Homolog-Dependent Repair Following Dicentric Chromosome Breakage in *Drosophila melanogaster*

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ABSTRACT Double-strand DNA breaks are repaired by one of several mechanisms that rejoin two broken ends. However, cells are challenged when asked to repair a single broken end and respond by: (1) inducing programmed cell death; (2) healing the broken end by constructing a new telomere; (3) adapting to the broken end and resuming the mitotic cycle without repair; and (4) using information from the sister chromatid or homologous chromosome to restore a normal chromosome terminus. During one form of homolog-dependent repair in yeast, termed break-induced replication (BIR), a template chromosome can be copied for hundreds of kilobases. BIR efficiency depends on Pif1 helicase and Pol32, a nonessential subunit of DNA polymerase δ. To date, there is little evidence that BIR can be used for extensive chromosome repair in higher eukaryotes. We report that a dicentric chromosome broken in mitosis in the male germline of *Drosophila melanogaster* is usually repaired by healing, but can also be repaired in a homolog-dependent fashion, restoring at least 1.3 Mb of terminal sequence information. This mode of repair is significantly reduced in *pif1* and *pol32* mutants. Formally, the repaired chromosomes are recombinants. However, the absence of reciprocal recombinants and the dependence on Pif1 and Pol32 strongly support the hypothesis that BIR is the mechanism for restoration of the chromosome terminus. In contrast to yeast, *pif1* mutants in *Drosophila* exhibit a reduced rate of chromosome healing, likely owing to fundamental differences in telomeres between these organisms.

KEYWORDS break-induced replication; BIR; DNA repair; recombination; chromosome; dicentric; double-strand break; telomere; healing; pif1; pol32

DNA double-strand breaks (DSBs) are a serious problem for cell survival, and if left unrepaired, can lead to genomic instability, aneuploidy, and cancer (Ceccaldi *et al.* 2016). DSBs may occur spontaneously during normal cell metabolism or be produced by exposure to exogenous agents, such as DNA-damaging chemicals or radiation. A number of mechanisms have evolved to repair DSBs (Shibata 2017). Nonhomologous end joining can join two broken ends, and may produce insertions or deletions of several base pairs (Ceccaldi *et al.* 2016). A DSB may also be repaired by homologous recombination (HR), a form of gene conversion that copies homologous DNA sequences from a sister chromatid, homolog, or other matching sequence (Ceccaldi *et al.* 2016; Shibata 2017). During repair by HR, broken ends are processed to generate 3′ single-stranded DNA overhangs. These 3′ single-strand tails can invade homologous sequences, form a D-loop, and initiate DNA synthesis. After a relatively short stretch of DNA synthesis (usually a few hundred or a few thousand base pairs), the invading strand(s) are removed from the D-loop and anneal to complementary sequences on the other broken end. Repair is completed through a combination of synthesis, trimming, and ligation. Single-strand annealing, in which complementary bases on single-stranded tails anneal, is another basis for repairing broken ends and is accompanied by deletions of varying lengths. Although DSBs can be efficiently repaired by any of these mechanisms, they only work if two broken ends are available. Difficulties arise when only a single broken end is present. This situation may occur from a failure to repair a DSB before cell division, from the erosion of telomeres, or by breakage of a dicentric chromosome during anaphase.

The most frequent outcome for a cell with a single broken end is death by apoptosis (Ahmad and Golic 1999; Titen and Golic 2008). Alternatively, a cell can choose to ignore the broken chromosome and continue to divide in a process called adaptation (Mersaoui *et al.* 2015). Adaptation is problematic due to the continued presence of an unrepaired chromosome end, which can fuse with other DSBs, including its

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sister chromatid after replication, to form a variety of chromosome rearrangements or new dicentric chromosomes (Mason and McEachern 2018). One solution that actually repairs the broken end is called healing, in which a new telomere is constructed upon the broken end (McClintock 1939; Haber and Thorburn 1984; Mason *et al.* 1984; Matsumoto *et al.* 1987; Pologe and Ravetch 1988; Levis 1989; Flint *et al.* 1994; Melek and Shippen 1996; Ahmad and Golic 1998; Sprung *et al.* 1999; Rong and Golic 2003; Pennaneach *et al.* 2006; Gao *et al.* 2008; Fortin *et al.* 2009). Although healing can prevent further chromosome fusion events, it is also likely to produce aneuploidy due to the loss of genetic information distal to the site of telomere healing. All these outcomes exact a significant cost, either through the killing of one or more cells, or through the generation of genome instability and aneuploidy.

An alternative that can restore the full length of a truncated chromosome has been demonstrated in yeast (Malkova *et al.* 1996; Morrow *et al.* 1997). Break-induced replication (BIR) begins when a DNA strand from a broken chromosome invades homologous DNA sequences and initiates DNA synthesis (Llorente *et al.* 2008; Anand *et al.* 2013; Malkova and Ira 2013). If a sister chromatid or homolog is used as the template for replication, and replication proceeds to the end of the chromosome, the full length of the chromosome is restored. BIR is also used in phages and bacteria for the initiation and repair of replication forks (Mosig 1998), and is considered an important mechanism for the repair of stalled or collapsed replication forks during S phase in eukaryotes (Haber 1999; Michel 2000; Sotiriou *et al.* 2016; Ait Saada *et al.* 2018). BIR can elongate the ends of chromosomes in the absence of functional telomerase (McEachern and Haber 2006; Doksani and de Lange 2014) and is implicated in a process called alternative lengthening of telomeres (ALT; Min *et al.* 2017; Gaspar *et al.* 2018; Zhang *et al.* 2019), which maintains telomeres in cancer cells that lack telomerase (Henson *et al.* 2002; Cesare and Reddel 2010). Therefore, BIR is an important component of the mechanisms that cells and organisms use to preserve genome integrity by ensuring the completion of replication, by precluding the formation of terminal deficiencies, and by combatting telomere erosion.

Experiments in yeast have found that BIR uses most components of the DNA replication machinery (Lydeard *et al.* 2010); however, there are several key differences between S-phase DNA replication and DNA synthesis by BIR. First, BIR replication is conservative (Donnianni and Symington 2013; Saini *et al.* 2013; Wilson *et al.* 2013; Kramara *et al.* 2018). Second, BIR is much more error-prone than normal replication (Deem *et al.* 2011). Third, BIR involves long fork progression, and is able to synthesize hundreds of kilobases of DNA from a single origin to restore the end of a chromosome (Morrow *et al.* 1997; Saini *et al.* 2013). Finally, Pol32, a nonessential subunit of Polδ, and Pif1 helicase, though not required for normal chromosome replication, are important for the synthesis of new DNA by BIR, particularly long-range synthesis (Johansson *et al.* 2004; Lydeard *et al.* 2007; Smith *et al.* 2009; Saini *et al.* 2013; Wilson *et al.* 2013).

Although BIR has been well documented as a mechanism to restore a chromosome end in yeast, to the best of our knowledge, there has been no systematic study of whether BIR may perform a similar function in higher eukaryotes. One study designed to detect BIR in plants concluded that it was extremely rare or possibly nonexistent. The authors speculated that BIR might not occur in organisms with chromosome arms longer than ~1 Mb, the length of the longest arm in *Saccharomyces cerevisiae* (Schubert *et al.* 2011). But, the fact that BIR has been implicated in several processes in higher eukaryotes suggests that its involvement in the restoration of chromosome termini is worth further exploration, especially in animal models.

We designed experiments to test whether chromosomes with a single broken end could be repaired by BIR in *Drosophila melanogaster*, which has chromosome arms that are ≥30 Mb in length. We found that healing was the predominant form of repair for such chromosomes, but that a significant minority of chromosomes was repaired in a process that utilized the homologous chromosome. Furthermore, mutations in *pif1* and *pol32*, which reduce BIR efficiency in yeast, also diminish the frequency of homolog-dependent repair (HDR) in *Drosophila*, although they do not entirely eliminate it. These results show, for the first time in a higher eukaryote, that the homologous chromosome can provide information that restores a long terminal segment to a broken chromosome, and that this likely occurs through BIR.

