Comprehensive analysis of *cis*- and *trans*-acting factors affecting Break-Induced Replication

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Abstract

Break-induced replication (BIR) is a highly mutagenic eukaryotic homologous DNA recombination pathway that repairs one-ended DNA double strand breaks such as broken DNA replication forks and eroded telomeres. While searching for cis-acting factors regulating BIR efficiency, we found that BIR efficiency is the highest close to chromosome ends. The variations of BIR efficiency as a function of the length of DNA to replicate can be described as a combination of two decreasing exponential functions, a property in line with repeated cycles of strand invasion, elongation and dissociation that characterize BIR. Interestingly, the apparent processivity of BIR depends on the length of DNA already synthesized. BIR is more susceptible to disruption during the synthesis of the first ~35-40 kb of DNA than later, notably when the template chromatid is being transcribed or heterochromatic. Finally, we show that the Srs2 helicase promotes BIR from both telomere proximal and telomere distal regions in diploid cells but only from telomere proximal sites in haploid cells. Altogether, we bring new light on the factors impacting a last resort DNA repair pathway.

Introduction

Break induced replication (BIR) is a eukaryotic one-ended homologous DNA recombination (HR) process (Kramara et al., 2018; Llorente et al., 2008). It is thought to deal with one-ended DNA double strand breaks (DSBs) such as those generated by the encounter of replication forks with single strand DNA (ssDNA) breaks, as well as other one-ended DSBs like eroded telomeres that can be repaired also outside S phase. Notably, a BIR-like mechanism is responsible for the alternative lengthening of telomeres (ALT) in absence of telomerase in 10–15% of all cancers (Dilley and Greenberg, 2015). A similar mechanism is responsible for the emergence of survivors

in the absence of telomerase in *Saccharomyces cerevisiae* (Lundblad and Blackburn, 1993). BIR can therefore take place between homologous loci located at allelic positions on sister chromatids or non-sister chromatids, as well as at non-allelic positions. The first steps of BIR are common to any canonical HR reaction, up to the generation of the D-loop and the initiation of DNA repair synthesis from the 3' invading end by DNA polymerase delta. Two-ended DSB repair by HR requires generally rather short tracts of DNA repair synthesis that initiate by DNA polymerase delta from both 3' ends of the DSB after they anneal to a complementary template (Nassif et al., 1994; Pâques and Haber, 1999; Szostak et al., 1983). In the case of BIR, the unique 3' invading end allows DNA polymerase delta to prime DNA repair synthesis (Donnianni et al., 2019; Liu et al., 2021; Lydeard et al., 2007). BIR associated DNA replication proceeds by migrating this D-loop potentially up to the telomere. This results in conservative DNA replication where the two newly synthesized strands are on the same chromatid (Donnianni and Symington, 2013; Saini et al., 2013). Pif1 is the helicase responsible for branch migrating the D-loop over tens to hundreds of kilobases (Buzovetsky et al., 2017; Li et al., 2021; Wilson et al., 2013). After about 30 kb of DNA synthesis by DNA polymerase delta, DNA polymerases alpha and epsilon come into play. This likely stabilizes the nascent DNA strand and promotes synthesis of the second strand of the BIR product, a process still not well understood during BIR (Liu et al., 2021). While dispensable for DNA synthesis during S phase, for two-ended DSB repair and repair of short gaps, the Pol32 subunit of DNA polymerase delta is essential for BIR and for the repair of long gaps, likely promoting the processivity of this enzyme (Jain et al., 2009; Lydeard et al., 2007). Overall, BIR associated DNA synthesis deviates from classical DNA repair synthesis as well as from S phase DNA synthesis both mechanistically and through the proteins involved.

BIR associated DNA synthesis is highly mutagenic, with high rates of point mutations, frameshifts and gross chromosomal rearrangements. Point mutations are mainly the result of the long ssDNA region generated behind the migrating D-loop ensuring DNA replication during BIR. These ssDNA regions can suffer base damage which promote subsequent mutagenesis when replicated by the translesion DNA polymerase zeta (Elango et al., 2019). The replication protein A (RPA) is important to protect these long ssDNA regions and ensure accurate BIR (Ruff et al., 2016). Interestingly, in addition to its D-loop disrupting activity (Piazza et al., 2019), the Srs2 helicase is important during BIR to prevent Rad51 to mediate toxic interactions involving these long ssDNA regions (Elango et al., 2017). DNA polymerase delta was shown to generate a high level of unrepaired frameshift mutations during BIR (Deem et al., 2011). Gross chromosomal rearrangements can form in the early phase of the BIR reaction where frequent template switching events were observed both between homologous chromosomes and between ectopic repeats (Smith et al., 2007). This observation led to the suggestion that the early phase of BIR undergoes more frequent cycles of strand invasion, elongation and dissociation than the late phase of BIR, but the mechanism remains unknown (Elango et al., 2019; Smith et al., 2007). Gross chromosomal rearrangements can also occur when the BIR template is damaged or when the DNA synthesis step is hampered such as in the absence of Pol32 or in the Pol3-ct mutant (Deem et al., 2008; Smith et al., 2009; Vasan et al., 2014). Notably, BIR intermediates are destabilized by interstitial telomeric repeats which can abort BIR reactions (Liu et al., 2021; Stivison et al., 2020). This leads to stable aborted BIR products thanks to the action of telomerase on the dissociated end.

Several other factors related to the structure of the template to replicate also inhibit BIR. The BIR reaction is blocked by a converging replication fork when initiated from a

one-ended DSB generated during S phase by the passage of a replication fork through an inducible ssDNA break (Mayle et al., 2015). The BIR reaction is also blocked by RNA-DNA hybrids normally resolved by RNases H; by RNA polymerase I transcription at the rDNA locus (Amon and Koshland, 2016), and eventually by converging RNA Polymerase II transcription close to the BIR initiating point (Liu et al., 2021). In addition, BIR is impaired when the template to replicate is heterochromatic (Pham et al., 2021). Last but not least, BIR efficiency, defined as repair efficiency by BIR reactions, seems to be inversely correlated to the length of DNA to replicate, but the nature of such a relationship is still unclear (Anand et al., 2014; Donnianni and Symington, 2013; Lydeard et al., 2007).

Here, while searching for cis-acting factors regulating BIR, we notably found that the variations of BIR efficiency as a function of the length of DNA to replicate can be modelled as a combination of decreasing exponential functions, in agreement with a mechanism involving repeated cycles of strand invasion, extension and dissociation. More precisely, our data support two phases during BIR, a first phase with low apparent DNA synthesis processivity followed by a second phase with higher apparent DNA synthesis processivity, likely supported by the DNA polymerases involved (Liu et al., 2021). Consequently, BIR initiated close to chromosome ends is much more efficient than BIR initiated at a more distal position, and we bring a definitive demonstration of this using CRISPR-Cas9-induced reciprocal translocation to modulate the length of DNA to synthesize from a unique BIR initiating locus. This BIR property, combined with all the other factors impeding BIR, participates to make BIR a last resort DSB repair mechanism.

Results

BIR efficiency is higher close to chromosome ends

In order to look for cis-acting factors regulating BIR efficiency, we initiated BIR along the one megabase-long right arm of chromosome IV, the longest *S. cerevisiae* chromosome arm apart from the right arm of chromosome XII that contains the rDNA locus. We took advantage of the yeast deletion library in which the *KANMX4* cassette is the selection marker that replaces every ORF (Winzeler et al., 1999), in order to use this cassette as a unique homologous region to initiate BIR with the same chromosome fragmentation vector from virtually any genomic locus (Figure 1) (Costelloe et al., 2012; Davis and Symington, 2004; Morrow et al., 1997). More precisely, the transcription orientation of the *KANMX4* cassette being the same as the deleted ORFs, we targeted BIR only at positions where the transcription orientation of the *KANMX4* cassette is directed toward the telomere using a single chromosome fragmentation vector (Morrow et al., 1997). We expressed BIR efficiency as the number of transformants with the linearized chromosome fragmentation vector initiating BIR from *KANMX4* normalized by the number of transformants with the linearized chromosome fragmentation vector initiating BIR from the control *BUD3* locus located ca. 100 kb away from the left telomere of chromosome III (Costelloe et al., 2012; Davis and Symington, 2004). We initiated BIR from 11 loci spread roughly every 100 kb between the centromere and the telomere of chromosome IV (TEL04R), the closest locus to the telomere being *YDR541c* whose 3' end is located ca. 11 kb away from TEL04R. BIR efficiency was roughly constant for all loci except for the *YDR541c* locus for which it was significantly higher (Figure 1B). Using the same strategy, we further observed an elevated BIR efficiency on the right ends of chromosomes V, IX and XII compared to more telomere distal regions located ca. 100 kb away from the corresponding telomeres (data not

shown). Overall, these observations show that telomere proximity behaves as a cis-acting factor facilitating BIR.

