that GAL-POL1 strains have increased levels of DNA replication and/or recombination intermediates. Using two-dimensional gel electrophoresis, Zou and Rothstein [30] showed that certain mutants of DNA polymerases alpha and delta resulted in increased levels of an rDNA structure (termed ‘‘xDNA’’) that had the properties expected for a recombination intermediate.

Low Pol1p Dramatically Increases Meiotic rDNA Recombination

Meiotic recombination between rDNA clusters on homologous chromosomes is greatly suppressed. Although the rDNA is about 10% of the genome and the yeast genome has a genetic length of about 4200 M, the rDNA cluster is only 2.5 M in length [8,20]. Unequal sister-chromatid meiotic recombination is less suppressed, with loss of an internal marker occurring in up to 10% of tetrads [31]. To evaluate the effect of Pol1p levels on meiotic rDNA recombination, we sporulated the wild-type and GAL-POL1 strains...
on plates containing either high or low galactose. Tetrads were dissected and scored for parental diatype (PD), non-parental diatype (NPD) and tetrateype (T) for three intervals: HPH-TRP1, TRP1-URA3, and HPH-URA3. The genetic distances between markers were calculated by standard procedures [32] and are shown in Table 2. As expected, recombination in the rDNA in wild-type cells was extremely low, 1 cM for the entire cluster (HPH-URA3 interval). Since the difference in recombination rates between the wild-type cells sporulated in high and low levels of galactose was not significant, the data were combined.

In contrast, the genetic distance between HPH and URA3 was increased to 28 cM in cells with low Pol1p (p<.0001). In the equation used to calculate map distances, in two-point crosses, NPD events are assumed to reflect four-strand double meiotic crossovers between markers [32]. For a two-point cross, however, an NPD event could also be a consequence of a mitotic crossover prior to meiosis. In general, the frequency of mitotic crossovers was lower than the observed frequency of meiotic NPD events, suggesting that at least some of the NPD tetrads reflect double meiotic crossovers. For example, in the strain with low levels of alpha DNA polymerase for the HPH-TRP1 interval, we observed a rate of mitotic crossovers of 1% (Table 1), whereas the rate of NPDs for the same interval was 6% (Table 2). In addition, as will be discussed further below, in analyzing the HPH-URA3 interval, we detected NPD tetrads that had one crossover in the HPH-TRP1 interval and a second crossover in the TRP1-URA3 interval, demonstrating that some NPD tetrads reflect meiotic exchanges. Nonetheless, we also calculated map distances for the three intervals excluding all of the NPD tetrads that could represent mitotic crossovers (values shown in parentheses in Table 2). Even with this conservative assumption, the genetic distance in the rDNA cluster is more than 10-fold elevated in the strain with low levels of alpha polymerase compared to the wild-type (p<.0001).

The genetic distances in the rDNA in cells with low Pol1p were not additive since the HPH-TRP1 distance is 30 cM, the TRP1-URA3 is 12 cM, and the HPH-URA3 distance is only 28 cM. There are two likely interpretations of this non-additivity. First, as described above, some of the NPD tetrads used in calculated the HPH-TRP1 and TRP1-URA3 distances may reflect mitotic crossover events. Second, since the equation used to calculate map distance [32] is based on the assumption that the interval examined has two or fewer crossovers, map distances for intervals that have more than two crossovers are underestimated. We observed four tetrads from low Pol1p cells that had marker segregation patterns consistent with triple crossovers surrounding TRP1; no such tetrads were found in wild-type cells or in GAL-POL1 cells sporulated on high galactose.

We also noted a significant increase in the HPH-URA3 distance in GAL-POL1 cells sporulated on high galactose relative to the wild-type strain, 11 and 1 cM, respectively (p<.0001). Pol1p is overexpressed about three-fold relative to wild-type under these conditions [26]. It is possible that the overexpression of this single unit of DNA polymerase alpha complex perturbs its assembly. We previously observed that overexpression of Pol1p resulted in elevated levels of chromosome rearrangements and chromosome loss [26].