Materials and Methods

Fly husbandry and stocks

All flies were raised on standard cornmeal medium at 25°. The stock *y w*; *P{FrTr} (3Lt) 61A5*, *Sb*/TM6, *Ubx* provided the insertion of inverted FRTs near the tip of *3L* (Titen and Golic 2010). A *y w*; *P{nosGal4}1C P{UASFLP}*/TM6, *Ubx* stock was used to provide FLP expression in the male germline. The *nosGal4* and *UASFLP* transgenes have been described elsewhere (Doren *et al.* 1998; Duffy *et al.* 1998). The following y^+ template chromosome markers were obtained from the Bloomington *Drosophila* Stock Center: *P{EPgy2}EY16041* (38.5 kb), *P{EPgy2}EY15596* (40.6 kb), *P{EPgy2}EY04470* (71.6 kb), and *P{EPgy2}scf[EY01124]* (1.3 Mb). Each of these insertions was recombined onto the *nosGal41C UASFLP* chromosome to generate BIR template chromosomes. The stock *y w* $corp^{95B}$; *eyelessFLP/*S^2 *CyO* was used for genetic testing of FRT sites (Kurzhals *et al.* 2011). *Drosophila* strains are available from the authors upon request.

HDR analysis

To test for HDR in the male germline, we crossed virgin *y w*; *FrTr(3Lt) Sb*/TM6, *Ubx* females to *y w*; y^+ *nosGal41C UASFLP*/TM6, *Ubx* males, where the males carried one of the four y^+ marker insertions on chromosome 3. The *y w*; *FrTr(3LT) Sb/*y^+ *nosGal41C UASFLP Sb*$^+$ sons were crossed to *y w* virgin females to examine the fate of chromosomes transmitted through their germline. Progeny were examined

Table 1 Recovery of repaired chromosomes after dicentric breakage in the male germline

Parental males[a]				Progeny								
					Sb							
					No change		Terminal deficiency		HDR			
Distance of y^+ from telomere	Genotype	*N*	Fertile (%)	Sb^+ *n*	*n*	%	*n*	%	*n*	%	Total *Sb*	Sb:Sb^+
38.5 kb	+	778	75	28,587[b]	7017	32	13,246	60	1668	7.6	21,931	0.77
	$pif1^{167}$	228	72	8,624	3849	52	3,364	45	225	3.0	7,438	0.87
	$pol32^{L2}$	596	25	4,975	1332	32	2,646	64	134	3.3	4,112	0.83
40.6 kb	+	567	79	19,207	4075	28	9,217	64	1189	8.2	14,481	0.75
	$pif1^{167}$	238	76	8,826	3423	45	3,879	50	376	4.9	7,678	0.87
	$pol32^{L2}$	615	28	7,535	1967	36	3,281	60	235	4.3	5,483	0.73
71.6 kb	+	484	70	18,770	3377	25	9,120	67	1099	8.0	13,596	0.72
	$pif1^{167}$	195	72	8,793	3878	51	3,519	46	253	3.3	7,650	0.87
	$pol32^{L2}$	698	28	9,264	995	30	2,281	69	44	1.3	3,320	0.35
1.3 Mb	+	476	84	21,061	4212	28	10,065	68	580	3.9	14,857	0.71
	$pif1^{167}$	242	75	10,448	5063	54	4,027	43	323	3.4	9,413	0.91
	$pol32^{L2}$	663	16	3,053	1246	42	1,711	57	25	0.8	2,982	0.98

HDR, homolog-dependent repair.

[a] Cross to assess outcomes is shown in Figure 2A.

[b] In this cross, 28,587 were y^+ Sb^+ and 0 were *y* Sb^+.

for telomere healing, HDR, or unchanged chromosomes (Table 1). Null alleles $pif1^{167}$ (Alexander *et al.* 2016) and $pol32^{L2}$ (Kane *et al.* 2012) were obtained from Mitch McVey. These mutations were combined with *y w*; *FrTr (3Lt) 61A5*, *Sb*/TM6, *Ubx* and with each of the *y w*; y^+ *nosGal41C UASFLP/TM6*, *Ubx* stocks. Both *pif1* and *pol32* are on chromosome 2. *pif1* and *pol32* homozygous males were tested for BIR with each of the four different templates.

Deletions in both *pif1* and *pol32* were confirmed by PCR. For the *pif1* mutant, a 1.7-kb deletion was confirmed by using the forward (5′-GTCTGGTATCCGGCAAGGATC-3′) and reverse primers (5′-CTAATCGAGACCAGCGGCTTG-3′) that flank the deletion site. In wild-type DNA, this primer set generates a 2.8-kb product. In the *pif1* mutant, these primers generate a 1.1-kb product. A second set of primers, 5′-CACCTGTGCGGTGAACATGG-3′ (forward) and 5′-CGTTCCTTCTCCTCCTTGAGC-3′ (reverse), was used to amplify DNA within the 1.7-kb deletion. This second primer set generates a 532-bp product in wild-type flies and no product in the *pif1* mutants.

For the *pol32* mutant, a 1.6-kb deletion was confirmed by using the forward (5′-GTCAAGGCAAATATCGTGCTAGAG-3′) and reverse primers (5′-GCTATTGTAGATGAACGGCGAT-3′) that flank the deletion site. In wild-type DNA, this primer set generates a 2.8-kb product. In the *pol32* mutant, these primers generate a 1.2-kb product. A second set of primers, 5′-GTTTGGTTTTCGGCGCATCC-3′ (forward) and 5′-ATAGCTTCTTCACAGCGGCG-3′ (reverse), was used to amplify DNA within the 1.6-kb deletion. This second primer set generates a 604-bp product in wild-type flies and no product in the *pol32* mutants.

Inverted FRT genetic test

HDR and terminal deficiency (TD) chromosomes were rebalanced over the TM6B,*Tb* balancer chromosome. Males from the recovered HDR and TD stocks were crossed with *yw*, $corp^{95B}$; *eyelessFLP/S*^{2} *CyO* flies (Chakraborty *et al.* 2015). Progeny from this cross were examined for the presence of FRTs using the eye apoptosis test described previously (Titen and Golic 2010; Kurzhals *et al.* 2011).

Genomic DNA preparation

Genomic DNA from five males and five females was isolated by homogenizing the flies in a 200-μl solution of 0.1 M Tris-HCl (pH 9.0), 0.1 M EDTA, 1% SDS, and 0.5% DEPC, and then incubated for 20 min at 70°. After incubation, 14 μl of 8 M potassium acetate was added and homogenates were left on ice for 30 min. Samples were centrifuged for 15 min at 4°. Supernatants were transferred to a fresh tube and genomic DNA was precipitated with 0.5 vol isopropanol, and then centrifuged for 5 min at room temperature. Pellets were washed with 70% ethanol, centrifuged for another 5 min at room temperature, air dried, and dissolved in 100 μl of water. DNA concentration was measured with a Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA).

Quantitative PCR for gene copy number analysis

Quantitative PCR (qPCR) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The FAM-labeled probe and primers were ordered from Integrated DNA Technologies. After 40 cycles of PCR amplification, the Ct values were evaluated for *mthl8* (24 kb from the *3L* telomere) and a sequence we called "Proximal" (77 kb from the *3L* telomere). The *mthl8* forward and reverse primer sequences are 5′- AACCAATCCCAGTTCGATCC-3′ and 5′-GGGCTGAACACCAAGTAGATAA-3′, with a probe sequence of 5′/56-FAM/aatgttgtg/ZEN/gcctttgtgcctagc/3lABkFQ/-3′. The Proximal forward and reverse primers are 5′-CCTTAGGTCTGGTAGCAGTTATTT-3′ and 5′-CCGTTTGACA GCTTGTGTTTC-3′, with a probe sequence of 5′-/56-FAM/

acaacttgc/ZEN/tcgctgtttgttccc/3lABkFQ/-3′. Primers and probes were made for Checkpoint Kinase 2 (Chk2, encoded by *loki*) as an internal control. The Chk2 forward and reverse primer sequences are 5′-CGAGAAGCCTACACCAAGAAA-3′ and 5′-CACTCAGGCTGCAAGATATGA-3′, respectively, with a probe sequence of 5′/56-FAM/tggagcttg/ZEN/ggagtggtgc-tattt/3lABkFQ/-3′. Control DNA from *y w* had two copies of both *mthl8* and *Chk2*. We found that the balancer chromosomes TM6B,*Tb* and TM6,*Ubx* have a *mthl8* mutation, which is undetected by qPCR (0 copy of *mthl8*). When heterozygous with these balancers, the HDR and TD chromosomes were distinguished by *mthl8* copy numbers of one and zero, respectively. Chromosomes repaired by homolog copying directed toward the centromere should have a copy number of three for the Proximal DNA sequence, 77 kb from the end of *3L*.