BIR efficiency from a chromosome fragmentation vector fits two exponential functions of the DNA length to synthesize

The efficiency of BIR initiated at the *KANMX4* locus located 115 kb away from TEL04R is lower than the efficiency of BIR initiated at the *BUD3* locus located at a similar distance from the left telomere of chromosome III (Figure 1B). This suggests that BIR is less efficient when using *KANMX4* as a homology region than when using *BUD3*. This likely results from the longer homology region of the *BUD3* cassette compared to the *KANMX4* cassette (ca. 5 kb and 1.5 kb, respectively) as previously observed (Lydeard et al., 2007).

To optimize the analysis of BIR efficiency as a function of the distance to the telomere, we switched to ca. 5kb homology regions cloned into the chromosome fragmentation vector instead of *KANMX4*. In addition to the regions encompassing *YDR541c* and *YDR479c* corresponding to the previous loci located 11 and 115 kb away from the telomere, respectively, we analyzed ten other loci (Figure 2A and Table S3). Seven of these loci are located between 166 and 26 kb from TEL04R. In order to test longer distances away from the telomere without involving additional BIR initiating loci which could have increased the probability of including local effects on BIR efficiency, we generated a reciprocal translocation that exchanged the 10 kb of chromosome IV proximal to TEL04R with the 291 kb of chromosome VII proximal to TEL07R. We performed the chromosome translocation as described by Fleiss and Fischer using CRISPR-Cas9-induced DSBs and oligonucleotides overlapping the translocation breakpoints as templates (Fleiss et al., 2019). The loci originally located 10, 57 and

117 kb from TEL04R are located 291, 338 and 398 kb from the right telomere in this translocated strain. For simplicity, we refer to the BIR initiating loci by their chromosome arms and the distance in kb to the telomere. Overall, these loci range from IVR-10 to IVR-398 (Figure 2A).

We measured BIR efficiency for these twelve loci as the ratio between the number of transformants with the linearized chromosome fragmentation vector initiating BIR, and the number of transformants with a circular vector (Figure 2). Overall, we observed that BIR efficiency decreases with the size of DNA to synthesize, but the relationship is not linear. Since BIR is likely characterized by repeated cycles of strand invasion, elongation and dissociation (Smith et al., 2007), we thought that BIR efficiency, hereafter designated $E(dist)$ as a function of the DNA length to synthesize ($dist$), should be best described by exponential functions. The data were poorly fitted by a single exponential function (value of Akaike's Information Criterion [AIC] equal to -584.2), so we tested combinations of two exponential functions describing separately the results for the first x and for the last $12-x$ distances (in increasing order) with x varying between 2 and 10. Similar fits with low, close AIC values were observed for combinations of two exponential functions when the first function fitted the data either from IVR-10 to IVR-26, or from IVR-10 to IVR-36, or from IVR-10 to IVR-41, or from IVR-10 to IVR-46 (AIC values equal to -622.5, -622.5, -623.0, and -623.6, respectively, see Figure S1). Figure 2 shows the curves corresponding to the first exponential function fitting the data from IVR-10 to IVR-36; this combination of exponential curves presents the additional advantage that the ends of the two curves meet without any shift.

The good fit of the data with a combination of two exponential functions can be interpreted by the following model invoking two successive DNA replication modes during BIR. The BIR efficiency, $E(dist)$, represents the probability that a DSB end with

homology to a chromosome region located at $dist$ kb away from the telomere is properly repaired by BIR, that is the probability that BIR proceeds as far as the distance $dist$. Let k be the probability that BIR is initiated at the DSB end, and let's consider first short distances (the first exponential curve). If we assume that DNA synthesis for these short DNA lengths goes on with a constant probability p_1 for each kb (which means that it has a probability $1-p_1$ to be irreversibly disrupted at each kb), the probability that $dist$ kb are replicated in a row is p_1^{dist} . $E(dist)$ is thus equal to the probability of initiation k multiplied by p_1^{dist} , hence the equation of the first fitting exponential curve is :

$$E(dist) = kp_1^{dist}.$$

Replication characteristics change after the synthesis of a certain DNA length. The value of this threshold, T , can be estimated at about 35-40kb, at the point where the two fitting curves meet (Figures 2 and S1). After this threshold, DNA synthesis goes on with a higher, constant probability p_2 to proceed for each kb, and we have, for $dist > T$, the equation :

$$E(dist) = kp_1^T p_2^{(dist-T)},$$

which corresponds to the second exponential fitting curve.

The fitting curves shown in Figure 2 correspond to values of k , p_1 and p_2 equal to 0.71 +/- 0.07, 0.935 +/- 0.009, and 0.995 +/- 0.002, respectively. According to our model, this means (i) that BIR has a probability of 0.71 to be initiated for cells transformed with the linearized chromosome fragmentation vector (if we assume that transformation efficiencies are identical for the circular vector and for the linearized chromosome fragmentation vectors, and that BIR is the only limiting factor for the maintenance of this artificial chromosome), and (ii) that the probability of BIR reactions to be irreversibly disrupted at each kb decreases after the synthesis of ~35-40 kb of DNA by a factor of 13, from 0.065 (1-0.935) to 0.005 (1-0.995).

Importantly, BIR events from telomere proximal and telomere distal regions strongly depend on *POL32* and to a lesser extent on *PIF1*, supporting long tract DNA repair synthesis and therefore *bona fide* BIR from all studied loci (Figure S2). This is further supported by PFGE analysis of clones transformed with the chromosome fragmentation vectors that revealed chromosome fragments at the expected size for BIR products (Figure S3).

Direct evidence that BIR efficiency depends on the size of DNA to synthesize

In order to directly demonstrate that the length of DNA to synthesize determines BIR efficiency independently of the initiation locus, we performed targeted chromosome translocations to modify the distance between chromosome IV BIR initiating loci and TEL04R (Figure 3). We generated the IVR-10_VIIR-101 translocated strain where the 101 kb telomeric fragment from the right arm of chromosome VII replaces the 10 kb telomeric fragment from the right arm of chromosome IV. In this strain, the length of DNA to synthesize for BIR events initiated upstream of the translocation point on chromosome IV increases by 91 kb (Figure 3A). BIR efficiency from the most telomere proximal IVR-10 locus shows a clear drop in this translocated strain compared to the parental strain. This latter locus shows a BIR efficiency comparable to the BIR efficiency measured from the IVR-117 locus in the parental strain (Figures 3A and 2). We also generated the IVR-113_XVIR-12 translocated strain where the 12 kb telomeric fragment from the right arm of chromosome XVI replaces the 113 kb telomeric fragment from the right arm of chromosome IV (Figure 3B). In this strain, the length of DNA to synthesize for BIR events initiated upstream of the translocation point on chromosome IV decreases by 101 kb, positioning the IVR-117 locus 16 kb away from the telomere. BIR efficiency from this locus increases in the translocated strain to 25

+/- 4% compared to 3 +/- 1% in the parental strain. In this translocated strain, the distance to the telomere of the IVR-57 and IVR-10 loci is unchanged and the corresponding BIR efficiencies are similar between the translocated and the parental strain (Figure 3B).

Overall, these results show that the length of DNA to synthesize during BIR directly influences BIR efficiency independently of the initiating locus.

Transcription and heterochromatin of the template impair BIR efficiency

In addition to the length of DNA to synthesize, other cis-acting chromosomal parameters may affect BIR efficiency. Both RNA polymerase I and II mediated transcription were shown to impair BIR (Amon and Koshland, 2016; Liu et al., 2021). We therefore tested the effect of transcription on BIR in our assay system by comparing BIR efficiency from the same locus with and without downstream transcription. The thiamine regulon composed of *THI5*, *SNO3* and *SNZ3* located in the left subtelomere of chromosome VI is transcribed in the absence of thiamine in the medium and switched off in the presence of thiamine (Llorente et al., 1999) (Figure S4A). We performed a reciprocal translocation to position this regulon just downstream of the IVR-10 locus (Figure 4A). In the translocated strain, the IVR-10 locus is positioned about 16 kb away from the telomere. BIR efficiency from this locus in the translocated strain is 29 +/- 2% in the presence of thiamine compared to 13 +/- 2% in the absence of thiamine (Figure 4A). We conclude that transcription downstream of a BIR initiating locus impairs BIR initiated from a chromosome fragmentation vector.

The Silent Information Regulator Sir proteins promote heterochromatinization of subtelomeres. We looked at the effect of *SIR3* deletion which encodes a structural component of the Sir complex. We found a significant increase in BIR efficiency from

the IVR-10 and IVR-57 loci in the absence of *SIR3* (Figure 4B). This is compatible with heterochromatin impairing BIR downstream and close to the BIR initiation locus only, but not when BIR events are initiated farther away.

