Formation of Complex and Unstable Chromosomal Translocations in Yeast

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Abstract

Genome instability, associated with chromosome breakage syndromes and most human cancers, is still poorly understood. In the yeast Saccharomyces cerevisiae, numerous genes with roles in the preservation of genome integrity have been identified. DNA-damage-checkpoint-deficient yeast cells that lack Sgs1, a RecQ-like DNA helicase related to the human Bloom’s-syndrome-associated helicase BLM, show an increased rate of genome instability, and we have previously shown that they accumulate recurring chromosomal translocations between three similar genes, CAN1, LYP1 and ALP1. Here, the chromosomal location, copy number and sequence similarity of the translocation targets ALP1 and LYP1 were altered to gain insight into the formation of complex translocations. Among 844 clones with chromosomal rearrangements, 93 with various types of simple and complex translocations involving CAN1, LYP1 and ALP1 were identified. Breakpoint sequencing and mapping showed that the formation of complex translocation types is strictly dependent on the location of the initiating DNA break and revealed that complex translocations arise via a combination of interchromosomal translocation and template-switching, as well as from unstable dicentric intermediates. Template-switching occurred between sequences on the same chromosome, but was inhibited if the genes were transferred to different chromosomes. Unstable dicentric translocations continuously gave rise to clones with multiple translocations in various combinations, reminiscent of intratumor heterogeneity in human cancers. Base substitutions and evidence of DNA slippage near rearrangement breakpoints revealed that translocation formation can be accompanied by point mutations, and their presence in different translocation types within the same clone provides evidence that some of the different translocation types are derived from each other rather than being formed de novo. These findings provide insight into eukaryotic genome instability, especially the formation of translocations and the sources of intrachromosomal heterogeneity, both of which are often associated with human cancers.

Introduction

Structural changes to chromosomes, such as translocations, terminal fusions, insertions, inversions or deletions, are often detrimental to normal cell proliferation and are commonly associated with cancers, accelerated aging and genomic disorders [1,2,3]. They are thought to result from non-homologous endjoining (NHEJ) of double-strand breaks (DSBs) or from erroneous homologous recombination (HR) between dispersed, nonallelic repeats (NAHR). HR events are initiated by 3’ end invasion of identical duplex DNA, normally on a homologous chromosome or a sister chromatid or, accidentally, nonallelic sequences. Break-induced replication (BIR) has been invoked as an HR mechanism for the repair of one-sided DSBs that may arise when a replication fork collapses at a nick in the template or when telomeres erode [4,5,6,7]. BIR is a Rad52-dependent mechanism and requires long homology for successful strand invasion; however, BIR requiring only microhomology has recently been proposed as a mechanism for generating copy number variation in the human genome [8]. In addition, recent evidence from yeast suggests that broken replication forks may also be substrates for an HR-protein independent, replication-based template-switching mechanism that is mediated by microhomology or microsatellites [9]. Despite these recent advances, genetic and mechanistic understanding of the causes of genome instability in model organisms as well as in human genome instability syndromes and cancer is still lacking. With the identification of numerous genes and gene networks that are required for the maintenance of genome stability, including DNA damage checkpoints, DNA repair factors, proteins for processing of recombination substrates, as well as components of chromatin assembly factors, the budding yeast Saccharomyces cerevisiae has emerged as a model organism for the study of genome instability [10,11,12,13,14,15,16,17,18,19]. Members of the RecQ family of DNA helicases have been recognized as important regulators of genome integrity from bacteria to humans (reviewed in [20]). Yeast cells lacking the RecQ-like helicase Sgs1 accumulate gross-chromosomal rearrangements (GCRs), exhibit elevated levels of mitotic recombination, have a reduced lifespan and are sensitive to chemicals that alkylate DNA or slow replication forks [14,20,21,22,23,24]. In vitro, Sgs1 is capable of unwinding various DNA substrates, but prefers Holliday junctions, consistent with its proposed role in recombination [25]. Sgs1 has also been shown to facilitate formation of the 3’ overhang during the processing of DSBs in preparation for
Materials and Methods

Identification of Translocations Involving CAN1, LYP1 and ALP1

Clones with spontaneous gross-chromosomal rearrangements (GCRs) that originate in a 12-kb nonessential region of chromosome V, which contains CAN1, were obtained exactly as previously described [34]. To identify GCR clones with translo-

Table 1. Saccharomyces cerevisiae strains used in this study.

| Strain number | Genotype |
|---------------|----------|
| KHSY1530      | MATa, ura3-52, trp1Δ63, his3Δ1200, leu2Δ11, lys2Δ8gl, hom3Δ10, ade2Δ1, ade8Δ, hxt13::URA3, sgs1::TRP1, mec3::HIS3 |
| KHSY2098      | MATa, ura3-52, trp1Δ63, his3Δ1200, leu2Δ11, lys2Δ8gl, hom3Δ10, ade2Δ1, ade8Δ, hxt13::URA3, sgs1::TRP1, mec3::HIS3, alp1::loxP |
| KHSY2147      | MATa, ura3-52, trp1Δ63, his3Δ1200, leu2Δ11, lys2Δ8gl, hom3Δ10, ade2Δ1, ade8Δ, hxt13::URA3, sgs1::TRP1, mec3::HIS3, alp1::loxP, ALP1::loxP |
| KHSY2612      | MATa, ura3-52, trp1Δ63, his3Δ1200, leu2Δ11, lys2Δ8gl, hom3Δ10, ade2Δ1, ade8Δ, hxt13::URA3, sgs1::TRP1, mec3::HIS3, alp1::loxP, ALP1::loxP, hom3::HIS3, ade8::HIS3, ade2::HIS3 |
| KHSY3114      | MATa, ura3-52, trp1Δ63, his3Δ200, leu2Δ11, lys2Δ8gl, hom3Δ10, ade2Δ1, ade8Δ, hxt13::URA3, sgs1::TRP1, mec3::HIS3, lys1-MUTABCDEF::loxP |