Polytene chromosome analysis

Polytene chromosome analysis was performed as described previously (Lefevre 1976). Polytene images were acquired with a Zeiss ([Carl Zeiss], Thornwood, NY) Axioskop equipped with phase contrast 40× and 100× objectives. Photographs were captured with an Olympus EVOLT E-330 camera.

Statistical treatments

To compare either results between different marker insertions or different genotypes, 2 × 2 contingency tests were used.

Data availability

Drosophila strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Results

Healing is the predominant mode of repair for a single broken chromosome end in Drosophila

Chromosomes with a single broken end were produced in the male germline by dicentric breakage (Figure 1). FLP-mediated recombination between inverted FRTs in the *P{FrTr}* element on sister chromatids generates a dicentric chromosome and an acentric chromosome. During anaphase, segregation of sister centromeres on the dicentric chromosome forms a bridge. When this bridge breaks in mitosis, each daughter cell receives a chromosome with a single broken end. A *white*$^+$ (w^+) transgene located adjacent and distal to the FRTs ends up on the acentric chromosome that is not stably maintained. The inverted FRTs were inserted near the tip of *3L* on a chromosome that also carries the dominant mutation *Stubble* (*Sb*) on *3R*. The homologous chromosome was marked with *Sb*$^+$, and carried *yellow*$^+$ (y^+) and w^+ in a *P* element inserted near the tip of *3L* (Figure 2).

If the broken *Sb* chromosome is healed by *de novo* telomere addition, a chromosome with a TD will be produced. By mating, these healed chromosomes can be recognized as yellow white Stubble (*y w Sb*) progeny. In our first experiment,

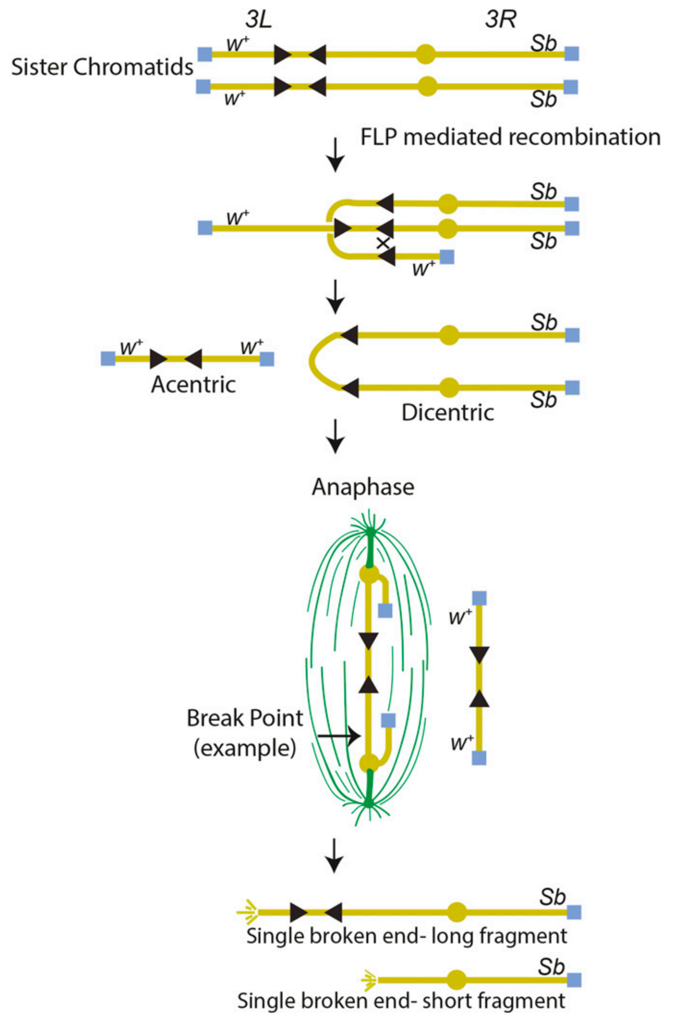


Figure 1 Dicentric chromosome formation and breakage. Schematic showing the generation of a dicentric chromosome 3. FLP induces recombination between inverted FRTs on sister chromatids to produce a dicentric chromosome and a small acentric chromosome. The FRT sites are located in the *P{FrTr}* element, 40.3 kb from the tip of *3L*, with a w^+ gene distal to the FRTs and the dominant *Sb* marker on *3R*. Dicentric formation is indicated by loss of w^+, which is located on the acentric chromosome piece. Asymmetric breakage of the dicentric chromosome, *e.g.*, as indicated by the arrow, produces daughter cells with short (no FRTs) and long chromosome fragments (with FRTs).

the *P* element carrying inverted FRTs, *P{FrTr}*, was inserted 40.3 kb from the tip of *3L*, while the y^+ w^+ *P* element was located 38.5 kb from the tip of *3L* on the *Sb*$^+$ homolog. We used *nosGal4* and *UASFLP* to drive the expression of FLP in mitotically dividing early germ cells of the male germline. The males carrying these elements were testcrossed to *y w* females and their progeny scored. Among the *Sb* progeny, healing was by far the most common repair event, with ~60% of the progeny receiving broken and healed chromosomes (Table 1).

By examining polytene chromosomes from flies heterozygous for terminal deficiencies, we attempted to determine the length of terminal deficiencies. In some cases, the TD was visible (Figure 3), though in most cases it was not. Since the *P*