Effect of trans-acting factors on BIR

In addition to cis-acting factors, other trans-acting factors may affect BIR and contribute to the low efficiency of BIR when it initiates far away from the telomere. A *DUN1*-dependent increase in the level of dNTPs is observed during BIR occurring in G2/M (Deem et al., 2011) as well as during the formation of type II survivors in the absence of telomerase (van Mourik et al., 2018). The fact that BIR requiring long DNA synthesis tracts is less efficient than BIR requiring short DNA synthesis tracts could indicate that the dNTP pool is a limiting factor for BIR in the former situation. We tested this possibility by deleting the ribonucleotide reductase inhibitor *Sml1* which is known to increase the dNTP pool (Deem et al., 2011). This did not increase BIR efficiency, but instead was associated with a slight decrease in BIR efficiency from the IVR-117 locus only (Figure 5A). On the contrary, in the absence of *Dun1*, which is required for *Sml1* degradation and therefore up-regulation of the ribonucleotide reductase activity, we observed a reduction in BIR efficiency from IVR-57 and IVR-10 loci (Figure 5A). Despite this decrease, BIR efficiency from IVR-10 is still much higher than BIR efficiency from IVR-57 in a wild type context, once again underlining the major contribution of telomere proximity to BIR efficiency.

Overexpression of *Rad51* and *Rad52* were shown to increase BIR efficiency using a chromosome based assay (Lydeard et al., 2010; Ruff et al., 2016). In our plasmid-based assay, overexpression of *Rad52* increases BIR efficiency from the IVR-117 and IVR-57 loci while the increase is significant only from IVR-117 when *Rad51* is

overexpressed (Figure 5B). The absence of effect of Rad51 and Rad52 overexpression on BIR initiated at the IVR-10 locus may come from shorter ssDNA intermediates during this BIR reaction that would not require extra Rad51 and Rad52 to be stabilized. Overall, BIR efficiency under these overexpression conditions is still low, and BIR initiated close to TEL04R from the IVR-10 locus still much higher than BIR efficiency from the IVR-57 and IVR-117 loci when Rad52 or Rad51 are overexpressed. In contrast to Rad51 and Rad52, the helicase Mph1 counteracts BIR (Mazón and Symington, 2013; Mehta et al., 2017; Pham et al., 2021). Consistent with this, in the absence of Mph1 we observed increased BIR efficiency from the IVR-117, IVR-57 and IVR-10 loci (Figure 5C).

Srs2 promotes BIR both at telomere proximal and distal sites in diploid cells, but only close to telomeres in haploid cells independently of Y' repeated elements

Srs2 is crucial to prevent Rad51 mediated toxic interactions involving the long ssDNA tract exposed during BIR (Elango et al., 2017). Therefore, Srs2 might be more important for BIR reactions initiated from telomere distal loci compared to telomere proximal loci because they may expose longer ssDNA tracts (Elango et al., 2019). To our surprise, in a haploid background, the absence of Srs2 decreases BIR efficiency only from the IVR-10 locus (Figure 6A). On the contrary, in a diploid background, BIR efficiency is decreased from all loci in the absence of Srs2 (Figure 6D). Because some Srs2 functions are affected by heterozygosity at the *MAT* locus but not by the ploidy itself (Heude and Fabre, 1993), we deleted the *MATa* locus from the previous diploid strain. In the absence of Srs2, BIR efficiencies were decreased in a diploid background

with and without *MATa*, but slightly less without *MATa* (Figure 6D). This shows that the BIR defect of a *srs2* null mutant in a diploid background is mainly due to the ploidy. The decrease of BIR efficiency from the telomere proximal locus in the haploid background in the absence of Srs2 may result from the presence of surrounding repeated sequences that may interfere with BIR. In the case of the right end of chromosome IV, these repeated sequences include the solo delta LTR YDRWdelta031 present in the IVR-10 locus that is used to initiate BIR from the fragmentation vector. In addition, from left to right the repeats from TEL04R comprise an X element core sequence, X element combinatorial repeats, a short stretch of telomeric repeats, and a long Y' element before the telomeric repeats (Figure 6A). To distinguish between the effect of the solo LTR and the subtelomeric repeats in the absence of Srs2, we took advantage of the translocated strain where the last right 113 kb of chromosome IV are replaced by the last right 12 kb of chromosome XVI (Figure 3B and 6B). In this translocated strain, the IVR-117 locus initially positioned 117 kb away from the telomere is now positioned 16 kb away from it. Importantly, this BIR initiating region is devoid of LTR, and the last right 12 kb of chromosome XVI also contain a X element core sequence and a short Y' element in addition to the telomeric repeats. In the presence of Srs2 in this translocated strain, BIR initiated from the IVR-117 locus is about five times (16.53/3.14) more efficient than in the absence of Srs2. This observation phenocopies what is observed in the parental strain and in the IVR-113_XVI-12 background itself at the most telomere proximal locus IVR-10. These results show that in the absence of Srs2 the defect in BIR efficiency at the most telomere proximal loci is independent of the LTR present in the BIR initiating region of chromosome IV. This BIR defect may therefore come from other repeats common to

TEL04R and TEL16R, which include the X element core sequence, Y' elements and telomeric repeats.

The X element core sequence and the telomeric repeats are common to all telomeres but not the Y' elements. To test the effect of the presence of Y' elements on BIR, we translocated the last 10 kb of the right end of chromosome VI comprising an X element core sequence and telomeric repeats only to replace the last 113 kb of the right arm of chromosome IV (Figure 6C). In the absence of Srs2, this translocated strain showed a BIR defect when it was initiated at the two telomere proximal loci IVR-117 and IVR-10. This shows that the effect of Srs2 on BIR initiated near telomeres is independent of Y' elements. Whether such an effect depends on the X element core sequence and / or the telomeric repeats remains to be determined.

Srs2 restrains ectopic recombination

The YDRWdelta031 LTR from the IVR-10 BIR initiating locus does not impact BIR efficiency in the absence of Srs2 since BIR efficiency is not significantly different for the IVR-117 and the IVR-10 initiating sites neither in the IVR-113_XVI-12 *srs2* strain nor in the IVR-113_VIR-10 *srs2* strain (Figure 6B and C). However, this element may promote ectopic interactions. We analyzed by PFGE followed by Southern blot the sizes of the BIR products from the chromosome fragmentation assay in various strain backgrounds (Figure S3). Initiating BIR at the IVR-10 locus, we observed 21% (15 out of 70 clones) of aberrant size products in a wild type background (Figure S3A), which come from either ectopic BIR due to the YDRWdelta031 or from template switching during the BIR reaction involving other repeats (Smith et al., 2007). The frequency of aberrant size products dropped to 2% (2 out of 89 clones) when BIR was initiated from the IVR-117 locus positioned 16 kb upstream of TEL16R in the translocated IVR-

113_XVI-12 strain (Figure S3C). This significant difference (p -value = 0.00012, Fisher's exact test) suggests that the presence of the LTR within the BIR initiating IVR-10 locus is responsible for most of the aberrant size BIR products.

In addition, BIR initiated from the IVR-117 locus in the wild type background shows only 3% (3 out of 97 clones) of abnormal BIR size products (Figure S3B). Since this BIR initiating locus and its surroundings are devoid of repeats, this significant difference (p -value = 0.00022, Fisher's exact test) with respect to the IVR-10 initiating locus suggests that abnormal size BIR products require repeated sequences at or in the downstream proximity of the BIR initiating locus. Indeed, downstream repeats far away from the BIR initiating locus such as the YDRWdelta031 LTR, as well as the other subtelomeric repeats including the long Y' elements from TEL04R do not promote as many detectable template switching events.

In the absence of Srs2, the frequency of aberrant size BIR products from the IVR-10 locus (28 out of 100 clones, Figure S3) is not statistically different from that observed in the presence of Srs2 (15 out of 70, Figure S3A) (p -value = 0.37; Fisher's exact test). This contrasts with the role of Srs2 in preventing template switching during recombination dependent replication at stalled replication forks in *Schizosaccharomyces pombe* (Jalan et al., 2019). However, the fraction of BIR products longer than expected is similar to the fraction of BIR products smaller than expected in the absence of Srs2 (15 versus 13, respectively, Figure S3D), while it is much lower in a WT background (2 versus 13, respectively, Figure S3A). This significant difference (p -value = 0.02; Fisher's exact test) suggests a qualitative difference in the mechanism generating abnormal size BIR products in the absence of Srs2. In addition, among the 100 *srs2* clones resulting from BIR initiated from the IVR-10 locus, we observed four clones where at least one endogenous chromosome did

not migrate at its expected size (Figure 7 and S3D). Such a situation was never observed in the presence of Srs2 out of 256 clones analyzed, which corresponds to a significant difference with respect to *srs2* clones (p -value = 0.006, Fisher's exact test). These 256 clones include the 70 clones where BIR was initiated from the IVR-10 locus (Figure S3A), the 97 clones where BIR was initiated from the IVR-117 locus (Figure S3B) and the 89 clones where BIR was initiated from the IVR-117 locus in the IVR-113_XVI-12 translocated strain. Furthermore, we did not observe any GCR out of 100 clones obtained by transforming the *srs2* null mutant with the circular chromosome fragmentation vector used as a control (Figure S3E). Although the numbers of GCR observed in *srs2* clones transformed with the linear chromosome fragmentation vector and the control vector are not significantly different (p -value = 0.1212, Fisher's exact test), these results suggest that the GCRs observed depend on the BIR reaction initiated in a *srs2* null background. These GCRs support the existence of recombination intermediates involving multiple chromosomes that have been resolved by structure specific nucleases (Dehé and Gaillard, 2017). Such intermediates can arise through template switching during BIR or through multi-invasions (Piazza et al., 2017; Smith et al., 2007), and are compatible with Srs2 undoing excessive Rad51-mediated ectopic DNA interactions during BIR in a wild type context.