Strand invasion [26]. In humans, lack of function of the RecQ-like DNA helicases BLM, WRN and RECQL4 is associated with Bloom’s, Werner and Rothmund-Thompson syndromes, respectively, which are characterized by chromosome abnormalities, increased cancer susceptibility and/or signs of premature aging [27,29,29]. Not unlike yeast cells lacking Sgs1, cells from Bloom’s syndrome patients exhibit aberrant and/or elevated levels of genetic exchange and chromosome instability. The most striking characteristics of cells from Bloom’s syndrome patients include elevated rates of sister-chromatid exchange, chromatid gaps, micronuclei and quadriradial structures [30,31]. In an ongoing effort to elucidate genetic and mechanistic determinants of chromosome instability in yeast, we previously identified various types of complex, recurring translocations between three homologous genes in yeast cells that lack Sgs1 and the DNA-damage sensor Mec3 [18]. A candidate screen revealed that deletion of other checkpoint components (Tel1, Rif5, Rad24) or deletion of chromatin assembly factors (Cac1, Asf1) also made sgs1Δ mutants susceptible to these recurring translocations [19]. We determined that these translocations originate in the CAN1 gene on chromosome V and target short stretches of identical sequences in the related genes LYP1 and/or ALP1 on chromosome XIV, which share 60-65% sequence identity with each other and with CAN1. Using the highly susceptible sgs1Δ mec3Δ mutant as a source for transfections, the goal of the present study was to gain insight into how the various simple and complex translocations between CAN1, LYP1 and ALP1, and possibly chromosomal translocations in general, are formed. For this purpose, we manipulated the location, copy number and level of sequence similarity of the translocation targets ALP1 and LYP1 and determined the effect of these changes on the accumulation, structure and stability of the translocation chromosomes. We find that complex, multipartite translocations only form if sequences of sufficient similarity are available on the same chromosome for template-switching, whereas translocation formation involving two successive interchromosomal rearrangements were not observed. Rather than giving rise to inviable cells, dicentric chromosomes provide a continuous source for new viable translocations and show signs of ongoing instability that leads to chromosome end erosion. Point mutations and DNA slippage events that accompany some rearrangements give further insight into the origin of stable translocations.

Yeast Strains, Plasmids and Media

All strains used in this study are derived from Saccharomyces cerevisiae strain S288C and are listed in Table 1. For GCR rate measurements, desired gene deletions were introduced into KHSY802 (MATa, ura3-52, trp1Δ63, his3Δ200, leu2Δ11, lys2Δ8gl, hom3Δ10, ade2Δ1, ade8Δ, hxt13::URA3, RDKY5027 [MAT2, ura3-52, trp1Δ63, his3Δ200, leu2Δ11, lys2Δ8gl, hom3Δ10, ade2Δ1, ade8Δ, hxt13::URA3]) by HR-mediated integration of PCR products by the LiAc method [32]. All haploid strains for GCR rate measurements were obtained by sporulating diploids heterozygous for the desired mutations. Spores were genotyped on selective media or by PCR. Media for propagating strains have been previously described [33]. ALP1 on chromosome XIV was deleted by inserting the loxP-kanMX6-loxP cassette from pUG6 (gift from S. Brill, Rutgers University) at ALP1, followed by Cre-recombinase-mediated removal of the kanMX6 cassette. To insert ALP1 into chromosome II, the ALP1 ORF was first inserted into pCR4 (Invitrogen) and a kanMX6 cassette was inserted downstream of ALP1 into the SAD site of pCR4, yielding plasmid pKHS332. The ALP1-kanMX6 cassette from pKHS332 was then amplified by PCR and inserted into chromosome II downstream of HIS7 between nucleotides 714703 and 714707 in a yeast strain that had ALP1 on chromosome XIV deleted (ALP1::loxP) to improve the targeting efficiency to chromosome II. For site-directed mutagenesis, LYP1 was inserted into pCR2.1 (Invitrogen) and base substitutions A879T, C885T, G902A, A906G, C927T, C937T, C981A (LYP1-MUTABCDEF) were introduced using the QuickChange protocol (Stratagene), to generate plasmid pKHS318. A lop-P::kanMX6-loxP cassette was inserted into the Petd site of PKHS318 and, together with lyp1-1-MUTABCDEF, used to replace the chromosomal LYP1. The kanMX6 cassette was excised from the chromosomal integration by transient Cre-recombinase expression. Unless noted otherwise, the CAN1 gene is in its wildtype location on chromosome V and a URA3 cassette was used to replace the HXT13 gene on chromosome V [10,34]. In the strain designated HR-1, the LIP1 and ALP1 genes are at their wildtype loci on chromosome XIV (KHSY1530). HR-1 is identical to HR-wt except that a second copy of ALP1 was inserted into chromosome II as described above (KHSY2147). In HR-2 LIP1 is in the wildtype location whereas ALP1 on chromosome XIV was deleted and a copy of ALP1 was inserted into chromosome II (KHSY2612). In HR-3, ALP1 on chromosome XIV was deleted and no other copy of ALP1 exists in this strain (KHSY2098). In HR-4 ALP1 is in its wildtype location whereas LIP1 was replaced with the mutant LIP1 allele containing A879T, C885T, G902A, A906G, C927T, C937T, C981A base substitutions (KHSY3114).
cations involving CAN1 and LYP1 and/or ALP1, GCR clones were screened by PCR. A primer pair that anneals to the 5' end of CAN1 and to the 3' end of LYP1 was used to amplify C/L translocations and a primer pair that anneals to the 5' end of CAN1 and the 3' end of ALP1 was used to amplify C/A and C/L/A translocations. PCR products were sequenced and analyzed by BLAST and Sequencher (GeneCodes) to distinguish between C/A and C/L/A translocations and to identify fusion sites. Translocations terminating in ALP1 on chromosome XIV were distinguished from those terminating in ALP1 on chromosome II by PCR using a primer pair that anneals to the 5' end of CAN1 and downstream of ALP1 ORF on chromosome XIV, or a primer pair that anneals to the 5' end of CAN1 and within the kanMX6 cassette linked to the ALP1 ORF insertion on chromosome II, respectively.