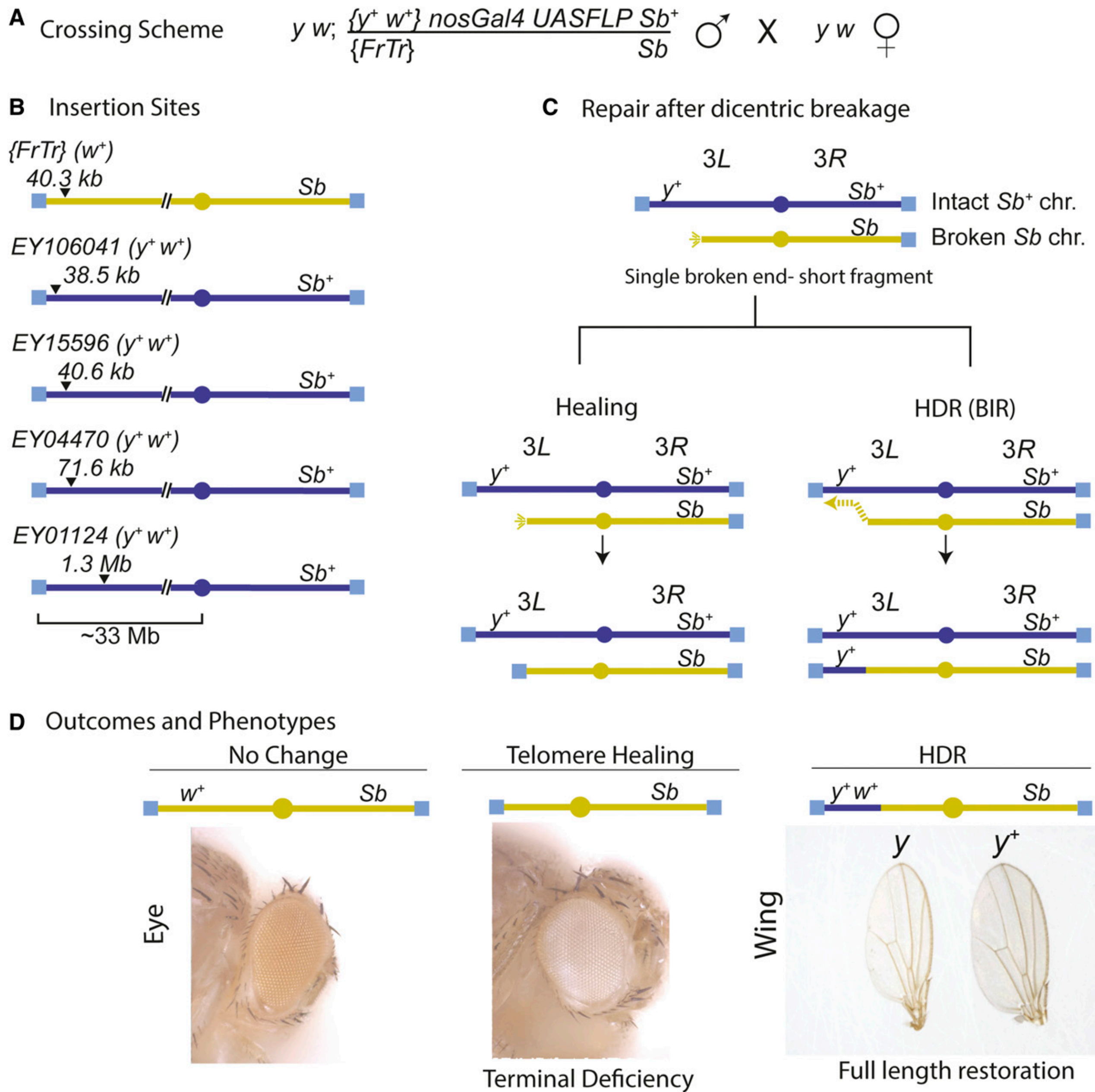


Figure 2 Experimental system to study HDR. (A) The crossing scheme for testing HDR. Crossing was used to generate males carrying chromosome *3* with *P{FrTr}* and *Sb*, heterozygous with a chromosome that carries y^+ at varying distances from the tip of *3L* along with Sb^+ and *nosGal4 UASFLP* [these elements, and w^+ of the template element on the Sb^+ chromosome, are not shown in (B and C) to save space]. (B) The locations of the *P{FrTr}* and y^+ insertions near the tip of *3L*. (C) The broken dicentric may be healed by telomere addition to produce a chromosome with a TD, or it may utilize information from the homolog. BIR is shown as an example of how sequence information may be transferred from the homolog to the terminus of the broken chromosome. (D) Phenotypes of *Sb* chromosomes: yellow and white$^+$ indicate an unchanged chromosome; healing that produces TD chromosomes is indicated by yellow and white; and chromosomes repaired by HDR are indicated by yellow$^+$ (and white$^+$). BIR, break-induced replication; chr., chromosome; HDR, homolog-dependent repair; TD, terminal deficiency.

{FrTr} insertion is very near the chromosome tip, a broken dicentric chromosome might lose only a small segment of chromosome, and this loss might not be visible. Additionally, large deficiencies will suffer reduced viability and reduced recovery because of segmental aneuploidy.

In fact, there is substantial evidence that large inviable deficiencies were generated in these experiments. Of the 3064 fertile males, 218 (Table 1) produced only y^+ w^+ Sb^+ progeny. These males occurred in all genotypes and with every template insertion. In contrast, in the control crosses shown in Table 2, every male transmitted both chromosome 3 homologs. Some of the males with no *Sb* progeny may have resulted from low fertility and small sample sizes (Table 1): 55/218 of these males had ≤10 progeny. However, 147/218

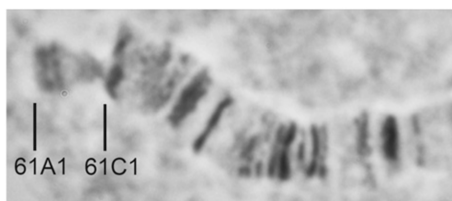


Figure 3 A heterozygous terminal deficiency. Polytene chromosome *3L* of an animal heterozygous for a terminal deletion of 61A-61B. The top homolog terminates at 61C1, while the bottom homolog is complete to the normal tip at 61A1.

of these males had ≥20 progeny and 28 of these 147 had >100 progeny each. Previous experiments have led to the conclusion that males with an intact Chk2 checkpoint do not transmit unhealed broken chromosomes (Titen *et al.* 2014). Chromosomes that are broken in mitotically dividing cells of the male germline must be healed by *de novo* telomere addition prior to meiosis if they are to be transmitted. In the experiments reported here, males without *Sb* progeny are most likely explained by breakage and healing to generate a TD that allows survival of germline cells, but which is too large to be viable in a whole animal.

HDR of broken chromosomes

If broken chromosomes are repaired using information from the homolog, they may acquire *P*-element sequences from that homolog. In these experiments, such chromosomes would be recognized as yellow$^+$ white$^+$ Stubble (y^+ w^+ *Sb*) progeny. A substantial fraction of the recovered *Sb* chromosomes did carry y^+. In the experiment with wild-type males, using the y^+ w^+ marker at 38.5 kb, ~8% of the *Sb* progeny were also y^+ w^+ (Table 1).

The *Sb* chromosome might acquire y^+ from the homolog either by recombination or by BIR. In wild-type males, there is essentially no recombination in the male germline (Hannah-Alava 1968; Ashburner et al. 2005). In control crosses to assess the relative viability and fertility of flies with chromosomes used in these experiments (Table 2), we also observed no male germline recombinants. Even when one homolog is efficiently cut by the I-*Sce*I meganuclease, recombination between homologs is very infrequent (0.3% recombination with ≥93% cutting; Rong and Golic 2003). Finally, there was not a single *y* Sb^+ reciprocal recombinant offspring among the 28,587 Sb^+ progeny in this experiment (which, should they occur, are expected to be w^+, since *nosGal4* and *UASFLP* are both marked with w^+), nor did we see *y* Sb^+ progeny from the other crosses listed in Table 1, although they were not examined as closely. It seems most likely that the *Sb* chromosomes acquire y^+ through BIR.

The broken chromosome can acquire information at least 1.3-Mb away from the tip of the homolog

To examine the lengths of HDR events, we used y^+ marker insertions at increasing distances from the tip of *3L*, up to 1.3 Mb from the end of the chromosome (Figure 2). As before, the insertion of inverted FRTs 40.3 kb from the chromosome tip was used to generate dicentrics. In all cases, healing was the most frequent repair event, constituting 60–70% of all recovered *Sb* chromosomes, but HDR events were also observed in each combination, constituting 4–8% of *Sb* chromosomes (Table 1). This result shows that HDR may extend to sequences ≥1.3 Mb from the tip of the chromosome.

Although there was no strict correlation between the location of y^+ on the template chromosome and the frequency with which it was acquired, there was a tendency for markers closer to the telomere to be acquired at a higher rate. Insertions 71.6 kb from the telomere or closer were acquired at a rate of ~8%, while the marker 1.3 Mb from the telomere was acquired at ~4%. The differences in efficiencies between markers at 38.5, 40.6, and 71.6 kb were either not significant or only marginally significant (38.5 kb *vs.* 40.6 kb, $P = 0.0356$). However, the three most distal y^+ insertions were acquired significantly more often than the y^+ marker at 1.3 Mb ($P < 0.0001$ in each case).