Discussion

Increased efficiency of BIR when initiated close to chromosome ends

Previous work already revealed a high BIR efficiency close to chromosome ends during the repair of I-SceI induced DSBs (Batté et al., 2017). Other reports showed an inverse correlation between the size of DNA to synthesize during BIR and BIR efficiency. Using

a chromosome fragmentation assay similar to ours, Davis and Symington (Davis and Symington, 2004) observed a higher BIR efficiency at telomere proximal compared to telomere distal positions when BIR was initiated on chromosomes II and III. In addition, they observed maximum BIR efficiency when initiation was in a Y' element. Although they related this observation to the repeated nature of the Y' element, the present study shows that the proximity to the telomere is the major determinant of this elevated BIR efficiency. Using a chromosome assay where BIR is triggered by DSB induction at an HO site, Lydeard et al. (Lydeard et al., 2007) made observations compatible with an inverse correlation between the size of DNA to synthesize during BIR and BIR efficiency, which was further supported by Donnianni and Symington (Donnianni and Symington, 2013). Using a more systematic approach monitoring BIR efficiency along the right arm of chromosome IV, we could establish that the inverse correlation between BIR efficiency and the size of DNA to synthesize during BIR is well fitted by a combination of two exponential functions. The quality of the fit by exponential functions of the size of DNA to replicate supports a model where BIR occurs by repeated cycles, in this case cycles of initiation, extension and dissociation.

From the fitting equations, we can notably extract a probability of DNA synthesis continuation after every kilobase of DNA synthesized. This latter parameter could be considered as an apparent processivity parameter of BIR-associated DNA synthesis. This processivity parameter increases after the synthesis of ~35-40 kb of DNA. This is reminiscent of the BIR reaction as proposed by Smith et al. (Smith et al., 2007) where the first part of the reaction is rather unstable with frequent template switches followed by a more stable part with fewer template switches. Our data suggest that the transition between these two stages of the BIR reaction takes place after the synthesis of ~35-40 kb DNA, while frequent template switching between homologs was restrained to

the first 10 kb downstream of the BIR initiation locus in (Smith et al., 2007). The reason for this apparent discrepancy is unclear. Interestingly, template switching during recombination dependent replication, a mechanism highly similar to BIR but induced by a replication block in *S. pombe* (Ait Saada et al., 2018), was observed up to 75 kb from the replication blocking lesion. This could indicate that the distance over which the BIR replisome is highly unstable was originally underestimated (Jalan et al., 2019). Overall, it is tempting to relate the biphasic BIR behavior we observe with recent findings showing that BIR relies first on DNA polymerase delta for about 25-30 kb prior to involving DNA polymerases alpha and epsilon to complete the reaction (Liu et al., 2021). The first unstable and consequently less processive phase of BIR would rely on DNA polymerase delta only. The second more stable and processive phase of BIR would rely on DNA polymerases delta, alpha and epsilon. A major unanswered question is how the transition between these two phases occurs. So far, DNA polymerase alpha has been shown to interact with Rad51 at least in *Xenopus laevis* (Kolinjivadi et al., 2017), with the Cdc13-Stn1-Ten1 complex (Lue et al., 2014), and with the Mcm2-7-GINS-Cdc45 complex (Georgescu et al., 2015). Whether any of these pathways is at play during long range BIR is unknown but deserves to be determined. Interestingly, the limited dataset for BIR initiated at a chromosomal HO induced DSB shows higher BIR efficiencies than those observed here for the chromosome fragmentation assay notably for the telomeric distal loci, suggesting a higher probability of BIR initiation from these loci (Donnianni and Symington, 2013; Ruff et al., 2016). This may illustrate the fact that the chromosome fragmentation vector is less stable than a broken natural chromosome presumably due to its sensitivity to the DNA end resection machinery (Costelloe et al., 2012), as well as the need to generate a full

telomere from the telomeric seed present at the opposite end from the one initiating BIR.

Cis- and trans-acting factors that inhibit BIR

i. Transcription. We found that induction of transcription right downstream of a BIR initiating locus decreases BIR efficiency. The successive encounters with transcribed genes likely participate to the instability of the BIR associated DNA synthesis. Importantly, the transcription effect on BIR efficiency applies only for BIR events initiated at proximity of the transcribed region, but not 47 kb away from it. This suggests that only the first and less stable phase of BIR is sensitive to transcription from the template DNA. While we did not test it here, recent work showed that the transcription inhibitory effect on BIR is specific to converging transcription (Liu et al., 2021). This may be particularly relevant when telomeres are maintained by BIR in the absence of telomerase. In this context, two classes of recombination can occur, among which the class I propagates Y' elements by BIR to all chromosome ends. Notably, for all the 17 out of 32 chromosome ends that contain at least one Y' element in the reference S288C strain, the transcription orientation of the Y' elements is systematically toward the telomere. Knowing that Y' elements are transcribed in strains deprived of telomerase activity (Yamada et al., 1998), the co-orientation between the directionality of the BIR DNA synthesis and the Y' transcription is likely under strong selection pressure.

ii. Heterochromatin. We observed increased BIR efficiency in the absence of Sir3 when BIR was initiated at 57 and 10 kb from the telomere. This suggests that BIR associated DNA synthesis is inhibited by heterochromatin, especially during the first and unstable phase of the reaction. These observations are in line with recent results showing a BIR

inhibition by Sir2 when Sir2 is loaded on the BIR template (Pham et al., 2021). In addition, subtelomeric heterochromatin prevents DNA end resection and therefore stabilizes the telomeric proximal DNA fragment and ensuing two-ended recombination (Batté et al., 2017). Overall, heterochromatin promotes two-ended recombination at the expense of BIR in heterochromatic subtelomeric regions by directly impairing BIR, likely at the DNA synthesis step, and by impairing DNA end resection which prevents the loss of the telomere proximal fragment.

iii. DNA modifying enzymes. In addition to transcription, replication and chromatin that affect BIR in cis, different enzymes impair BIR. Exo1 and Sgs1 mediated DNA end resection impairs BIR both in a plasmid-based chromosome fragmentation assay and in a chromosomal assay (Costelloe et al., 2012; Lydeard et al., 2010; Marrero and Symington, 2010; Ruff et al., 2016). The Mph1 helicase is also known to restrict BIR (Mazón and Symington, 2013; Mehta et al., 2017; Pham et al., 2021). Interestingly, we found a higher BIR increase in the absence of Mph1 at telomere distal compared to telomere proximal regions. This may be explained by the longer exposure of the running D-loop to the action of Mph1. This running D-loop is also likely sensitive to structure specific nucleases able to cleave it since the combined absence of Mus81, Yen1 and Rad1 yields optimal BIR frequency in a DSB repair chromosomal assay (Mazón and Symington, 2013).