We detected an elevation of the frequency of tetrads with one Trp+ and three Trp− spores (instead of the expected 2:2 marker segregation) in the GAL-POL1 strain. Loss of the TRP1 insertion can occur by unequal crossing-over between sister chromatids, intra-chromatid recombination, and gene conversion (either between homologues, or unequally between sister chromatids). Of the 280 four-spore tetrads from GAL-POL1 cells sporulated on high galactose, 20 had one Trp+ to three Trp− spores (7%). In GAL-POL1 cells sporulated on low galactose, this level was 25% (51 out of 204 tetrads). We observed only one tetrad that had three Trp+ to one Trp− spore, and this tetrad was from GAL-POL1 cells sporulated on low galactose. This bias toward TRP1 marker loss indicates that the majority of these events are intrachromatid events (for example, unequal crossovers between sisters), rather
Hyper-Recombination in rDNA

Increased Meiotic rDNA Recombination in Strains with Low Alpha DNA Polymerase Is Independent of the Fob1p-Dependent Replication Block

If the increased meiotic rDNA recombination in cells with low Pol1p is initiated from DSBs at the RFB site, we would expect the fob1 mutation to reduce this recombination. Instead, we observed the opposite: the rate of recombination in cells that lack Fob1p and that have low Pol1p is significantly greater than observed in cells with only low Pol1p (Table 3), with a total HPH-URA3 frequency of 0.04. In a sample of 134 tetrads examined in the GAL-POL1 strain, we expect five DCOs in the HPH-URA3 interval. We observed ten (Table S2). This calculation confirms that the crossovers observed in the rDNA in the GAL-POL1 strain with low levels of alpha polymerase have no interference or negative interference.

Cells with Reduced Pol1p Have No Crossover Interference in the rDNA

In S. cerevisiae, as in most other eukaryotes, crossovers in one region reduce the probability of a nearby crossover [33]. In organisms in which tetrad analysis is possible, interference can be calculated in two-point crosses by analyzing the relative frequencies of PD, NPD, and T tetrads [34]; modifications introduced by

Stahl [35]; also, http://molbio.uoregon.edu/~fstahl/). For most genetic intervals in S. cerevisiae, NPD tetrads (representing four-strand double crossovers) are significantly less frequent than expected on the basis of the number of T tetrads (representing single crossovers as well as certain types of double crossovers). We used two procedures to calculate the expected number of NPD tetrads. First, using our observed numbers of PD, T, and NPD tetrads, we calculated the expected number of NPD tetrads by a direct application of the equation described in the Stahl Web site (NPD exp in Table 2). We then used a chi-square analysis to compare the observed and expected numbers of tetrads in all three classes, converting the chi-square value to a p value (p in Table 2). We also calculated the degree of interference as 1−(NPDobserved/NPDexpected). For all three intervals in the strain with low levels of alpha DNA polymerase, interference was negative, suggesting that a crossover in one region of the rDNA increases the probability of a second crossover in the rDNA. This effect is specific to the rDNA, as a non-rDNA LYS2-TYR1 interval on chromosome II has significant crossover interference in both the wild-type and GAL-POL1 strains (Table 2).

Because (as discussed above), NPD tetrads in two-point crosses can reflect a mitotic exchange rather than two meiotic crossovers, we also examined interference in a more traditional way. From Table 2, we calculated that the frequency of tetratype tetrads (mostly representing single crossovers) in the HPH-TRP1 interval in the GAL-POL1 strain with low levels of DNA polymerase is about 0.25 (32 of 126 tetrads, excluding NPD tetrads from the total). The frequency of tetratype tetrads in the TRP1-URA3 interval is 0.15. Thus, the expected frequency of tetrads with crossovers in both intervals (assuming no interference) is 0.04. In a sample of 134 tetrads examined in the GAL-POL1 strain, we expect five DCOs in the HPH-URA3 interval. We observed ten (Table S2). This calculation confirms that the crossovers observed in the rDNA in the GAL-POL1 strain with low levels of alpha polymerase have no interference or negative interference.

than “classic” gene conversion events. We did not observe TRP1 marker loss in any tetrads from wild-type cells (total of 362 four-sporule tetrads). This result differs from an earlier report in which an internal rDNA marker was lost in ~10% of wild-type tetrads [31]. This variation may be due to differences in strain background or in the location of the inserted marker.