Pif1 and Pol32 participate in HDR in Drosophila

In yeast, BIR efficiency depends on Pol32, a nonessential subunit of DNA polymerase δ (Lydeard *et al.* 2007; Deem *et al.* 2008; Kane *et al.* 2012; Donnianni and Symington 2013; Vasan *et al.* 2014; Vasianovich *et al.* 2014), and the Pif1 helicase (Kane *et al.* 2012; Wilson *et al.* 2013; Sakofsky *et al.* 2014; Vasianovich *et al.* 2014; Buzovetsky *et al.* 2017). These proteins do not appear to affect the initial steps of BIR (strand invasion and D-loop formation), but do promote the processivity of replication during this mode of repair. Although BIR still occurs in the mutants, it is much less efficient. To determine if Pif1 and Pol32 contribute to HDR in *Drosophila*, we measured the efficiency of HDR in males that carried homozygous null mutations in these two genes. As shown in Table 1, the *pif1* and *pol32* mutants exhibited reduced rates of HDR relative to wild-type for all the template homologs that we tested ($P < 0.0001$ for all combinations except for *pif1* in the 1.3-Mb experiment where $P = 0.063$). In *Drosophila*, Pif1 and Pol32 contribute to the efficiency of HDR, but are not absolutely required for it.

We also discovered that the *pif1* mutant strongly and consistently reduced the recovery of TD chromosomes in all four sets of experiments ($P < 0.0001$ for each). Despite TDs constituting a smaller fraction of *Sb* chromosomes recovered from *pif1* mutants, there was an overall increase in the recovery of *Sb* chromosomes from *pif1* fathers. As discussed above, since healed chromosomes are missing genes from the end of the chromosome, they are likely to have reduced viability, and will be recovered at a reduced rate as adults because of death sometime prior to eclosion. If germline cells with a broken chromosome do not heal that chromosome, and these cells are eliminated prior to meiosis, it would tend to equalize the proportions of *Sb* and Sb^+ progeny. There was no consistent or strong effect of *pol32* on the frequency of chromosome healing, although *pol32* males did show a high rate of sterility in our experiments.

Table 2 Fertility of *P{FrTr}* and y^+ template chromosomes

Genotype	Distance from *3L* telomere	*N* (fertile males)	*N* (infertile males)	y^+ or *FrTr* chromosome		Wild-type chromosome		Total progeny
				n	%	*n*	%	
P{FrTr}[a]	40.3 kb	27	2	1167	50	1171	50	2338
y^+ template chromosomes[b]	38.5 kb	29	2	1397	53	1222	47	2619
	40.6 kb	26	4	1158	54	982	46	2140
	71.6 kb	28	2	1253	55	1011	45	2264
	1.3 Mb	21	9	1082	55	870	45	1952

[a] Crosses were *y w; P{FrTr} Sb/+* males x *y w* females.
[b] Crosses were *y w; P{y⁺ w⁺} nosGal4 UASFLP Sb⁺/+* x *y w* females.

Chromosomes repaired by HDR are typically complete

We next asked whether chromosomes repaired by HDR are full-length chromosomes or whether they might be shorter than wild-type chromosomes. To do this, we used qPCR to assay the copy number of a DNA sequence more distal than the y^+ marker gene. The most distal ~20 kb (or more) of *3L* consists of highly repetitive sequence that is also found on other chromosomes, making it unsuitable for this test. Instead, we chose to assay a segment of the *mthl8* gene, the most distal gene on *3L*, lying ~24 kb from the tip. We generated fly stocks from single F_1 males carrying a y^+ w^+ *Sb* chromosome and balanced these over the *TM6B,Tb* balancer. To ensure that we assayed independent events, we used only a single F_1 male from any given parent. We then determined whether these chromosomes possessed the most-distal gene on *3L*, *mthl8*. TaqMan qPCR was used to measure the *mthl8* gene copy number in each of these recovered stocks. Fortuitously, we discovered that the *TM6B* balancer has a polymorphism that rendered its copy of *mthl8* undetectable by our qPCR method. Therefore, if BIR copied the most-distal gene, a single copy of *mthl8* would be detected by qPCR. If BIR terminated early, no copies of *mthl8* would be found.

We tested 59 y^+ w^+ *Sb* chromosomes recovered from wild-type flies and found that 58/59 had copied the *mthl8* segment (Table 3). Although the numbers of HDR chromosomes recovered in the presence of mutations in *pif1* and *pol32* were far fewer, we saw little evidence to indicate that these mutations changed the outcome of HDR: 29/30 tested chromosomes had acquired the *mthl8* sequence (Table 3). Neither did we detect any significant difference in the fraction of chromosomes that were full length in experiments using markers that were different distances from the tip of the chromosome. One of the incomplete events was found in experiments with the y^+ template at 38.5 kb in a wild-type background and one with the template at 1.3 Mb in a *pif1* background.

Overall, 98% (87/89) of HDR chromosomes acquired *mthl8*. In 2/89 cases, it appears that the broken *Sb* chromosome acquired only a portion of the homolog and must then have acquired a telomere by healing. An alternative explanation, albeit unlikely, is that perhaps these two cases represent instances of BIR that were complete to the chromosome tip but, because of the mutagenic nature of BIR, *mthl8* was copied inaccurately leading to a failure of the qPCR reaction. In any event, when a broken chromosome is repaired by HDR, that repair is usually complete to the end of the chromosome. This is true in wild-type, *pif1*, and *pol32* males.

Some apparent healing events are cryptic HDR events

To recognize an HDR event in our experiments, a y^+ marker gene must be acquired from the homolog. Repair events that initiate from breakpoints distal to that marker will initially be scored as TDs. For instance, if a dicentric chromosome were to break at a site 100 kb from the normal end of the chromosome, HDR could acquire the distal 100 kb from the homolog. However, if the homologous chromosome used a y^+ marker >100 kb from the tip, then this event would be scored as a TD. To determine how often this might occur, we assayed several putative TD chromosomes (*y w Sb*) for the presence of *mthl8*. Overall, 8 of 174 putative TDs were found to possess *mthl8*, and must represent chromosomes that experienced HDR (Table 4). These cryptic HDR events were found in wild-type and mutant genotypes, and were, as one would expect, more frequent in experiments with y^+ markers further from the telomere (1/35 with y^+ at 38.5 kb, 1/51 at 40.6 kb, 2/42 at 71.6 kb, and 4/46 at 1.3 Mb), but with these small numbers the differences were not statistically different.

The real frequency of HDR events in these experiments is therefore higher than indicated by the chromosomes that acquired y^+. If ~1/10th (4/46) of the chromosomes scored as TD in the 1.3-Mb experiment were actually HDR events, their total frequency would be ~10%, making it comparable to similarly corrected frequencies in the experiments with markers closer to the telomere. While the small number of chromosomes tested in Table 4 do not justify any certainty about the magnitude of such corrections, it seems likely that the actual frequencies of HDR events within each wild-type or mutant genotype are similar, but appear to vary simply because y^+ markers that are further from the telomere cannot detect all HDR events.

One of the chromosomes that was collected as a TD in the experiment with y^+ at 38.5 kb acquired *mthl8* but did not acquire y^+. This chromosome is difficult to account for, since the 38.5-kb position is closer to the telomere than any possible breakpoint of the dicentric. Perhaps the broken end invaded the homolog at a site distal to the y^+ marker using a

Table 3 Molecular characterization of y^+ w^+ *Sb* chromosomes

Distance of y^+ from *3L* telomere	Genotype	N^a	Complete HDR (*mthl8*$^+$)		Terminal *Df* (*mthl8*$^-$)	
			n	%	*n*	%
38.5 kb	+	19	18	95	1	5
	pif1^{167}	4	4	100	0	0
	pol32^{L2}	3	3	100	0	0
40.6 kb	+	12	12	100	0	0
	pif1^{167}	6	6	100	0	0
	pol32^{L2}	4	4	100	0	0
71.6 kb	+	14	14	100	0	0
	pif1^{167}	4	4	100	0	0
	pol32^{L2}	2	2	100	0	0
1.3 Mb	+	14	14	100	0	0
	pif1^{167}	6	5	83	1	17
	pol32^{L2}	1	1	100	0	0
Total		89	87	98	2	2

HDR, homolog-dependent repair.
[a] *N* = number of independent y^+ w^+ *Sb* chromosomes tested.