Factors that promote BIR

BIR relies on the homologous recombination machinery, the polymerases delta, alpha and epsilon, and the Pif1 helicase. Factors protecting the long ssDNA generated behind the migrating D-loop also promote BIR. These factors include RPA, as well as Rad51 and Rad52, whose overexpressions increase BIR efficiency (Lydeard et al.,

2010; Ruff et al., 2016). We recapitulated this latter property in our assay at the IVR-117 and IVR-57 BIR initiating loci but not at the IVR-10 locus, likely because of shorter ssDNA tracts not requiring additional Rad51 or Rad52 to be stabilized (Figure 5B). The Srs2 helicase is another factor that was shown to prevent toxic DNA interactions involving the long ssDNA generated behind the migrating D-loop (Elango et al., 2017). Consistently, we found that Srs2 promotes BIR at any initiating locus tested in diploid cells. Unexpectedly, we observed this facilitation only when BIR is initiated 10 kb upstream of the telomere in a haploid background, while Elango et al. observed a BIR defect in the absence of Srs2 in a haploid background disomic for chromosome III (Elango et al., 2017). This apparent discrepancy may come from different experimental conditions, such as the use of a chromosome fragmentation vector versus a chromosome III disome, or the use of a transformation-based assay involving asynchronous cells versus G2-arrested cells where two copies of the template chromatid are present. Suspecting that the BIR defect we observed in the absence of Srs2 in haploid cells resulted from the presence of subtelomeric repeats, we further showed that Srs2 facilitates BIR independently of the LTR present in the IVR-10 BIR initiating locus, and independently of the subtelomeric Y' elements. The most likely hypothesis, still to be formally demonstrated, postulates that Srs2 promotes BIR in a haploid background close to telomeres by preventing ectopic recombination between the repeated X elements core sequences and / or between telomeric repeats themselves. Such a scenario would explain the defect in formation of survivors in the absence of telomerase observed in a *srs2* null mutant (Kockler et al., 2021). The link between the BIR defect in the absence of Srs2 and the presence of repeated sequences is further supported by the fact that the BIR defect in a diploid *srs2* null background is not suppressed by knocking out the *MATa* allele and therefore results

from the ploidy of the cell. This diploid specific BIR phenotype observed in a *srs2* null background is in line with the diploid specific lethality of the *srs2K41A* helicase-dead mutant that accumulates toxic inter-homolog joint molecule intermediates (Keyamura et al., 2016). It is also in line with the higher sensitivity to genotoxic agents of a *srs2* null diploid strain compared to a *srs2* null haploid strain (Bronstein et al., 2018). Finally, the fact that this Srs2 phenotype is restricted to BIR events initiated in the vicinity of the telomere further supports the biphasic nature of the BIR-associated DNA synthesis step, with ssDNA being more accessible during the first phase of BIR.

In conclusion, multiple factors act in parallel to inhibit BIR and promote two-ended recombination when possible. Rad52, Rad59, RPA, Mph1 and MRX promote capture of the second end at two-ended DSBs which facilitates two-ended recombination at the expense of one-ended recombination (Pham et al., 2021). Here we show that BIR efficiency is further impaired by the extent of DNA to replicate, and by multiple chromosome features including transcription, heterochromatin and DNA repeats. In this context, it is important to note that even abortive BIR events promote genomic rearrangements like segmental duplications when coupled to joining with a chromosome fragment capped by a telomere (Hastings et al., 2009; Koszul et al., 2004; Li et al., 2021; Yang et al., 2020). Last but not least, it was recently shown that *S. cerevisiae* completes DNA replication after metaphase by a process reminiscent of mitotic DNA synthesis (MIDAS) observed in mammalian cells exposed to replication stress (Minocherhomji et al., 2015), and that this process primarily affects subtelomeric regions (Ivanova et al., 2020). *S. cerevisiae* DNA replication therefore relies on an efficient MIDAS-like mechanism to replicate the last tens of kb of its chromosomes, which is precisely the range of size over which we found BIR to be the most efficient.

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Figures legends

Figure 1: BIR efficiency along the right arm of chromosome IV. **A.** The BIR assay used in this study is based on the transformation into yeast cells of a linearized chromosome fragmentation vector (CFV). This CFV contains a telomere seed (black arrowhead), the *URA3* auxotrophic marker, the centromere from chromosome IV (CEN4) and a region of homology with the genome to initiate BIR (*KANMX4* or *BUD3*). This CFV being deprived of any origin of replication, the only way it can be maintained into the cell is after undergoing BIR to incorporate an origin of replication from the chromosome as well as a telomere to be stabilized. The number of transformants obtained using the linearized plasmid reflects BIR efficiency. **Top left:** the region of homology with the genome is the *KANMX4* selection cassette. This cassette has been used to systematically replace individually all the *S. cerevisiae* ORFs keeping the same orientation of transcription as the endogenous ORFs (Winzeler et al., 1999). **Top right:** the region of homology with the genome encompasses *BUD3*. **B.** Ratio between BIR initiated from the *KANMX4* selection cassette from different loci along the right arm of chromosome IV and BIR initiated from *BUD3*. Mean ratios of five (1,080; 981; 875; 11),

four (781; 678; 383; 282; 182) and three (480; 115) independent experiments are shown. Error bars represent standard deviation.

Figure 2: A. Diagram representing twelve BIR initiating loci used to generate Figure 2B (Table S3). Top: wild type strain with nine BIR initiating loci on the right arm of chromosome IVR. Bottom: translocated strain with three BIR initiating loci IVR-398, IVR-338 and IVR-291 corresponding to the IVR-117, IVR-57 and IVR-10, respectively, from the parental non-translocated strain. **B.** BIR efficiency modelled as a combination of two exponential functions of the length of DNA to synthesize. BIR efficiency is expressed as the ratio between the number of transformants from the linearized chromosome fragmentation vector and the number of transformants from the circular chromosome fragmentation vector containing an autonomous replicating sequence (ARS). The two red curves represent exponential law fits. See text for details.

Figure 3: BIR efficiency in translocated strains where the distance between the BIR initiating loci and TEL04R was modified. **A.** The translocation exchanges the last 101 kb of the right arm of chromosome VII with the last 10 kb of the right arm of chromosome IV, increasing by 91 kb the length of DNA to synthesize for BIR initiated at the IVR-117, IVR-57 and IVR-10 loci. WT: reference (wild type) strain. **B.** The translocation exchanges the last 12 kb of the right arm of chromosome XVI with the last 113 kb of the right arm of chromosome IV. This decreases by 101 kb the length of DNA to synthesize for BIR initiated at the IVR-117, but does not modify the distance to the telomere of the IVR-57 and IVR-10 loci. The WT points are the same in the two graphs and represent the means of 49 independent experiments. Values for the translocated strains represent the means of three independent experiments. Error bars

represent standard deviations. Indicated are Student's *t*-test *p*-values <0.05 when comparing a given point from a translocated strain with its equivalent from the parental strain without translocation.

Figure 4: BIR efficiency is decreased by cis-acting factors including transcription and heterochromatin of the template chromatid. **A.** A chromosome translocation positioned the last 16 kb of the left arm of chromosome VI downstream of the IVR-10 BIR initiating locus. This region contains *THI5*, *SNZ3* and *SNO3* where transcription is active in the absence of thiamine in the growth medium and inactive in its presence. The arrows indicate the transcription orientation of the corresponding genes. Values are the means of three independent experiments in the presence of thiamine and four experiments in the absence of thiamine. Indicated is the Student's *t*-test *p*-value <0.05 when comparing BIR efficiency with or without thiamine in the growth medium. **B.** BIR efficiency from the IVR-117, IVR-57 and IVR-10 loci in a *sir3* null mutant. WT values are identical to Figure 3. The *sir3* values correspond to the means of five independent experiments. Indicated are Student's *t*-test *p*-values <0.05 when comparing BIR efficiency from a given locus in a mutant strain with the BIR efficiency from the same locus in the WT strain. Error bars represent standard deviations.

Figure 5: BIR efficiency is affected by the trans-acting factors Dun1, Rad51, Rad52 and Mph1. Indicated are Student's *t*-test *p*-values <0.05 when comparing BIR efficiency from a given locus in a mutant strain with the BIR efficiency from the same locus in the WT strain. Error bars represent standard deviations. **A.** BIR efficiency of the null mutants *dun1*, *sml1* and *dun1 sml1* from IVR-117, IVR57 and IVR-10 loci. WT values are identical to Figure 3. Mutant values are the means of four independent

experiments for *dun1* and three independent experiments for *sml1* and *dun1 sml1*. **B.** Similar to A with *RAD51* and *RAD52* overexpression (OE) from a multicopy vector. Values are the means of three independent experiments for each condition. **C.** Similar to A with the *mph1* null mutant. Values are the means of seven independent experiments for IVR-117 and IVR-10 and six independent experiments for IVR-57.

Figure 6: BIR efficiency in the absence of Srs2. **A.** Parental strain configuration. The IVR-10 locus overlaps a Ty1 LTR (YDRWdelta31). The telomeric region of the right arm of chromosome IV TEL04R is composed of an X element core sequence (XC), a ca. 475 bp sequence common to all chromosome ends; X element combinatorial repeats (XR); an internal stretch of telomeric repeats (TR); a long Y' element (Y'-L) and a terminal stretch of telomeric repeats (right arrowhead). In the absence of Srs2, BIR efficiency decreases from the IVR-10 locus only. **B.** The last 113 kb of the right arm of chromosome IV were replaced by reciprocal translocation with the last 12 kb of the right arm of chromosome XVI. The telomeric region of the right arm of chromosome XVI TEL16R is composed of a XC; a short Y' element (Y'-S) and a terminal stretch of telomeric repeats. The IVR-117 locus is 16 kb away from the telomere in the translocated strain and does not overlap any LTR. In the absence of Srs2, BIR efficiency decreases from the IVR-117 and IVR-10 loci that are located 16 and 10 kb away from the flanking telomeres TEL16R and TEL04R, respectively. **C.** The last 113 kb of the right arm of chromosome IV were replaced by reciprocal translocation with the last 10 kb of the right arm of chromosome VI. The telomeric region of the right arm of chromosome VI TEL06R is composed of a XC and a terminal stretch of telomeric repeats. The IVR-117 locus is 14 kb away from the telomere in the translocated strain and does not overlap any LTR. In the absence of Srs2, BIR efficiency decreases from

the IVR-117 and IVR-10 loci that are located 14 and 10 kb away from the flanking telomeres TEL06R and TEL04R, respectively. **D.** Effect of ploidy and mating type heterozygosity on BIR efficiency. WT values in panels A are the same as in Figure 3. The *srs2* values of panels A and the values of panel C are the means of three independent experiments. The values of panels B and D are the means of four experiments. Indicated are Student's *t*-test *p*-values <0.05 when comparing BIR efficiency from a given locus in a mutant strain with the BIR efficiency from the same locus in the relevant reference strain. Error bars represent standard deviations.