Comparative Genome Hybridization (CGH)

Genomic DNA was extracted from a YPD culture inoculated with a single colony of the GCR clone. Proteins were removed by three rounds of phenol-chloroform-isomylalcohol extraction. Ten micrograms of genomic DNA at a concentration of 250 ng/μl were used per array. The parental strain RDKY3615 with an intact chromosome V was used as the reference genomic DNA. Hybridization, array scanning and data extraction are performed by NimbleGen Systems, Inc. The CGH array used for this analysis covers the S. cerevisiae genome using 45–85mer isothermal probes with a median probe spacing of 12 bp.

Results

Dependency of Complex Translocations on Intrachromosomal Template-Switching

Previously we showed that cells lacking Sgs1 and the DNA damage sensor Mec3 are particularly susceptible to translocations between CAN1 and ALP1 (C/A), CAN1 and LYP1 (C/L), or even all three related genes (C/L/A) [19]. Unexpectedly, the more complex tripartite C/L/A translocations arise as frequently as the simple C/A translocations, leading us to hypothesize that intrachromosomal rearrangements between the LYP1 and ALP1 genes, which are located on the same arm of chromosome XIV, may promote tripartite translocation formation. Here, to elucidate the formation of these tripartite translocations, the ALP1 and LYP1 loci were modified in an sgs1A mec3A mutant and the effect of these manipulations on the rate and type of translocations as well as on gene fusion site selection was determined. In addition to the yeast strain with CAN1, LYP1 and ALP1 in their wildtype locations (HR-wt), four new strains were constructed (Figure 1). The first strain, HR-1, contains a second copy of ALP1 on chromosome II in the same orientation and at a distance from the telomere similar to that of ALP1 on chromosome XIV. In this strain, ALP1 on chromosome XIV competes with ALP1 on chromosome II as a translocation target for LYP1. While ALP1 on chromosome XIV can be utilized for intrachromosomal rearrangements with LYP1, ALP1 on chromosome II can be utilized for interchromosomal rearrangements with LYP1. Thus, in theory, the complex C/L/A translocations in HR-1 can arise either by rearrangement between chromosomes V and XIV, or by rearrangement between three different chromosomes. The standard GCR assay, which selects for clones that had suffered a spontaneous DNA break within a 12 kb region on chromosome V that also includes the CAN1 gene [34], was used to collect 423 clones from HR-1 with various chromosome V rearrangements, which may include de novo telomere additions, insertions, inversions, large interstitial deletions as well as translocations. Among those 423 clones, 65 clones in which a broken CAN1 gene on chromosome V had rearranged with LYP1 and/or ALP1, were identified (Table 2). Translocations targeting ALP1 on chromosome II were distinguished from those targeting ALP1 on chromosome XIV using a PCR primer that anneals downstream of ALP1 on chromosome II, but not on chromosome XIV. The frequency of all CAN1/LYP1/ALP1 translocations in HR-1 (15%, 65/423) was similar to that of the wildtype strain (13%, 20/150) and C/A and C/L/A translocations terminating in chromosome XIV formed readily. However, no C/L/A translocations terminating in ALP1 on chromosome II were found. This lack of C/L/A translocations with chromosome II is not due to unavailability of ALP1 on chromosome II as a suitable translocation target since C/A translocations involving ALP1 on chromosome II were frequent (45/65). Instead, it demonstrates that intrachromosomal rearrangements between LYP1 and ALP1 do not form. To verify this finding, a second strain was constructed, HR-2, in which ALP1 on chromosome II was kept, but the second copy of ALP1 on chromosome XIV was deleted so that there was no competition between two ALP1 copies (Figure 1, HR-2). Indeed, when we screened chromosome V rearrangements in HR-2, no C/L/A translocations were observed (0/166). This absence of C/L/A translocations with chromosome II suggests that tripartite translocation formation depends on an intrachromosomal, secondary rearrangement, such as template-switching between similar DNA sequences. Thus, we reasoned that facilitating this

![Figure 1](https://example.com/figure1.png)

**Figure 1. Modification of location, copy number and sequence similarity of ALP1 and LYP1.** In unmodified cells (HR-wt), CAN1 (blue) is on chromosome V and LYP1 (green) and ALP1 (red) are in opposite orientations on the same arm of chromosome XIV. Two copies of ALP1 were present on chromosome XIV and II in HR-1, ALP1 was moved from chromosome XIV to II in HR-2, ALP1 was deleted in HR-3 and sequence similarity between HER-II of ALP1 and LYP1 was increased from 96% to 100% in HR-4.