Table 4 Molecular characterization of *y w Sb* chromosomes

Distance of y^+ from *3L* telomere	Genotype	N^a	HDR (*mthl8*$^+$)		Terminal *Df* (*mthl8*$^-$)	
			n	%	*n*	%
38.5 kb	+	19	1	5	18	95
	pif1^{167}	7	0	0	7	100
	pol32^{L2}	9	0	0	9	100
Total		35	1	3	34	97
40.6 kb	+	18	0	0	18	100
	pif1^{167}	10	0	0	10	100
	pol32^{L2}	23	1	4	22	96
Total		51	1	2	50	98
71.6 kb	+	17	1	6	16	94
	pif1^{167}	7	1	14	6	86
	pol32^{L2}	18	0	0	18	100
Total		42	2	5	40	95
1.3 Mb	+	19	3	16	16	84
	pif1^{167}	12	0	0	12	100
	pol32^{L2}	15	1	7	14	93
Total		46	4	9	42	91

HDR, homolog-dependent repair.
[a] *N* = number of independent *y w Sb* chromosomes tested.

limited stretch of homology before carrying out BIR (Hastings *et al.* 2009), but more detailed molecular characterization would be required to determine whether or not this is the case.

Chromosomes with terminal inverted repeats were not recovered

When the dicentric chromosome breaks, it will typically generate one short chromosome that lacks a variable portion of the chromosome tip and a complementary long chromosome lacking the very tip, which also carries an inverted duplication of material proximal to the FRTs (Figure 1). If this long chromosome is healed and transmitted, it should still carry FRTs and be capable of making dicentric chromosomes again when exposed to FLP. To test for the presence of inverted FRTs on TD chromosomes we performed a somatic test. The *eyFLP* transgene expresses FLP in the eye throughout larval development. If a fly with *eyFLP* also carries a chromosome with adjacent inverted FRTs, dicentric chromosomes will be produced in the eye at a high rate, triggering apoptosis, and generating adults with small and rough eyes (Kurzhals *et al.* 2011). The original *Sb* chromosome carrying the *P*{*FrTr*} element with inverted FRTs consistently produces flies with small rough eyes in this experiment.

We crossed flies carrying 64 independent TD chromosomes to flies carrying *eyFLP*, but found no cases of TD chromosomes that still carried inverted FRTs. This stands in contrast to a previous experiment in which we did recover both the short fragments without FRTs and long fragments with FRTs (Titen *et al.* 2014). One significant difference between those and the current experiments may account for the difference: a heat shock was used to produce a short pulse of FLP expression in experiments where both types were recovered, but here we used *nosGal4 UASFLP* to express FLP continuously in germline stem cells. Thus, in the current experiments, long-fragment chromosomes carrying inverted FRTs may be subject to repeated rounds of dicentric formation and breakage, and suffer elimination. We observed similar results in a previous set of experiments using *nosGal4 UASFLP* (Titen and Golic 2010).

Another explanation for why such chromosomes were not recovered among the apparent TD chromosomes is that they tend to be repaired by HDR rather than by healing. In this case, the broken long fragment would acquire sequences closer to the centromere (Figure 4). If this were the result of a simple exchange event, a dicentric chromosome would be generated and likely continue the bridge-breakage cycle. However, if BIR was initiated, then terminated, and the end healed, a chromosome partially repaired by replication toward the centromere would be produced. If it did not acquire y^+ from the homolog, either because it initiated copying at a more proximal site or because it copied itself, it would appear as a TD with inverted FRTs and, as discussed, these were not recovered. However, a chromosome that acquired y^+ from the homolog by partial centromere-directed BIR followed by healing would show up as an HDR chromosome that lacks *mthl8*.

Certain aspects of results already presented argue against the possibility that *mthl8*-minus chromosomes represent centromere-directed BIR. First, chromosomes repaired by BIR and that initiate replication toward the centromere should carry inverted FRTs, and be subject to negative selection in the presence of prolonged FLP synthesis. Second, if centromere-directed BIR events that acquire y^+ account for any of the recovered BIR chromosomes, they could not be among those where the template y^+ marker lay distal to the insertion of *FRT*s, yet one of the *mthl8*-minus chromosomes was from this group. Third, they likely would be most often found among BIR events that acquired the most centromere-proximal marker at 1.3 Mb from the tip, but only 1/21 of these was *mthl8*-minus.

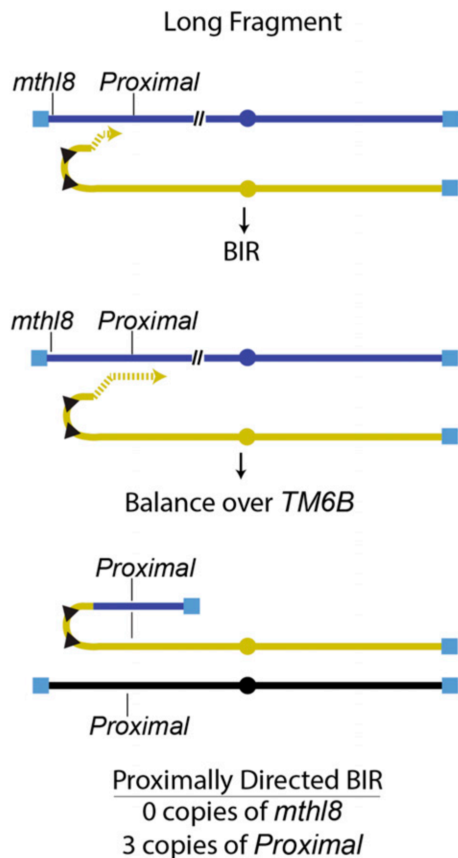


Figure 4 Repair of a long-fragment chromosome by centromere-directed BIR. A long chromosome fragment with FRTs and an inverted duplication at the broken end would be expected to fold back onto itself due to sequence homology. BIR from a long fragment would be directed proximally toward the centromere, and could be distinguished from distally directed BIR by measuring the gene copy number of *mthl8* (located 24 kb from the tip of *3L*) and a DNA sequence called "Proximal," which is 77 kb from the tip of *3L*. A proximally directed BIR is expected to have no copies of *mthl8* and is likely to have three copies of Proximal. BIR, break-induced replication.

To further test the possibility that some HDR events were products of centromere-directed BIR, we tested them in two ways. First, we used the *eyFLP* test to check for the presence of inverted FRTs. Forty-eight independent HDR chromosomes were tested (including one of the *mthl8*-minus chromosomes), and none showed a rough-eye phenotype, indicating that they did not carry inverted FRTs. Second, both *mthl8*-minus HDR chromosomes were tested with qPCR to assay the copy number of a region of DNA 77 kb from the tip of *3L* (indicated as Proximal in Figure 4). We expect that a chromosome that picked up y^+ by centromere-directed BIR should carry two copies of this segment, and that in the segmentally aneuploid fly there should be three copies. We found that flies carrying either of the two HDR y^+ *Sb* chromosomes that lacked *mthl8* had only two copies of this segment, further confirming that that these chromosomes did not carry a terminal inverted repeat.

Discussion

These experiments were designed to determine whether a broken dicentric chromosome could be restored to its full length by using information from the homolog in *D. melanogaster*. Dicentric chromosomes were produced in the male germline by FLP-mediated exchange between inverted *FRT*s on sister chromatids. This is a very efficient process and dicentric chromosomes can be generated at frequencies that approach 100%. When the dicentric breaks in mitosis it delivers a chromosome with a single broken end to each daughter cell. By suitably marking each chromosome, we then asked whether the broken chromosome might acquire sequences from its homolog during the process of repair. We found that HDR constituted a significant fraction, probably ~10%, of all broken chromosome repair events.