Figure 7: PFGE and Southern blot of BIR products initiated at the IVR-10 locus in the absence of Srs2. Ethidium bromide stained gels (left) and corresponding radioactive signal from Southern blot using a *URA3* probe specific of the chromosome fragmentation vector (right) are shown. Arrowheads indicate abnormal size chromosome fragments (CF) resulting from BIR initiated by the chromosome fragmentation vector. Gross chromosomal rearrangements (GCR) are indicated by arrows. Roman numbers on the left correspond to *S. cerevisiae* chromosome numbers.

Materials and Methods

Yeast strains and growth conditions

Yeast strains used in this study are derivatives of *S. cerevisiae* S288C and are listed in Table S1. Standard media, growth conditions and genetic methods are as described in (Amberg et al., 2005). Genomic targeting experiments were performed by PCR-mediated gene replacement (Baudin et al., 1993), followed by PCR analysis for discriminating correct and incorrect targeting. Details of the primers used for gene

disruption and confirmation are available on request. The UV hyper-sensitivity of the *srs2* null mutants was verified. Translocations were constructed according to (Fleiss et al., 2019). Briefly, two CRISPR-Cas9 mediated DSBs were generated at the two translocation breakpoints. Repair of these DSBs using two donor fragments containing the two translocation junctions yields the desired translocation. Induction of two DSBs is achieved by cloning two guide RNAs in the pGZ110 plasmid expressing Cas9. Donor fragments are 90 base pairs (bp) long DNA molecules containing two 45 bp regions flanking the translocation point. Translocations were confirmed by PCR and by pulse field gel electrophoresis as described in (Smith et al., 2007). Oligonucleotides used for translocations are in Table S2.

Plasmids

Chromosome fragmentation vectors (CFVs) all derive from the CFV pLS192 described in (Marrero and Symington, 2010). All these plasmids contain a centromere, the *URA3* selection cassette, a telomere seed and a region of homology to a genomic locus to initiate BIR after linearization by *Sna*BI digestion (Figure 1). The plasmid pADW17 is identical to pLS192 but, in addition, contains an ARS (autonomously replicating sequence) to allow self-replication after transformation in yeast. The plasmid pADW17 is used as a control for yeast transformation efficiency.

CFVs were built by replacing the D8B genomic region containing *BUD3* from pLS192 by another genomic locus after *Eco*NI-*Bgl*II double digest (Davis and Symington, 2004; Marrero and Symington, 2010). Regions of interest were PCR amplified using a high-fidelity DNA polymerase generating blunt ended fragments, with one oligonucleotide containing either a *Bam*HI or a *Bgl*II restriction site for subsequent semi-blunt cloning. The *Eco*NI site was made blunt after T4 DNA polymerase treatment. Plasmid pBL003

contains the *KANMX4* cassette. All other CFVs contain ~5kb long genomic regions located on the right arm of chromosome IV. Table S3 lists all the CFVs used.

Plasmid pGZ110_synth4 was used as a substrate to generate novel combinations of pairs of guide RNAs cloned in a the Cas9 expressing plasmid pGZ110 and generate on-demand chromosomal translocations (Fleiss et al., 2019). *RAD51* and *RAD52* overexpression was done using the high-copy-number plasmids pRS423_*RAD51* and pRS423_*RAD52*, respectively (Ruff et al., 2016), and was checked by RT-qPCR (Figure S4B).

BIR assay

Chromosome Fragmentation Vectors (CFVs) were digested with SnaBI for linearization and 100ng were used to transform competent yeast cells (Costelloe et al., 2012; Davis and Symington, 2004; Morrow et al., 1997). Cells were plated on synthetic medium lacking uracil to select for Ura⁺ transformants. In parallel, competent yeast cells were transformed with the circular CFV pADW17 plasmid to control for transformation efficiency. BIR efficiency corresponds to the number of transformants obtained with a linearized CFV divided by the number of transformants obtained with the circular pADW17 using the same amount of DNA. BIR efficiency of the *pif1m2* mutant was determined by using the pRS416 circular plasmid as a control. Indeed, pADW17 did not yield any transformant in this genetic background, likely because of the telomeric sequence it contains. Yeast transformants were counted after 3 days of growth at 30°C. BIR efficiencies were determined at least three times for each strain and the mean values were used to determined statistical significance using Student's *t*-test.

Induction of transcription in the absence of thiamine

Thiamine-free medium was prepared using Yeast Nitrogen Base Vitamin Free medium (FORMEDIUM), which composition is based upon the formulation of Yeast Nitrogen base except that all vitamins are omitted, and complemented with biotin 0.002 mg/l, Ca-panthotenic acid 0.4 mg/l, inositol 2 mg/l, p-aminobenzoic acid 0.2 mg/l. Thiamine 0.4 mg/l was added when required. Yeast cells were grown to stationary phase overnight with or without thiamine prior dilution the next morning in the same medium and grown to exponential phase. Yeast cells were transformed with the CFVs and transformants were selected on synthetic medium lacking uracil in the presence or absence of thiamine.

RNA isolation and qPCR

Total RNA was isolated using the hot acid-phenol method (Schmitt et al., 1990), treated with RNase-free DNase I (Qiagen) and reverse transcribed using the Superscript III reverse transcriptase (Life technologies) with random hexanucleotide primers (Sigma-Aldrich). Quantitative PCR amplification of cDNA was carried out using the SYBR Premix Ex Taq II (Ozyme) using the following oligonucleotides: THI5_F (5'-AAGACTACACCGCCGTCA-3') and THI5_R (5'-AGCAAGCCAACTTGTCAATTC-3') for *THI5*; RAD51_cDNA_F (5'-TTCTTCCACCACGCGATTAG-3') and RAD51_cDNA_R (5'-AGATCGCGAACACACATTCA-3') for *RAD51*; RAD52_cDNA_F (5'-CTGCTAGCTCAAACCCAGAG-3') and RAD52_cDNA_R (5'-ACTCGCTGGAATATGCTTGG-3') for *RAD52*; ACT1_F (5'-CTATGTTACGTCGCCTTGGA-3') and ACT1_R (5'-TTTGGTCAATACCGGCAGAT-3') for *ACT1* used as a control.

Statistical analysis and modelling

All analyses were performed with the R environment (<http://www.R-project.org/>). The relationship between BIR efficiency and the length of DNA to synthesize (Figures 2 and S1) was fitted using the nonlinear least squares method implemented in R function `nls()`. The goodness of fit of the models was estimated using Akaike's Information Criterion calculated with R function `AIC()`. Student's *t*-tests were performed using R function `t.test()` with the two-sided option. Differences in proportions were tested using Fisher's exact test for count data implemented in R function `fisher.test()`.

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A.

BIR from *KANMX4*

BIR from *BUD3* control locus

B.

BIR from *KANMX4* / BIR from *BUD3*

Figure 2

Figure 3

A.

B.

Figure 4

Figure 5

Figure 6

A.

B.

C.

D.

Figure 7

Supplementary Figures

Figure S1: Fitting the relationship between BIR efficiency and the length of DNA to synthesize. Combinations of two exponential functions are presented, with the first function fitting the data either from IVR-10 to IVR-26, or from IVR-10 to IVR-36, or from IVR-10 to IVR-41, or from IVR-10 to IVR-46.

Figure S2: BIR efficiency in *pol32* and *pif1m2* mutants. Note that the pADW17 plasmid containing a telomeric seed was used as a transformation control for the *pol32* mutant (left graph) but could not be used in the *pif1m2* mutants (right graph). Instead, the pR4316 plasmid was used. The transformation efficiency of pRS316 is higher than the transformation efficiency of pADW17. This likely explains the difference in BIR efficiency between the WT strains from the two graphs. Indicated are Student's *t*-test *p*-values <0.05 when comparing BIR efficiency from a given locus with its equivalent from the corresponding WT. WT values from the left graph are the same as in Figure 3 and represent the means of 49 independent experiments. The *pol32* values from the left graph are the means of three independent experiments. WT values from the right graph are the means of four independent experiments, and mutant values from the right graph are the means of three independent experiments. Error bars represent standard deviations.