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intrachromosomal rearrangement between LIP1 and ALP1 by increasing sequence identity between LIP1 and ALP1 should lead to an increase in the formation of C/L/A translocations. To test this possibility, seven single nucleotides in LIP1 were changed to perfectly match ALP1, extending the length of identical sequences between the two genes, which range from 5–41 bp in the wildtype genes, to a single region of 173 identical base pairs in strain HR-4 (Figure 1). Surprisingly, neither the overall GCR rate (1.1 × 10⁻⁷) nor the rate of CAN1/LIP1/ALP1 translocations (9.4 × 10⁻⁹) increased in HR-4 when compared to HR-wt (GCR rate: 1.3 × 10⁻⁷, CAN1/LIP1/ALP1 translocation rate: 1.7 × 10⁻⁸). That increasing the similarity of LIP1 and ALP1 did not affect translocation rates or translocation types suggests that the conversion of dicentric C/L translocations into monocentric C/L/A translocations may not be the rate-limiting step in translocation formation. Instead, the success of the initial translocation between CAN1 and LIP1 may determine the translocation rate, and experiments are currently underway to test this possibility. Finally, we wanted to assess if C/L translocations were so rare because they were promptly converted into C/L/A translocations or because cells harboring dicentrics could not grow into colonies. For this purpose, ALP1 was deleted from the genome (Figure 1, HR-3) and GCR clones were screened for C/L translocations. That none were found suggests that most translocation chromosomes with C/L fusions do not survive unless ALP1 is available for a secondary rearrangement that converts the dicentric into a monocentric chromosome.

Breakpoint Site Selection does not Depend on Chromosomal Target Location but Shows a Positive Correlation with 5’ Homology Length

The CAN1, LIP1 and ALP1 genes share 60–65% overall sequence identity, and we previously reported that rearrangements between LIP1 and ALP1 more often occurred in longer stretches of identical sequences than expected by chance, consistent with a homology-driven, Rad52-dependent translocation mechanism [19]. In order to determine how the modifications of ALP1 and LIP1 had affected breakpoint selection, sites in CAN1 and LIP1 where translocations originate (donor sites) and sites in LIP1 and ALP1 at which translocations are aimed (target sites) were amplified and sequenced in all 93 translocation isolated from the unmodified and the modified strains. In this study, the term ‘breakpoint’ is used to describe the sites within the CAN1, LIP1 and ALP1 genes at which the nucleotide sequence of one gene is fused to the nucleotide sequence of another gene; thus the term ‘breakpoint’ most likely refers to the sites where recombination events were resolved rather than sites at which the initiating DNA lesion occurred. We first identified all sites in CAN1 at which translocations originate and found that 89% of them cluster within two regions, which together span only 283 bp of the 1773-bp CAN1 gene (Figure 2 A). The first cluster spans 110 bp and is hereafter referred to as homologous region I, HER-I. While CAN1 and LIP1 share 83% of HER-I sequence, only 63% similarity exists with ALP1 (Figure 2 E). Moreover, the CAN1-LIP1 alignment also shows fewer gaps and longer continuous stretches of matching sequences, suggesting that the 5’ end of LIP1 may be the preferred target for CAN1 invasion (Figure S1). The second breakpoint cluster, HER-II, was noticed in our previous study. It spans 173 bp, with CAN1 sharing 78% of sequence with both LIP1 and ALP1, but with LIP1 and ALP1 sharing 96% with each other (Figure 2 E). When sorted by translocation type, it emerged that HER-I facilitates C/L/A translocations (Figure 2 C) and HER-II facilitates C/A (Figure 2 B) and C/L translocations (Figure 2 D). Not a single one of the 47 C/A translocations originated in HER-I, suggesting that the 63% sequence similarity between the HER-I regions of CAN1 and ALP1 is not sufficient for an interchromosomal translocation, whereas 83% identity between the HER-I regions of CAN1 and LIP1 appears sufficient. Taken together, this finding demonstrates that a 110-bp region of 83% sequence identity and with homology blocks not exceeding 14 bp in length is sufficient for Rad52-dependent break-induced replication in yeast cells lacking Sgs1 and Mec3, but not in wildtype cells or in the single mutants, in which these translocations are not observed.

Next we asked why some translocations from CAN1 to LIP1 undergo a secondary rearrangement with ALP1 to form C/L/A translocations whereas other translocations from CAN1 to LIP1 terminate as C/L translocations. We found that the sites in CAN1 at which C/L translocations originate (Figure 3 A, blue) were downstream of sites in CAN1 at which C/L/A translocations originate (Figure 3 A, red), and the LIP1 target sites in C/L translocations were downstream of all LIP1 target sites in C/L/A translocations (Figure 3 B). This finding suggests that sequence similarity between LIP1 and ALP1 downstream of these C/L breakpoints is insufficient for an rearrangement with ALP1. Thus, a translocation from CAN1 to LIP1 only results in a viable

### Table 2. Effect of changes in translocation target location, copy number and sequence identity on structure of spontaneous translocations involving the CAN1, LYP1 and/or ALP1 loci.