To clarify whether reduced Pol1p resulted in elevated meiotic crossovers in non-rDNA regions of the genome, we also investigated meiotic crossovers in a non-rDNA interval on chromosome II, between LYS2 and TYR1. The LYS2-TYR1 genetic distance in our wild-type strain is 32 cM, in agreement with the 35 cM average distance between these loci reported in the Saccharomyces Genome Database. In cells with low Pol1p, the distance between these markers was 41 cM (Table 2). Although this increase relative to wild-type was statistically significant (p = 0.02 by a Chi-square test), it is far less dramatic than that observed within the rDNA.

Figure 4. CHEF gel analysis of the size of rDNA arrays in cells with high and low levels of polymerase alpha. Individual colonies of a GAL-POL1 strain were sub-cultured twice (about 50 cell divisions) in media with high or low levels of galactose. We subsequently isolated DNA from these derivatives in agarose plugs, treated the plugs with BamHI (which does not cleave in the rDNA), and separated the resulting fragments by CHEF gel electrophoresis. The separated DNA molecules were transferred to membranes and hybridized to an rDNA-specific probe. The derivatives in each lane were: 1 (GAL-POL1 strain before subculturing), 2 (GAL-POL1 derivative sub-cultured once on high galactose), 3–7 (five individual derivatives sub-cultured twice on high galactose), 8 (GAL-POL1 strain sub-cultured once on low galactose), 9–13 (five individual isolates sub-cultured twice on low galactose).

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Increased Spo11p-Mediated Cleavage and Decreased Sir2p Binding of rDNA in Meiotic Cells with Low Pol1p

Meiotic recombination is initiated in S. cerevisiae by Spo11p-dependent DSBs [29]. The number of Spo11p-dependent DSBs in the rDNA is low, as expected based on the genetic data [22]. The hyper-Rec phenotype associated with low DNA polymerase could reflect either Spo11p-independent DSBs (perhaps generated during the meiotic S-period) or increased Spo11p-dependent DSBs. To distinguish between these two possibilities, we sporulated our strains under low-galactose conditions and used chromatin immunoprecipitation to purify Spo11p-associated DNA, followed by quantitative real-time PCR analysis. Since the chromatin immunoprecipitation experiments were done without formaldehyde treatment of the chromatin, these experiments monitor the covalent attachment of Spo11p to target DNA, reflecting Spo11p catalyzed DSBs. In both POL1 and GAL-POL1 cells, Spo11p-catalyzed DSBs were at same level at HIS4, a previously identified hotspot for Spo11p-mediated DSBs [36], with

Table 3. Meiotic segregation patterns in strains with the fob1 mutation.

| Interval | Strain       | PD | T  | NPD | cM  | NPD exp* | p   | Int |
|----------|--------------|----|----|-----|-----|----------|-----|-----|
| HPH-TRP1 | POL1 fob1    | 460| 22 | 0   | 2   | 0        | nd  | nd  |
|          | POL1 fob1, High Gal | 218| 22 | 0   | 5   | 0        | nd  | nd  |
|          | GAL-POL1 fob1, Low Gal | 125| 36 | 3   | 17 (11) | 1.4 | 0.15 | −1.1 |
| TRP1-URA3| POL1 fob1    | 463| 19 | 0   | 2   | 0        | nd  | nd  |
|          | GAL-POL1 fob1, High Gal | 203| 35 | 2   | 10 (7) | 0.8 | 0.17 | −1.5 |
|          | GAL-POL1 fob1, Low Gal | 113| 43 | 8   | 28 (13) | 2.8 | .001 | −1.9 |
| HPH-URA3 | POL1 fob1    | 445| 37 | 0   | 4   | 0        | nd  | nd  |
|          | GAL-POL1 fob1, High Gal | 192| 45 | 3   | 13 (11) | 1.4 | 0.15 | −1.1 |
|          | GAL-POL1 fob1, Low Gal | 86 | 61 | 17 | 50 (30) | 7.8 | <.0001 | −1.2 |