Figure S3: PFGE analysis of BIR products. Individual transformants were used to prepare DNA plugs that were analyzed by PFGE. Each gel lane corresponds to a unique transformant. Ethidium bromide stained gels (left) and corresponding radioactive signal from Southern blots using a *URA3* probe specific of the chromosome fragmentation vector (right) are shown. Arrowheads indicate abnormal size chromosome fragments (CF) resulting from BIR initiated by the chromosome fragmentation vector. Gross chromosomal rearrangements (GCR) are indicated by arrows. **A.** Reference strain BY4741 transformed with the chromosome fragmentation vector initiating BIR at the IVR-10 locus. **B.** Reference strain BY4741 transformed with the chromosome fragmentation vector initiating BIR at the IVR-117 locus. **C.** Translocated IVR-113_XVI-12 strain transformed with the chromosome fragmentation vector initiating BIR at the IVR-117 locus. Note that chromosome IV from this strain is 101 kb shorter than its normal size. This is translated by a longer migration in the gel and a longer distance from chromosome XII as compared to all PFGE using non-translocated strains. **D.** *srs2* null strain transformed with the chromosome fragmentation vector initiating BIR at the IVR-10 locus. Note that GCRs involving chromosome XII that contains the rDNA locus were not considered. **E.** *srs2* null strain transformed with the circular chromosome fragmentation vector. Ethidium bromide stained gels only are shown since no BIR takes place in this context. The rationale of this experiment was to test for potential GCR in the absence of Srs2. No GCR were detected out of 100 clones.

A.

B.

C.

D.

E.

A.

THI5/ACT1

B.

RAD51/ACT1 *RAD52/ACT1*

Figure S4: Quantification of mRNA levels by RT-qPCR using *ACT1* for normalization. **A.** Relates to Figure 4A. IVR-10_VIL-16 is the translocated strain containing *THI5* translocated downstream of the IVR-10 kb locus. Mean ratios of *THI5* and *ACT1* RT-qPCR quantifications from two independent experiments are indicated. Error bars indicate the deviation from the mean. **B.** Relates to Figure 5B. Ratios of *RAD51* and *RAD52* RT-qPCR quantifications normalized by the RT-qPCR quantification of *ACT1*. Overexpression of *RAD51* and *RAD52* were verified on one experiment only.

Table S1. List of strains

strain number	genotype	origin
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(Winzeler et al., 1999)
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr541c::KANMX4 (11kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr479c::KANMX4 (135kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr443c::KANMX4 (182kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr387c::KANMX4 (282kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr338c::KANMX4 (383kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr294c::KANMX4 (480kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr198c::KANMX4 (378kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr146c::KANMX4 (781kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr101c::KANMX4 (875kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr046c::KANMX4 (81kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ydr001c::KANMX4 (1080kb from TEL04R)[#]</i>	(Winzeler et al., 1999)
BLY344	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pol32::HPH</i>	This study
BLY1183	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ura3Δ::NATMX pif1m2</i>	This study
BLY1487	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pol32::HPH ura3Δ::NATMX pif1m2</i>	Gift from V. Borde
BLY1218	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sir3::kanMX</i>	This study
BLY1491	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dun1::HPH</i>	This study
BLY1493	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sml1::HPH</i>	This study
BLY1552	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dun1::HPH sml1::kanMX</i>	This study
BLY433	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mph1::kanMX</i>	This study
BLY1575	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 srs2::kanMX</i>	(Winzeler et al., 1999)
BLY1575_1	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 srs2::kanMX</i>	This study
BLY1598	<i>MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 srs2::kanMX/srs2::kanMX</i>	BLY1575 x BLY1575_1
BLY1597	<i>MATa::NAT/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 srs2::kanMX/srs2::kanMX</i>	This study
BLY1576	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> Translocation IVR-10_VIIR-101	This study
BLY1577	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> Translocation IVR-113_XVIR-12 (No LTR)*	This study
BLY1620	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> Translocation IVR-10_VIIR-291	This study
BLY1622	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 srs2::kanMX</i> Translocation IVR-113_XVIR-10 (No LTR)*	This study
BLY1651	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> Translocation IVR-10_VIR-10 (No Y' element)*	This study
BLY1656	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 srs2::kanMX</i> Translocation IVR-10_VIR-10 (No Y' element)*	This study
BLY1639	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> Translocation IVR-113_VIR-10 (No LTR, No Y' element)*	This study
BLY1654	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 srs2::kanMX</i> Translocation IVR-113_VIR-10 (No LTR, No Y' element)*	This study
BLY1599	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> Translocation IVR-10_VIL-16**	

[#] Strain used to initiate BIR from the *KANMX4* cassette, which location from the right telomere of chromosome IV TEL04R) is given in parentheses

* "No LTR" and "no Y' element" refers to the absence of LTR or Y' element in the chromosome fragment used as BIR template

** VIL chromosome fragment contains the thiamine regulon composed of *SNO3*, *SNZ3* and *THI5*

Table S2. Oligonucleotides used to generate reciprocal translocations

BLY1576: Reciprocal translocation IVR-10_VIIR-101			
Name	Sequence 5' – 3'	Type	Description
TU002	TCGAACGCTCTTCAATCAACTACTATCTTACTTCCACGTTTTAGAGCTAGAAATA GCAAG	gRNA	gRNA targeting the IVR-10 locus
TU003	CTAGACGCTCTTCAAACCTTTTTGTTGGAATGTTAAGCGATCATTTATCTTTCACT GCGG	gRNA	gRNA targeting the VIIR01 locus
TU010a	AAATATTTAATTGCATGTTATTTTGTTTAACTACTATCTTACTTCAAAAGGAAAAA TAAACAATAATTTTCGTTCCCCATCTATCCTTCG	donor	45bp of IVR-10 locus + 45bp of VIIR01 locus
TU010b	CGAAGGATAGATGGGGAACGAAATTATTGTTTTATTTTCTTTTGAAGTAAGAT AGTAGTTAAACAAAATAACATGCAATTAAATATTT	donor	reverse complement of TU010a
TU011a	AGTAGAGCAGATCTTTTAGCAAGGATCCGCTTAACATTCCAACAACACTGGTCC ACCGGACCCTCAGGCTTCCTTGCCCTGTGGAACCAG	donor	45bp of IVR-10 locus + 45bp of IVR-10 locus
TU011b	CTGGTTCACAAAGGCAAGGAAGCCTGAGGGTCCGGTGGACCAGTGTGTTGG AATGTTAAGCGGATCCTTGCTAAAAGATCTGCTCTACT	donor	reverse complement of TU011a
TU014	AGGCAATGTTCTGAGTAAGT	translocation validation	forward IVR-10 locus
TU015	TCATGTGCCAAGGTTTTGTGT	translocation validation	reverse IVR-10 locus
TU016	TACCGCTGTTGATACCAAGGA	translocation validation	forward VIIR01 locus
TU017	TAAAAAGCACGGAAGGAACGC	translocation validation	reverse VIIR01 locus
BLY1577: Reciprocal translocation IVR-113_XVIR-12 (No LTR, presence of Y' element)			
Name	Sequence 5' – 3'	Type	Description
TU004	TCGAACGCTCTTCAATCGCGATACAGTACGTGTCATGGTTTTAGAGCTAGAAAT AGCAAG	gRNA	gRNA targeting the IVR-113 locus
TU005	CTAGACGCTCTTCAAACCTCAGTGTCAGCATGAGTATTGATCATTTATCTTTCACT GCGG	gRNA	gRNA targeting the XVIR-12 locus
TU012a	AAAACCTCGCCTGCACTCCGAAACGAAATGCGATACAGTACGTGTCTGAAGGAT TATTGGTATTGGCCGTTGTGTTTTTCTATACATAG	donor	45bp of IVR-113 locus + 45bp of XVIR-12 locus
TU012b	CTATGTATAGAAAAACACAACCGCCAATACCAATAATCCTTCAGA CACGTACTGTATCGCATTTTCGTTTCGGAGTGCAGGCGAGTTTT	donor	reverse complement of TU012a
TU013a	GGGCTTACAGCTCCTATGTGGATATGTGAATACTCATGCTGACACATGCGGTT AGTATATATACATATATATATATATATATATATATTTCAA	donor	45bp of XVIR-12 locus + 45bp of IVR-113 locus
TU013b	TTGAATATATATATATATATATATATATATATATATACTAACC GCATGTGTCAGCATG AGTATTCACATATCCACATAGGAGCTGTAAGCCC	donor	reverse complement of TU013a
TU018	ACCACAACGGAGACCCAATC	translocation validation	forward IVR-113 locus
TU019	CGAATACTACCATGAGTTGACCA	translocation validation	reverse IVR-113 locus
TU020	TACTGTTTTGTGGTTGGGAT	translocation validation	forward XVIR-12 locus