The mechanism of HDR

There are two clear possibilities for how a homolog might be used to repair a broken chromosome. One possibility, which is well documented in yeast, is BIR, where a strand from the broken end invades the homolog and initiates DNA replication that proceeds to the end of the chromosome. Alternatively, the intact homolog might donate its terminus to the broken chromosome via an exchange event. As mentioned previously, mitotic recombination in the male germline is very infrequent. Even when one of the two homologs is cut by the I-*Sce*I meganuclease, the rate of recombination is much less than 1%. On its face, it seems unlikely that HDR, which occurred at rates of ~8% in these experiments, could be accounted for by recombination. However, a break produced by I-*Sce*I cutting is fundamentally different from the DSB produced by dicentric breakage: the former presents the cell with two broken ends, but the latter only one. It is conceivable that the necessity of coping with a single broken end could increase the propensity to repair by recombination.

In yeast, an alternative to BIR repair of a one-ended break is an exchange that moves the end of an intact homolog to the broken chromosome, leaving the donor with a TD. These events are called half-crossovers (HCOs). An HCO chromosome is genetically indistinguishable from a chromosome repaired by BIR. In yeast, these repair types are distinguished by whether the original donor chromosome is maintained (BIR) or lost (HCO). In a typical experiment, a disomic strain will be used and the HO endonuclease will induce a DSB on one of the two homologs. If the chromosome that was cut is repaired by BIR, it will be seen as loss of heterozygosity distal to the cut site, but the cells will retain both copies of that chromosome. In some cases, an identical repaired chromosome is produced but in a monosomic condition. In such cases, it is concluded that the intact homolog donated its distal segment to the broken chromosome by exchange and that the donor was then lost because it lacked a telomere. This is supported by observations in yeast that healing of a broken end is a relatively rare event and that chromosomes without telomeres are generally lost (Kramer and Haber 1993;

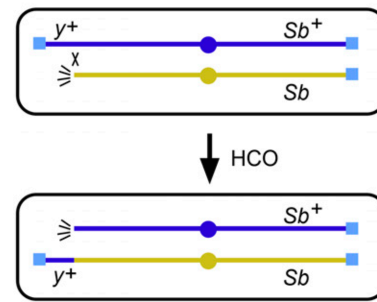
Sandell and Zakian 1993). The chromosome that remains is the HCO chromosome.

If HCOs occurred after dicentric breakage in our experiments, a y^+ *Sb* chromosome and a *y* Sb^+ chromosome would be produced (Figure 5; we ignore w^+ in this discussion for simplicity). The Sb^+ chromosome might then be healed to produce a TD. Since healing is the most common outcome in these experiments, we would expect to see many *y* Sb^+ chromosomes if HCOs were at all frequent, and we saw none among the progeny listed in Table 1. Moreover, we have previously observed two healing events in a single cell, when a ring chromosome opened to form a linear chromosome, suggesting that the need for multiple repair events in a cell does not restrict such an outcome (Hill and Golic 2015).

Alternatively, if the donor chromosome of an HCO were not healed, we would expect to recover neither the HCO chromosome nor the broken homolog, because chromosomes with unhealed broken ends act as dominant cell lethals in the germline of wild-type males (Titen *et al.* 2014; Kurzhals *et al.* 2017). Complete loss of one copy of chromosome 3 would also likely be a dominant cell lethal and prevent the recovery of the HCO chromosome. In further support of these points, 89 of the parental males listed in Table 1 transmitted *Sb* chromosomes which were all y^+ (Table 5). All but one of these males also transmitted Sb^+ chromosomes that were all y^+ as well and the average Sb:Sb^+ ratio in these males was 0.93. Apart from the single exceptional male, which could also be explained by the spontaneous occurrence of a dominant lethal mutation on the Sb^+ chromosome, this provides little support for the view that a germline cell could carry an unrepaired Sb^+ chromosome and still produce functional y^+ *Sb* gametes that resulted from an HCO.

If a broken end persisted into G2 of the cell cycle, then additional outcomes would be possible. First, any repair events would have to compete with end joining of the broken sister chromatids and the ensuing bridge–breakage–fusion cycle. But, if exchange between a broken chromosome and its intact homolog did occur in G2, the segregation patterns of these products must be considered. As shown in Figure 6A, if mitotic recombination occurs between two intact chromosomes at a site in the middle of a chromosome arm in G2, whether induced by X-rays or by site-specific recombination, the recombinant chromatids almost always segregate from one another in the following mitosis in what is called X segregation (Pimpinelli and Ripoll 1986; Beumer *et al.* 1998). This occurs because, after such an exchange, sister chromatid cohesion distal to the site of exchange maintains the coupling of homologs in a mitotic bivalent, which directs their attachment to the mitotic spindle. On the other hand, if exchange occurs near the end of a chromosome, then preferential X segregation does not occur: X and the alternative Z segregation are equally frequent. Based on the recovery of cryptic HDR events, it appears that one-half or more of the recovered HDR chromosomes initiated repair within the most distal 1.3 Mb of *3L*. A similar small amount of chromatin distal to a

A HCO in G1 after dicentric breakage



cell fate
gametes

B Healing

C No healing and cell death

= centromeres
= telomeres
= DSB

Figure 5 Consequence of HCO in G1. (A) When an HCO occurs in G1 of the cell cycle it transfers y^+ to the *Sb* homolog (w^+ not shown to save space), leaving the Sb^+ chromosome with a TD. (B) If the TD is healed, both the HCO and its reciprocal product, the TD, can be recovered. (C) If the TD is not healed, the broken end kills the cell and neither homolog is transmitted through gametes. DSB, double-strand break; HCO, half-crossover; TD, terminal deficiency.

point of mitotic exchange is not sufficient to drive preferential X segregation (Beumer *et al.* 1998). If these distally initiated repair events occurred by HCO, X and Z segregation should be equally frequent (Figure 6, B and C). Following X segregation, the TD that is the reciprocal product of an HCO might not be recovered because it is in a cell with a homozygous TD. However, following Z segregation, both the HCO and the reciprocal TD should be recovered. Therefore, regardless of whether an HCO arose by recombination in G1 or recombination in G2, the reciprocal product should also be produced and recovered.

Since a chromosome repaired by BIR and one repaired as an HCO are genetically indistinguishable, it is not possible to state with complete certainty that none of the y^+ *Sb* chromosomes recovered (listed in Table 1) are HCOs. However, if they are, they must be only a very small fraction of the total. For recombination to completely explain the recovery of y^+ *Sb* chromosomes, it must be an order of magnitude more frequent following dicentric breakage than it is following I-*Sce*I cutting, it must occur entirely in G2, and it must always be followed by X segregation. Although our experiments cannot determine whether repair occurs in G1 or G2, if HCOs