Tetrads were examined from AMC156 (POL1 fob1) and AMC160 (GAL-POL1 fob1) sporulated on plates containing either high or low galactose levels. Column headings, abbreviations, and methods of analysis are identical to those in Table 2.

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no significant difference between these strains ($p = .487$). There is an approximately 4-fold increase in Spo11p-associated rDNA in cells with low Pol1p compared to wild-type cells ($p = .0039$) (Figure S2 A–C). Thus, low levels of Pol1p disrupt mechanisms required for suppression of Spo11p entry into the rDNA array, leading to increased Spo11p-catalyzed DSBs.

Loss of the histone deacetylase Sir2p results in elevated rates of unequal meiotic [23] and mitotic unequal crossing over in the rDNA, and increased levels of Spo11p cleavage in the rDNA in mitotic cells [22]. We used quantitative real-time PCR of immunoprecipitated meiotic DNA to measure Sir2p in the GAL-POL1 strain sporulated in low levels of galactose. In logarithmically-growing cells, there are two sites of Sir2p binding in each rDNA unit, one near the RFB site, and the other at the 5′ end of the 35S transcript [37]. We found that there is a significant decrease in Sir2p bound near the RFB site in GAL-POL1 meiotic cells as compared to wild-type meiotic cells ($p = .022$) (Figure S2D). We also investigated Sir2p binding in logarithmically-growing cells with low Pol1p; we did not find a significant decrease in the level of Sir2p (data not shown). Lastly, by chromatin immunoprecipitation, we looked for an alteration in the binding of the cohesin subunit Mcd1p in vegetative wild-type cells and in cells with low Pol1p. We found no significant difference (data not shown).

**Discussion**

We show that reduced levels of DNA polymerase alpha result in elevated mitotic recombination and greatly elevated meiotic recombination within the yeast rDNA. Unlike most previous studies of rDNA recombination, we used markers within and flanking the rDNA, allowing us to quantitate both sister-chromatid and homologue recombination. As described below, we suggest that the hyper-Rec phenotypes resulting from low alpha DNA polymerase in mitosis and meiosis reflect two fundamentally different mechanisms.

**Mitotic rDNA Recombination**

The rate of reciprocal mitotic crossovers (RCOs) between homologues in the 120 kb CEN3–CAG1 interval of chromosome V is about $4 \times 10^{-3}$ per cell division [38]. Assuming this rate is representative of mitotic recombination throughout the genome, we would expect the rate of RCOs in the rDNA, which is about ten times larger than the CEN5–CAG1 interval, to be approximately $4 \times 10^{-4}$ per cell division. Since we observe a rate of RCOs of about $3 \times 10^{-3}$ per cell division in the wild-type strain, mitotic crossovers in the rDNA are not suppressed and, in fact, appear somewhat elevated relative to non-rDNA sequences. In cells with low Pol1p, rDNA recombination between both clusters on homologues and clusters on sister chromatids was increased about five-fold. To determine whether stalling of replication forks at the RFB site is responsible for the elevated rDNA recombination, we examined strains that lacked the RFB-binding Fob1p. Although loss of Fob1p reduces the hyper-Rec rDNA phenotype associated with sgs1 and dna2 helicase mutants [13,39–41], the fob1 mutation did not decrease mitotic recombination in our strains with low polymerase. We also directly compared the level of DSBs at the RFB in wild-type and low Pol1p strains, and found no difference.