| Translocation Type | HR-wt | HR-1 | HR-2 | HR-3 | HR-4 |
|--------------------|-------|------|------|------|------|
| CAN^{wt}           | 7     | 6    | n.a. | n.a. | 1    |
| CAN^{mut}          | n.a.  | 46   | 1    | n.a. | n.a. |
| C/L                | 3     | 0    | 1    | 0    | 0    |
| C/L/A^{mut}        | 7     | 12   | n.a. | n.a. | 4    |
| C/L/A^{wt}         | n.a.  | 0    | n.a. | n.a. |      |
| Other              | 3     | 1    | 1    | 0    | 0    |
| Translocation Frequency | 13% (20/150) | 15% (65/423) | 1.8% (3/166) | 0% (0/45) | 8% (5/60) |

*Translocation types CAN^{wt} and CAN^{mut} refer to CAN1 translocations terminating in ALP1 on chromosome XIV or II, respectively. Translocation types C/L^{wt} and C/L^{mut} refer to C/L/A translocations terminating in ALP1 on chromosome XIV or II, respectively. HR-wt, HR-1, HR-2, HR-3 and HR-4 refer to strains KHSY1530, KHSY2147, KHSY2612, KHSY2098, and KHSY3114, respectively. n.a., not available.

**Other** refers to clones with translocation types other than one of the three major types of C/A, C/L/A and C/L translocations, including clones with multiple translocation type.

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chromosome if the initiating breakpoint in CAN1 is located in the HER-I region or at the extreme 5’ end of the HER-II region, so that most of the 96% identical HER-II region is available for a secondary rearrangement between LYP1 and ALP1. Interestingly, we found one translocation that originated in CAN1 and targeted HER-I of LYP1 (Figure 3 A, B, labeled *), but failed to go on to become a C/L/A translocation, even though the entire HER-II region was available for a secondary rearrangement with ALP1. That this C/L translocation was identified in the HR-2 strain, in which LYP1 and ALP1 were on two different chromosomes, demonstrates that translocations from CAN1 to LYP1 get stuck in HER-I when ALP1 is not available on the same chromosome for a secondary rearrangement. This finding is consistent with our conclusion above that secondary rearrangements between LYP1 and ALP1 only occur intrachromosomally.

To determine how moving ALP1 to a different chromosome had affected breakpoint selection in ALP1, we compared the breakpoint target sites in ALP1 on chromosome II with those in ALP1 on chromosome XIV. This analysis revealed that the chromosomal location of ALP1, while affecting translocation type, did not influence target site selection within ALP1 or donor site selection within CAN1 (Figure 3 C, D). C/A translocations, no matter whether they target chromosome XIV (red) or II (blue), originate from nearly identical sets of CAN1 sites. Similarly, the sites in ALP1 on chromosome XIV and ALP1 on chromosome II that are targeted by CAN1 also match. Thus, we can conclude that while translocation rates are determined by the chromosomal location of the target genes, breakpoint selection is not. This breakpoint analysis also revealed that the location of the breakpoints in CAN1 exactly predicts the location of the target sites in ALP1, as evidenced by the matching patterns of donor sites in CAN1 and target sites in ALP1 (Figure 3 C and D). This predictability of breakpoint patterns extends to C/L/A translocations (Figure 3 E–H), where donor sites in CAN1 predict the target sites in LYP1 (Figure 3 E–F) and donor sites in LYP1 predetermine the target sites in ALP1 (Figure 3 G–H). However, no connection appears to exist between LYP1 sites targeted by CAN1 and LYP1 sites serving as a donor for the L/A rearrangement. Requirement of the downstream HER-II region in the conversion of dicentric C/L translocations to monocentric C/L/A translocations indicates 5’ to 3’ directionality of the recombination process. This directionality is further supported by the positive correlation between the length of homology between CAN1 and ALP1 as well as between CAN1 and LYP1 upstream of the C/A and C/L breakpoints, respectively, and the number of breakpoints observed at that site, whereas no correlation exists for the downstream sequence (Figure 4). There was only a weak positive correlation between the length of homology between LYP1 and ALP1 and the number of interchromosomal L/A rearrangements observed at that site ($r = 0.38$), suggesting that the interchromosomal rearrangement is HR-dependent whereas the intrachromosomal rearrangement may be HR-independent and/or affected by additional constrains.

Figure 2. Breakpoint locations in CAN1. CA, C/L/A and C/L translocations were sequenced and the last nucleotide of the CAN1 gene was mapped to the 1773-bp CAN1 gene. (A) The vast majority of breakpoints fall within Homologous region I (HER-I) or Homologous region II (HER-II), whereas further analysis reveals that (B) C/A translocations originate from HER-II, and (C) C/L/A translocations originate from HER-I of CAN1. (D) CAN1 breakpoints of dicentric C/L translocations can fall into HER-I or HER-II depending on availability of ALP1 for a secondary rearrangement. (E) Location and shared sequence identity of the breakpoint clusters HER-I and HER-II in CAN1, LYP1 and ALP1. HER-I spans 110 bp and shows greater similarity between CAN1 and LYP1 than ALP1. HER-II spans 173 bp and shows 96% sequence identity between LYP1 and ALP1 whereas CAN1 shares only 78% sequence identity with LYP1 and ALP1 in that region. HER-I and HER-II are the two largest regions of greatest sequence identity present in these three genes. Over the entire ORF, CAN1, LYP1 and ALP1 share 60–65% of their sequence. Sequences are shown to scale and are aligned at the HER-I and HER-II regions.