TU021	ATGGGTCCAGCGTACAACCTC	translocation validation	reverse XVIR-12 locus
BLY1620: Reciprocal translocation IVR-10_VIIR-291			
Name	Sequence 5' – 3'	Type	Description
TU002	TGCAACGCTCTTCAATCAACTACTATCTTACTTCCACGTTTTAGAGCTAGAAATA GCAAG	gRNA	gRNA targeting the IVR-10 locus
TU105	CTAGACGCTCTTCAAACACCTTTCCAACATGTCGCTGGATCATTTATCTTTCACT GCGG	gRNA	gRNA targeting the VIIR-291 locus
TU107	AAATATTTAATTGCATGTTATTTTGTTTAACTACTATCTTACTTCGGTCGGGCAG TAAAGCAGAATTTAGAAATACTAGTACGATTGTT	donor	45bp of IVR-10 locus + 45bp of VIIR-291 locus
TU108	AACAATCGTACTAGTATTTCTGAAATTCTGCTTTACTGCCCGACCGAAGTAAGA TAGTAGTTAAACAAAATAACATGCAATTAATATTT	donor	reverse complement of TU107
TU109	TACCCGACTCTGAAGAAAAACAAAAACAGCGACATGTTGGAAACACTGGTC CACCGGACCTCAGGCTTCCTTGCCTTGTGGAACCAG	donor	45bp of VIIR-291 locus + 45bp of IVR-10 locus
TU110	CTGGTTCACAAGGCAAGGAAGCCTGAGGGTCCGGTGGACCAGTGTTCCTCA CATGTCGCTGTTTTTTGTTTTCTTCAGAGTCGGGTA	donor	reverse complement of TU109
TU014	AGGCAATGTTTCGTGAGTAAGT	translocation validation	forward IVR-10 locus
TU015	TCATGTGCCAAGGTTTTGTGT	translocation validation	reverse IVR-10 locus
TU115	TAGCGCCTATACGAAGCAGC	translocation validation	forward VIIR-291 locus
TU116	TCTAATCAATGCAACGTGTTTGAC	translocation validation	reverse VIIR-291 locus
BLY1639: Reciprocal translocation IVR-113_VIR-10 (No LTR, No Y' elements)			
Name	Sequence 5' – 3'	Type	Description
TU004	TGCAACGCTCTTCAATCGCGATACAGTACGTGTCATGGTTTTAGAGCTAGAAAT AGCAAG	gRNA	gRNA targeting the IVR-113 locus
TU127	CTAGACGCTCTTCAAACATTGTCAATGAGCAGGTTGAGATCATTTATCTTTCAC TGCGG	gRNA	gRNA targeting the VIR-10 locus
TU140	AAAACTCGCCTGCACTCCGAAACGAAATGCGATACAGTACGTGTCAAT <u>IGG</u> AT TATTTTTGGCTGTTCAAGATTAACAGACTCAGTAG	donor	45bp of the IVR-113 locus + 45bp of the VIR-10 locus
TU141	CTACTGAGTCTGTTTTAATCTTGAACAGCCAAAAATAATCCAATTGACACGTACT GTATCGCATTTTCGTTTCGGAGTGCAGGCGAGTTTT	donor	reverse complement of TU140
TU142	ATGTCCATCAAGACTAGCGTAATAAAAAACAACCTGCTCATTGACATGCGGTTA GTATATATACATATATATATATATATATATATTTCAA	donor	45bp of the VIR-10 locus + 45bp of the IVR-113 locus
TU143	TTGAATATATATATATATATATATATATATATATATATACTAACC GCATGTCAATGAGCA GGTGATTTTTATTACGCTAGTCTTGATGGACAT	donor	reverse complement of TU142
TU018	ACCACAACGGAGACCCAATC	translocation validation	forward IVR-113 locus
TU019	CGAATACTACCATGAGTTGACCA	translocation validation	reverse IVR-113 locus
TU132	ACACACAGAGACCACCGTTTA	translocation validation	forward VIR-10 locus
TU133	AGCCAGGGTATCTTCTGTGAG	translocation validation	reverse VIR-10 locus

BLY1599: Reciprocal translocation IVR-10_VIL-16. Locates <i>SNO3</i> , <i>SNZ3</i> and <i>THI5</i> thiamine regulon on chromosome IV.			
Name	Sequence 5' – 3'	Type	Description
TU002	TCGAACGCTCTTCAATCAACTACTATCTTACTTCCACGTTTTAGAGCTAGAAATAGCAAG	gRNA	gRNA targeting the IVR-10 locus
TU081	CTAGACGCTCTTCAAACGTTCTCAAATACAGTGCTAAGATCATTTATCTTTCACTGCGG	gRNA	gRNA targeting the VIL-16 locus
TU087	AAATATTTAATTGCATGTTATTTGTTTAACTACTATCTTACTTCTCAAATACAGTGCTAATAATCACATGGTTCGTAGAGGGCGTGAA	donor	45bp of the IVR-10 locus + 45bp of the VIL-16 locus
TU088	TTCACGCCCTCTACGAACCATGTGATTATTAGCACTGTATTTGAGGAAGTAAGATAGTAGTTAAACAAAATAACATGCAATTAAATATTT	donor	reverse complement of TU087
TU089	CTGGTTCCACAAGGCAAGGAAGCCTGAGGGTCCGGTGGACCAAGTGAACAGGTCCGGTGATCATTTTCTACAATTTTGTACATAGAAAATT	donor	45bp of the VIL-16 locus + 45bp of the IVR-10 locus
TU090	AATTTTCTATGTACAAAATTGTAGAAAATGATACACCGACCTGTTCACTGGTCCACCGGACCCTCAGGCTTCCTTGCCTTGTGGAACCAG	donor	reverse complement of TU089
TU014	AGGCAATGTTCTGTGAGTAAGT	translocation validation	forward IVR-10 locus
TU015	TCATGTGCCAAGGTTTTGTGT	translocation validation	reverse IVR-10 locus
TU091	GCAAGGCAAGCAGATGCAAT	PCR trans. validation	forward VIL-16 locus
TU092	ACGTGGTATAATAGGCTCAAGGA	PCR trans. validation	reverse VIL-16 locus

Table S3. Chromosome fragmentation vectors (CFVs) used

Plasmid name	Distance from telomere (kb)	Chromosome coordinates of BIR initiating region	Oligonucleotides sequences used to amplify the corresponding genomic loci and clone them into EcoNI-BglII double digested pLS192
CA50	166	1360939 - 1365875	TACAGGGTTTCAGGGATAAATGGA TGTCT <u>AGATCT</u> CTGCTAAGGTGTTTGTACCCGA
pBL013	117	1409510 - 1414996	TTGTCTCCAATTGGCATACG TTCCCGCTGCTGGTACTATT
CAG	99	1428154 - 1433026	CCAATCTTGGCTTCCTCCTA TCAAG <u>GGATCC</u> AATGCAGAGGCGATGAAGC
CAM	57	1469940 - 1474882	ATCTCCGTAAATGAGCAAG TCAT <u>GGATCC</u> GTACGAATTGAAATGAAATGT
TU480	46	1480926 - 1485534	GATGCCAGCCATCATGTCAA TCGAAC <u>GGATCC</u> ATGCATGAGTTTTTGTTCATTTC
TU485	41	1485838 - 1490734	TCTCTGCTTGAGCATTTGGG TCGAAC <u>GGATCC</u> TCAGACCAGGGAAAAACCT
TU491	36	1491010 - 1495847	ATCGCTGTCGGCTAAGTAAA TCGAAC <u>GGATCC</u> GTGCAGAATAACTTGGCGAAAA
CAD	26	1500792 - 1505691	CTGGAAGATATTGTTTATCCG TGTCT <u>AGATCT</u> CATTATTTAATCCTTATATTCCA
pBL014	10	1517068 - 1521977	AAGGTTTTCTGTGGTAGTTTC AGTCAGTGTGACCCAATAAGAA
pLS192 pBL003	97	96821-102096 <i>KANMX4</i>	U2 : CGTACGCTGCAGGTGAC D2 : ATCGATGAATTCGAGCTCG

All CFVs except pLS192 and pBL003 are meant to initiate BIR from chromosome IV regions, which coordinates are from the *Saccharomyces* Genome Database (SGD). pLS192 is meant to initiate BIR from the left arm of chromosome III (Marrero and Symington, 2010). pBL003 contains the *KANMX4* cassette amplified from the BY4741 *rad27::KANMX4* strain using the U2 and D2 oligonucleotides (Winzeler et al., 1999) (http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html). The

resulting CFV is meant to initiate BIR from any *KANMX4* cassette which transcription orientation is from the telomere to the centromere.

BamHI (GGATCC) and BglII (AGATCT) sites are underlined. Note that the regions amplified to construct pBL013 and pBL014 contain a BamHI and a BglII site, respectively.