In previous studies, an elevated level of unequal sister-strand mitotic recombination in the rDNA was observed in strains lacking Sir2p [14,23]. It is unlikely that the hyper-Rec effect of low alpha polymerase in mitotic cells reflects a reduction in the level of Sir2p for several reasons. First, loss of Sir2p specifically elevates rDNA recombination [23] but, as discussed below, loss of alpha polymerase elevates recombination in other regions of the genome. Second, the hyper-Rec phenotype caused by the sir2 mutation is dependent on Fob1p [14], unlike the hyper-Rec phenotype resulting from low DNA polymerase alpha. Third, Kobayashi et al. [14] showed that intragenic recombination within a single rDNA gene was not elevated in sir2 strains, although unequal sister-strand recombination was elevated. These researchers also found a defect in the level of the cohesin subunit Mcd1p in sir2 strains and suggested that the loss of sister-strand cohesion in sir2 strains led to elevated levels of unequal sister-strand recombination without an elevated level of recombinogenic lesions. Finally, we failed to see any effect of low alpha polymerase on the level of Sir2p binding in the rDNA in mitotic cells by chromatin immunoprecipitation experiments.

Although the hyper-Rec phenotype in our experiments is not correlated with elevated DSBs at the RFB, we suggest that the hyper-Rec phenotype is likely to reflect elevated DNA lesions (perhaps distributed randomly) based on several arguments. First, we found an elevated rate of RCO in an interval of chromosome XII (CEN12–HPH, Table 1) that does not contain rDNA, although only a small number of events were detected. Second, strains with low levels of alpha polymerase are hyper-Rec in non-rDNA regions; mitotic recombination in the CEN5–CAG1 interval is elevated about twenty-fold by low alpha DNA polymerase [26]. A general hyper-Rec phenotype is associated with mutations affecting many components of the DNA replication system [reviewed by [42]]. Third, an elevated level of DSBs in strains with low alpha DNA polymerase was physically demonstrated at a fragile site on chromosome III [26]. Fourth, increased levels of Holliday junctions, presumably representing repair of DNA lesions, are observed in the rDNA of polymerase alpha mutants [30]. Fifth, in analyzing intact chromosomal DNA samples by CHEF gels, chromosome XII was often trapped in the wells, characteristic of the behavior of branched DNA molecules. Strains that lack Rrm3 also have elevated levels of rDNA recombination and chromosome XII molecules that are trapped in the gel wells [43,44].

**Meiotic rDNA Recombination**

Low levels of Pol1p very substantially increase meiotic recombination in the rDNA between homologues and sister-chromatids. We also observe a small, but significant, elevation of recombination in the L152–TRY1 interval (Table 1). The much greater stimulation of recombination in the rDNA and the observed increase in Spo11p-mediated DSBs in the rDNA in strains with low levels of DNA polymerase argue that the stimulation is not primarily a consequence of DSBs associated with problems with DNA replication. The stimulation is also independent of Fob1p. In contrast to its effect in mitosis, loss of Fob1p results in increased rather than decreased meiotic recombination in the rDNA. Fob1p is involved in the recruitment of Sir2p to the rDNA [45]. Since we found that strains with low alpha DNA polymerase have somewhat reduced meiotic levels of Sir2p in the rDNA, two different methods of reducing the concentration of Sir2p in the nucleus result in a hyper-Rec meiotic phenotype.