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Unstable Dicentrics Give Rise to Complex Monocentrics and Intraclonal Heterogeneity

While C/L translocations can be identified by screening with primers that anneal to the 5' and 3' ends of \textit{CAN1} and \textit{LYP1}, respectively, primers that anneal to the 5' and 3' ends of \textit{CAN1} and \textit{ALP1} amplify both C/A and C/L/A translocations, which can only be distinguished by sequencing. The simultaneous presence of C/A and C/L/A translocations in the same clone is indicated by double peaks in the sequencing chromatogram at sites where the homologous regions in \textit{ALP1} and \textit{LYP1} differ. The identification of such clones (included in ‘Other’ in Table 2) that harbor multiple types of translocation between \textit{CAN1}, \textit{LYP1} and \textit{ALP1} (Figure 5 A) indicates instability of translocation chromosomes. To test this possibility, clone 1095 harboring C/L, C/A and C/L/A translocations was streaked on agar for single colonies with the expectation that we would obtain the three translocations in individual colonies if the translocations were stable. The colonies that were obtained after 3 days of growth were heterogeneous, ranging from tiny to large with round or irregular edges. Of 40 single colonies that were analyzed, 25 contained single translo-
tions that were identical to those found in the original clone. Among the other 15 single colonies, however, three novel translocations with breakpoints not seen in the original clone were identified as well as six new combinations of new and old translocations. Thus, instead of the expected three translocations, a total of six different translocations in nine different combinations were identified (Figure 5 B). Since all 40 colonies were derived from single cells, the identification of single colonies with multiple (old and new) translocations in several combinations indicates that at least one of the original translocations is unstable. The obligatory presence of the original C/L translocation in all colonies with multiple new and old translocations suggests that the dicentric C/L chromosome is unstable and subjected to cycles of rearrangement that lead to new translocations. In addition to clone 1095, evidence that C/L and C/L/A translocations present in the same clone may be derived from each other was also found in heterogeneous clone 1063, in which sequencing revealed that all translocations shared the same CAN1 breakpoint at nucleotide 800 of CAN1. Continued instability of chromosome V after having rearranged with LYP1 to form a dicentric C/L translocation is detectable by array-based comparative genome hybridization (array CGH) (Figure 5 C). The two clones with single C/L translocations analyzed here were obtained from sgs1A mutants with defects in the DNA damage checkpoint clamp (mej3A) or clamp loading (rad24A), which had previously been shown to yield C/L translocations [19]. While CGH on both clones showed that loss of chromosome V sequence is most noticeable distal of the CAN1 breakpoint (due to loss of this region in the original C/L translocation and hence its absence in all its derivatives), it also revealed further degradation beyond the CAN1 locus, indicating ongoing instability.

Error-Prone Break-Induced Replication Reveals Common Translocation Origin

Identical CAN1 breakpoints shared by multiple translocations in heterogeneous clones were suggestive of a common origin of the various translocations. Thus, we analyzed the DNA sequences downstream of CAN1 breakpoints in heterogeneous clones for additional shared features. Indeed, in heterogeneous clone 1063 an A879T substitution was identified in the C/L translocation 12 nucleotides downstream of the C/L breakpoint (Figure 6 A). Since translocations between CAN1, LYP1 and ALP1 are nonreciprocal, as indicated by the presence of intact LYP1 and ALP1 genes on chromosome XIV, analysis of the wildtype LYP1 gene was possible.

Figure 4. Positive correlation between the number of breakpoints and length of 5' homology. (A) 5' homology block length in HER-II of CAN1 shows a positive correlation to the number of C/A breakpoints at that site, whereas (B) 3' homology block length shows no correlation. (C) 5' homology block length in HER-I of CAN1 shows a positive correlation to the number of C/L/A translocations originating from that site, whereas (D) no correlation is found for 3' homology block length. The Pearson correlation coefficient (r) is indicated. doi:10.1371/journal.pone.0012007.g004
in this clone. Sequencing revealed that the A879T mutation was not present in the intact LYP1 gene of this clone, suggesting that the mutation occurred during translocation from CAN1 to LYP1. This A879T substitution could have resulted either from a polymerase error or from CAN1 invading and copying the nearby ALP1 locus (which contains a T at this location) prior to forming the C/L translocation (Figure 6 B). Upstream of this A/T mismatch LYP1 and ALP1 share 41 bp of perfect sequence identity, which could have stabilized such a transient template-switch. If the C/L/A translocation in the same clone was indeed derived from this C/L translocation, as already suggested by their common CAN1 breakpoint, the base substitution should also be present. That sequencing of the C/L/A translocation indeed identified the same base substitution suggests that these two translocations are derived from each other instead of arising independently from separate CAN1 invasions. A T521C substitu-
tion 21 nucleotides downstream of the C/A breakpoint was found in the C/A translocation of another clone (1840), but not in ALP1 of the intact chromosome XIV of that clone, suggesting it arose during translocation formation and C/L/A is derived from C/L. (B) Formation of the A879T change by template switching from LYP1 to ALP1 and back to LYP1. (C) A T521C change in the C/A translocation of clone 1840 was not present in ALP1 on the intact chromosome XIV in the same clone. T521C may have resulted from a replication error or template switching to a locus other than ALP1, LYP1 or CAN1 since none of them contains a C at this location. (D) Alignment of HER-I of CAN1 and LYP1, indicating seven breakpoints at which CAN1 and LYP1 recombine. Except for breakpoint 1, all are followed by a single mismatch. The (AG)4 repeats in CAN1 and LYP1 are indicated in red and green, respectively. (E–G) Looping out of a single AG unit leads to a longer perfect match between CAN1 and LYP1, and could explain why breakpoints 1 and 2 can be found fused to 5’-CCTT sequence of LYP1.