Based on our observations and those of others, a relatively simple model can be proposed. A reduction in the level of Sir2p in the rDNA results in a reduction in the level of the Pch2p. San-Segundo and Roeder [24] showed that Sir2p was required for the localization of Pch2p to the nucleolus and pch2 mutants had elevated rates of meiotic recombination. These researchers also showed that Pch2p excludes Hop1p from the nucleolus. Since hop1 strains have reduced levels of Spo11p-catalyzed DSBs [46], increased entry of Hop1p into the nucleolus would be expected to elevate Spo11p-induced DSBs.
There are several alternative explanations of our data. First, breaks in the rDNA of cells with low Pol1p during meiotic S-phase may be re-located outside the nucleolus for repair, and during this time of re-location, Spo11p-induced DSBs could be formed [47]. Second, DNA lesions (for example, single-strand nicks) in the rDNA in cells with low polymerase may recruit the Mre11p/Rad50p/Xrs2p complex that subsequently associates with Spo11p [48], resulting in an increased level of Spo11p-catalyzed DSBs. Finally, we cannot rule out the possibility that some of the meiotic recombinogenic lesions are a consequence of DNA lesions resulting from low DNA polymerase during meiotic recombination that are independent of Spo11p.

In summary, we suggest that *fob1* mutations have different recombination phenotypes in mitosis (hypo-Rec) and meiosis (hyper-Rec) because of the different effects of Sir2p. In mitosis, the primary recombination-related role of Sir2p is to help maintain sister-chromatid cohesion and loss of Sir2p results in elevated unequal sister-strand recombination. In meiotic recombination, Sir2p acts to prevent recombination-stimulating proteins such as Hop1p and Spo11p from entering the nucleolus and, consequently, loss of Sir2p elevates meiotic recombination.

These explanations leave two important questions unanswered. First, why does a low level of alpha DNA polymerase reduce Sir2p binding in the rDNA of meiotic cells? Second, why does low alpha DNA polymerase reduce Sir2p binding in meiotic, but not mitotic cells? Although we cannot provide definitive answers to either of these questions, it is possible that the role of Pol1p in chromatin assembly is relevant. Pol1p acts to prevent recombination-stimulating proteins such as Hop1p and Spo11p from entering the nucleolus and, consequently, loss of Sir2p elevates meiotic recombination.

Chromatin Immunoprecipitation

Immunoprecipitation and real-time PCR experiments were performed using the pYrr1G12 rDNA plasmid [38] with EcoRI. For Southern analysis of DSBs at the RFB (rDNA probe 3′), we performed PCR of genomic DNA with the primer pair: 5′ GTTAAGCAGTCCATTATG 3′ and 5′ TAGTTAACAGGACATGGCC 3′. Probes were labeled by random-prime labeling using Ready-To-Go DNA Labeling Beads (GE Healthcare). Southern hybridization and washing were standard. Membranes were exposed to a PhosphoImager screen for one to three days. Images were captured with a Typhoon imager (GE Healthcare) and quantification was performed using Quantity One analysis software (BioRad).

Statistical Analyses

Calculations of 95% confidence intervals and *p*-values using Chi Square and Fisher’s Exact tests were done using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). The number of NPDs expected in each interval was calculated using the Stahl Lab Online Tools “Better Way” calculator (http://www.mobio.uoregon.edu/~fstahl/ and also [35]). This calculation was used
instead of the traditional NPD = 1/2[1–T–(1–3T/2)/2/3] [34], because in the Papazian equation, the expected number of NPDs cannot be calculated when T exceeds 2/3. In the L1S2-TYR1 interval, T in some strains exceeds 2/3. The “Better Way” calculator is better for all values of T and can be applied when T>2/3.

Supporting Information

**Figure S1** Under-representation of chromosome XII in a strain with low levels of DNA polymerase alpha. (A) Analysis of chromosome migration by CHEF gel separation of genomic DNA from early log-phase cultures of wild-type cells in YPD (lane 1) and GAL-POL1 cells in YPR with low galactose (lane 2). (B) Southern blot of the gel shown in (A) with an rDNA-specific probe. Found at: doi:10.1371/journal.pgen.1000105.s001 (0.45 MB TIF)