Figure 6. Translocation formation is associated with single nucleotide changes and evidence of DNA slippage. (A) An A879T change was observed in the C/L and C/L/A translocations of clone 1063, but not in LYP1 of the intact chromosome XIV of that clone, suggesting it arose during translocation formation and C/L/A is derived from C/L. (B) Formation of the A879T change by template switching from LYP1 to ALP1 and back to LYP1. (C) A T521C change in the C/A translocation of clone 1840 was not present in ALP1 on the intact chromosome XIV in the same clone. T521C may have resulted from a replication error or template switching to a locus other than ALP1, LYP1 or CAN1 since none of them contains a C at this location. (D) Alignment of HER-I of CAN1 and LYP1, indicating seven breakpoints at which CAN1 and LYP1 recombine. Except for breakpoint 1, all are followed by a single mismatch. The (AG)4 repeats in CAN1 and LYP1 are indicated in red and green, respectively. (E–G) Looping out of a single AG unit leads to a longer perfect match between CAN1 and LYP1, and could explain why breakpoints 1 and 2 can be found fused to 5’-CCTT sequence of LYP1.

In addition to base substitutions, we detected possible evidence of DNA slippage (Figure 6 D–G). With a single exception, the CAN1 sequence in C/L and C/L/A translocations terminates at single base-pair mismatches within HER-I (Figure 6 D, breakpoints 2–7). Termination at breakpoint 1, which is not followed by a mismatch (Figure 6 D, breakpoint 1) was observed in one C/L/A translocation and could be explained by DNA slippage within a short AG repeat during strand invasion. Specifically, slippage of
the (AG)$_4$ repeat in CAN1 during annealing to the corresponding (AG)$_3$ repeat in LYP1 may have led to looping out of a single repeat unit in CAN1 (Figure 6 F) or, more likely, a shift in base pairing (Figure 6 G). As a result, a mismatched base may sometimes be located after (AG)$_3$ or after (AG)$_2$, thus making both sites susceptible to becoming CAN1 translocation donor sites. The increase in length of the base-paired region from six to ten matches as a result of this slippage event may have promoted termination of the CAN1 sequence at breakpoint 1.

Discussion

Although some RecQ-like helicases have been successfully purified so that their substrate specificity and enzymatic function could be determined in vitro, less is known about the mechanism by which RecQ-like DNA helicases preserve genome integrity or about the types of genome rearrangements that arise in cells lacking RecQ-like DNA helicases. In an ongoing effort to shed light on these questions, we previously showed that yeast double mutants lacking the RecQ-like helicase Sgs1 in addition to certain DNA damage checkpoint components (Mec3, Rad24, Tel1) accumulate recurring, Rad52-dependent, Rad51-independent translocations between the related CAN1, LYP1 and ALP1 genes [19]. Simple C/L and C/L translocations, but also more complex C/L/A rearrangements were found in these mutants, and the aim of the current study was to test models for the formation of these translocations, and to gain additional insight into the general mechanism of translocation formation. One possibility for C/L/A translocation formation was that they arise in a single event, in which CAN1 invades LYP1, but dissociates and reanneals to the nearby ALP1. Alternatively, C/L/A translocations could form as a result of two independent events; in the first event CAN1 translocates to LYP1 and forms a dicentric C/L chromosome, and in the second event, possibly during anaphase of mitosis, this C/L dicentric breaks and invades ALP1 to form a monocentric C/L translocation. Interestingly, the inability to form C/L/A translocations if all three genes are located on three different chromosomes and the observation of clonal instability point to the following two sources for C/L/A translocations. Translocation formation is initiated by a DNA break on chromosome V that leads to invasion of LYP1 on chromosome XIV, using the sequence homology provided by the HER-I sequence in CAN1 and LYP1. This HER-I-mediated invasion of LYP1 by CAN1 leads to initiation of DNA synthesis on chromosome XIV, which may then be subjected to dissociation and re-invasion cycles as previously described [6]. If the re-invading strand mistakenly anneals to the nearby ALP1, this time utilizing the 96%-identical HER-II sequence for an intrachromosomal template-switching event, a monocentric C/L translocation forms. If re-invasion occurs at the same site in LYP1 or BIR simply continues without dissociation, a dicentric C/L chromosome forms. This dicentric provides the second source for C/L/A translocation formation as it is likely to be susceptible to breakage in mitosis followed by renewed attempts at repair. This ongoing instability of dicentrics is supported by our finding of intrachromosomal heterogeneity of translocation types. Broken C/L dicentrics are likely to utilize LYP1 sequence contained in them to invade chromosome XIV at LYP1, forming either a another unstable dicentric or giving rise to a stable, monocentric C/L chromosome by undergoing an intrachromosomal template-switch to ALP1. Repeated cycles of breakage and repair of C/L dicentrics are the likely explanation for presence of multiple, different translocations in the same clone and continued formation of new gene fusions in our study.

Thus, while recombination between HER-I sequences of CAN1 and LYP1 leads to dicentrics that provide a source for C/L/A translocations, it appears that annealing of CAN1 with HER-II of LYP1 leads to C/L dicentrics that disappear from the population because they are unable to undergo a stabilizing rearrangement with ALP1 due to lack of downstream homology. In some cases, seemingly stable C/L translocations with breakpoints in HER-II could be obtained (Figure 3 A and B). In these cases it is likely that they underwent conversion to a monocentric chromosome, using chromosome XIV sequences other than ALP1, without disrupting the C/L fusion. In other cases, C/L translocations were found to be highly unstable, giving rise to new translocations. Although unstable C/L translocations were formed by annealing HER-II of CAN1 and LYP1, the CAN1 breakpoints were located at the very 5' end of HER-II, leaving almost all of HER-II available for rearrangements with ALP1 and formation of stable C/L/A translocations. Further evidence that C/L/A translocations and can be derived from unstable C/L dicentrics is provided by identical CAN1 breakpoints and the occurrence of single-nucleotide changes shared by multiple translocations in the same clone. Such base substitutions near translocation breakpoints may result from replication errors or re-invasion at similar sequences and could, combined with the potential for frameshifts due to slippage at the gene fusion site, be a source for mutations and loss of gene function even if recombination occurs between allelic sequences on sister chromatids or homologous chromosomes. These recombination-associated errors were rare, occurring in three of the 573 translocations between CAN1, LYP1 and ALP1 in HR-wt and HR-1 (0.005%).

Intrachromosomal, faulty template-switching between inverted repeats was recently also proposed in a study by Pack et al [35] to account for the formation of dicentric chromosomes in budding yeast, which, similar to the dicentrics in our study, proved to be unstable and substrates for further chromosomal rearrangements. These authors also reported that intrachromosomal template-switching is Rad52-independent, which could suggest that the Rad52-dependence of the CAN1/LYP1/ALP1 translocations studied here is due to the interchromosomal recombination event between CAN1 and LYP1 or CAN1 and ALP1, whereas intrachromosomal template switching between LYP1 and ALP1 may be Rad52-independent. Indeed, the weaker correlation between 5' homology block length and the number of L/A breakpoints observed at that site compared to that for C/L or C/A breakpoints suggests that additional factors affect template switching and may suggest a lesser role (or no role) for HR in the intrachromosomal template-switch between LYP1 and ALP1.

That translocations between CAN1, LYP1 and ALP1 form so frequently in cells lacking Sgs1 and a DNA damage sensor such as Mec3, but not in the single mutants, most likely stems from the independent roles of these factors in preventing different intermediates of translocation formation, such that in the double mutants increased lesion formation, aberrant lesion processing, greater tolerance for dicentrics and/or the products of their breakage and defective checkpoint activation combine to create conditions suitable for translocation formation. That C/L dicentrics are unstable and give rise to multiple new rearrangements suggests that dicentrics break during anaphase to fuse again, entering a cycle of repeated breakage and fusion until a stable translocation chromosome is generated, if ever. This process may be comparable to the futile breakage-fusion-bridge (BFB) cycle observed in multicellular eukaryotes. In cancers where intratumor heterogeneity is common, such as osteosarcoma, a positive correlation has been observed between the number of dicentrics and the frequency of BFBs, which are thought to be a source of
mitotic chromosome instability and may in some cases generate complex rearrangements involving multiple chromosomes [36]. Interestingly, increased presence of micronuclei, which are thought to contain chromosome fragments that have resulted from breakage of unresolved BFBs, has been reported for cells from Bloom’s syndrome patients and from BLM knock-out mice [37,38]. Indeed, the Hickson laboratory recently showed that BLM localizes to BFBs and to novel ultrafine bridges (UBFs), the latter of which commonly emerge from centromeric regions in normal cells [39,40,41]. BFBs and UBFs accumulate in BLM-defective cells, and the authors found evidence that BLM is required for efficient and proper resolution of bridge structures, most likely by decatenation, rather than the prevention of bridge formation prior to anaphase [40,41]. Thus, lack of BLM, or Sgs1 in yeast, may contribute to increased chromosome breakage and occasional large-scale rearrangements and DNA loss. Combining the lack of Sgs1/BLM with dysfunctional Tel1/ATM or Mec3/9-1-1 checkpoint pathways creates conditions under which mitotic chromosome breaks may not be efficiently detected and/or faithfully processed, allowing recurring, complex translocations and unstable dicentrics to arise and persist [19]. DNA lesions that give rise to translocations may be present at increased rates in cells lacking Sgs1, as Sgs1 has been shown to also have roles in the processing of DSBs [26], the resolution of unusual secondary DNA structures, such as G4 tetrads [42,43], resolution of recombination intermediates [22] and possibly in checkpoint activation itself [44]. Here, we have provided evidence how this increased genome instability can lead to the formation of complex translocations by intragenic, interchromosomal BIR that requires as little as 110 bp of 83% identity with homology blocks that do not exceed 14 bp, and by intrachromosomal template-switching that requires as little as 173 bp of 96% identity separated by 2445 bp. In addition, dicentric chromosomes are a source of intrachromal, and most likely intratumor, heterogeneity, giving rise to not only translocations with new breakpoints, but also cells with new combinations of these chromosome rearrangements. In Bloom’s syndrome and other human chromosome instability syndromes such ongoing genome instability is likely to contribute to increased cancer incidence at an earlier age and other characteristic signs of premature aging.

Supporting Information

Figure S1 Needleman-Wunsch alignments of CAN1, ALP1 and LIPI. Alignments of the 5’ ends of (A) CAN1 and ALP1 and (B) the 5’ ends of CAN1 and LIPI reveal greater sequence similarity and longer continuous regions of identical sequences between CAN1 and LIPI than CAN1 and ALP1.

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Author Contributions

Conceived and designed the experiments: KS. Performed the experiments: KS EV LD CL AR. Analyzed the data: KS. Contributed reagents/materials/analysis tools: KS. Wrote the paper: KS.

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