**Figure S2** ChIP analysis of Sir2p- and Spo11p-associated DNA in a wild-type strain and a strain with low levels of Pol1p. (A) Location of primer sets within the rDNA used for real-time PCR analysis of chromatin immunoprecipitations; primers used to generate the PCR products are in Supp. Table 5. A single rDNA unit is shown; PCR products are a selection of those published by Huang and Moazed [5] and are indicated by black horizontal bars. (B) and (C) Spo11p-associated DNA immunoprecipitated from wild-type (POL1) and GAL-POL1 strains sporulated in low galactose was quantified by real-time PCR. Spo11p binding at HIS3 (a known hotspot for Spo11p binding) and at rDNA location 19 were quantified relative to Spo11p binding at the CUP1 locus. Error bars represent the 95% confidence intervals. (D) Sir2p-associated DNA was immunoprecipitated from wild-type (POL1) and GAL-POL1 strains sporulated in low galactose. The binding was quantified by real-time PCR, relative to binding at rDNA location 3-3. Error bars represent the 95% confidence intervals. Found at: doi:10.1371/journal.pgen.1000105.s002 (0.33 MB TIF)

**Table S1** Frequencies of sectored colonies of various phenotypes reflecting mitotic recombination in a fob1 strain and in a fob1 strains with high and low levels of DNA polymerase alpha. Colonies of AMC156 (fob1) and AMC157 (fob1 GAL-POL1), grown on rich medium, were replica-plated to medium lacking uracil or tryptophan, or containing hygromycin. As in Table 1, the patterns of sectoring and other types of analysis (primarily tetrad analysis of the sectors) were used to classify the different types of recombination events. A full discussion of this classification is in the Table 1 legend. Found at: doi:10.1371/journal.pgen.1000105.s003 (0.03 MB DOC)

**Table S2** Numbers of two-, three-, and four-strand meiotic double crossovers (DCOs) in tetrad with a crossover in the HYH-TRP1 and TRP1-URA3 intervals. Found at: doi:10.1371/journal.pgen.1000105.s004 (0.03 MB DOC)

**Table S3** Haploid strain genotypes and constructions. *All strains were derived from MS71 (Δ ade5-1 his7-2 ura3-32 nap1-289) by transformation or crosses with isogenic strains. Only those markers that differ from the genotype of MS71 are shown. Some of our strains contain insertion of drug-resistant markers at genomic locations that are not within genes. For such markers, we indicate the chromosome containing the insertion and the SGD coordinate at the position of the insertion. For example, the marker XIH451250:HPH represents an insertion of the hygromycin-resistance gene on chromosome XII next to base 451250. **Strains constructed by transformation were made using PCR fragments to the targeted location. The template for PCR amplification is indicated. Primer sequences used in strain construction are shown with upper case letters corresponding to the targeted genomic regions and lower case letters corresponding to the selectable marker on the plasmid. Found at: doi:10.1371/journal.pgen.1000105.s005 (0.08 MB DOC)

**Table S4** Diploid strain genotypes and constructions. *All strains were derived by crosses of haploids that are isogenic with MS71 (described in Supp. Table 4). Only those markers that differ from the genotype of MS71 are shown. An illustration of the nomenclature for a diploid heterozygous for an insertion of a drug-resistant marker is: XIH451250:HPH/ XII451250. In this strain, one chromosome had an insertion of the HPH gene on chromosome XII at base 451250 (SGD coordinates) and the other chromosome did not. Found at: doi:10.1371/journal.pgen.1000105.s006 (0.03 MB DOC)

**Table S5** Primers for real-time PCR. Found at: doi:10.1371/journal.pgen.1000105.s007 (0.04 MB DOC)

**Text S1** Low levels of DNA polymerase alpha induce mitotic and meiotic instability in the ribosomal DNA gene cluster of Saccharomyces cerevisiae. Found at: doi:10.1371/journal.pgen.1000105.s008 (0.05 MB DOC)

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Author Contributions

Conceived and designed the experiments: AC TP. Performed the experiments: AC PM MG. Analyzed the data: AC TP. Wrote the paper: AC TP.

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