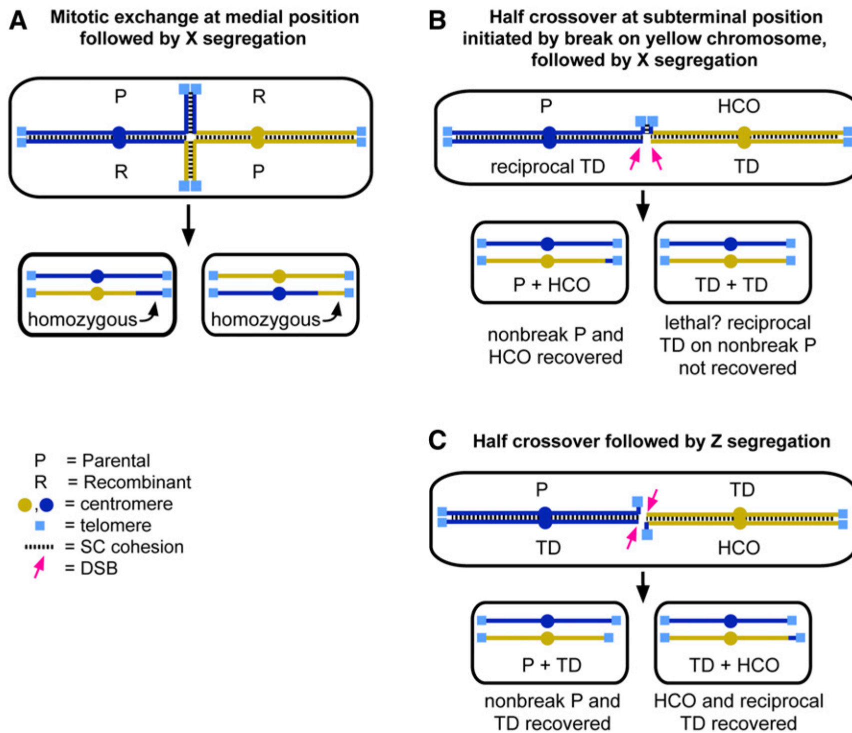


Figure 6 Recombination in G2. (A) Mitotic recombination in G2 in the middle of a chromosome arm is shown. Cohesion of the SCs distal to the site of exchange conjoins the homologs until they attach to the spindle and direct X segregation, with recombinant chromatids segregating to opposite daughter cells. (B and C) An HCO at a position near the telomere has very little SC cohesion to maintain conjunction of homologs. As a consequence, X and Z segregation are equally frequent. Outcomes of the different types of segregation are indicated below the daughter cells. Telomeres indicated by squares, SC cohesion by a dashed line, and centromeres by circles. HCO, half-crossover; P, parent; R, recombinant; SC, sister chromatid; TD, terminal deficiency.

occurred, the reciprocal product, a *y* Sb^+ TD chromosome, should also be recovered in either case. The fact that they were not argues that HCOs are not a viable repair event in male germline cells.

When considering repair outcomes in wild-type yeast, BIR is by far the most frequent mode of repair for a DSB where only one end can find homology for repair, outnumbering HCOs by an order of magnitude or more. If repair choices are similarly determined in *Drosophila*, BIR should predominate. Even in yeast *pol32* and *pif1* mutants, in which BIR efficiency is reduced, BIR can be nearly as, or even more, frequent than HCOs (Deem *et al.* 2008; Donnianni and Symington 2013; Wilson *et al.* 2013; Sakofsky *et al.* 2014; Vasan *et al.* 2014; Vasianovich *et al.* 2014; Buzovetsky *et al.* 2017). The observation that efficient HDR of a single broken end in flies is similarly dependent on Pif1 and Pol32 strengthens the proposition that HDR repair proceeds through a similar mechanism in yeast, and in flies.

Taken as a whole, these various lines of evidence support the conclusion that most, and likely all, of the HDR events recovered in these experiments were produced by BIR. BIR has been extensively documented in yeast, but it has not been established whether it might be used to restore long chromosome segments in higher eukaryotes. The work presented here shows that HDR can restore at least 1.3 Mb to the end of a chromosome, which likely represents the longest instance of BIR to date. It is possible that BIR restoration tracts could be much longer, but this remains to be tested. The observation that BIR can exceed 1 Mb is important due to the fact that BIR has been primarily studied in *S. cerevisiae*, where the longest chromosome arm is ~1 Mb.

HDR: distance vs. efficiency

The experiments summarized in Table 1 show a distance dependence for HDR, with events >1.3 Mb occurring about one-half as frequently as shorter events. However, this difference appears to arise from the distribution of dicentric chromosome breakpoints rather than the reduced efficiency of longer BIR events. When apparent TD chromosomes from the experiment with the 1.3 Mb marker were molecularly assayed for the acquisition of a distal sequence from the homolog, ~9% of such chromosomes were found to be HDR chromosomes, but which had initiated replication distal to the marker at 1.3 Mb. These results suggest that a large fraction of the recovered HDR events initiate from chromosome breaks in the region between 71.6 kb and 1.3 Mb. We have previously shown that dicentric chromosomes tend to break at a limited number of hotspots (Hill and Golic 2015) and there may be such a hotspot in this region. It is possible that HDR efficiency in *Drosophila* decreases with length, but a different experimental design would be required to test this.

In yeast, the efficiency of BIR appears to be strongly dependent on the distance from the site of initiation to the end of the chromosome, with 128-kb events occurring approximately one-half as frequently as 15–20-kb events (Lydeard *et al.* 2007; Donnianni and Symington 2013; Ruff *et al.* 2016). However, the yeast experiments were designed so that the only homologous region for the initiation of BIR was within a construct placed at a specific genomic location. As this segment of homology was placed at different loci, the chromosomal context was also altered. The effects of context and distance of polymerization have yet to be disentangled.

Table 5 Progeny from males whose *Sb* offspring were all y^+

Template position	Genotype	N^a	Progeny y^+ Sb^+	y^+ *Sb*	*Sb*:Sb^+
38.5 kb	+	26	1216	1051	0.85
	pif1	2	112	92	0.82
	pol32	3	21	25	1.19
40.6 kb	+	22	934	838	0.90
	pif1	5	263	249	0.95
	pol32	2	100	80	0.80
71.6 kb	+	11[b]	488	523	1.09
	pif1	2	103	109	1.06
	pol32	2	38	29	0.76
1.3 Mb	+	8	343	386	1.12
	pif1	4	231	200	0.87
	pol32	2	11	14	1.27
Total		89	3860	3596	0.93

[a] *N* = number of parental males.
[b] One male produced 54 y^+ *Sb* and 0 y^+ Sb^+.

Healing is the most frequent mode of repair for a one-ended DSB

Despite the fact that HDR is clearly an option, healing by telomere addition to the broken end was still the most frequent method of repair, occurring ~8× as often as HDR. This seems surprising since healing is associated with a loss of genetic material distal to the site of breakage, which may be extensive (*e.g.*, Figure 3), while HDR can restore the full length of the chromosome. How a cell chooses between healing or HDR is a critical question. In yeast, the availability of homologous sequences on both sides of an HO-induced break plays an important role in the choice of gene conversion over BIR (Mehta *et al.* 2017). The availability of homologous sequences may play a similar role in the choice between healing and HDR in *Drosophila*. The use of the homologous chromosome for repair is no doubt facilitated by the pairing of homologs that occurs in mitotically cycling cells in this species. Chromosomes are intimately paired along their lengths in interphase. In mitotic prophase this pairing is still evident, but it dissolves as mitosis proceeds. After division, chromosomes decondense and pairing is reestablished. The homologous chromosome can be efficiently used for the repair of DSBs by gene conversion generated by I-*Sce*I or *P*-element transposition (Rong and Golic 2003; Johnson-Schlitz and Engels 2006). However, the experiments presented here utilized FLP-mediated fusion of sister chromatids and breakage of the resulting dicentric bridge in mitosis to generate the uncapped chromosome end that requires repair. The homolog may not be immediately available until mitotic pairing is reestablished in interphase. It follows that there is probably some period when homologous sequences are not available for repair of the broken end. This may cause the observed bias for healing. Other variables, such as the location of terminal breaks and the required length of the repair tract, may also affect the efficiency of HDR. Further work is needed to determine what, if any, specific cellular mechanisms regulate the choice of healing *vs.* HDR.

Mixed mode repairs are rare or absent

Our experiments also addressed the question of whether HDR, specifically BIR, and healing might be used in combination. That is, might a chromosome begin repair by BIR, but at some point terminate replication prematurely and complete repair by healing? This sequence of events must be quite rare. In 98% of cases the broken chromosome was restored completely. However, critically, there were 2/89 cases where it appears that the broken chromosome was only partially restored and then healed. These infrequent cases of incomplete restoration lend even further support to the conclusion that BIR is the primary mechanism for HDR. They are readily explained by partial BIR and healing, but are not an expected consequence of an HCO. Nonetheless, such events are rare. Once a decision is made to utilize HDR, it seems to be an almost irreversible commitment. We conclude that HDR usually restores the full length of the chromosome or fails in such a way that it does not allow healing of the incompletely restored chromosome.

We are not aware of any cases in yeast where the repair of a broken end has involved both incomplete BIR and healing, though other outcomes do occur. These can result from events such as template switching during BIR, the use of small amounts of homology to initiate BIR at ectopic locations, or rearrangements involving an HCO donor chromosome (Mehta and Haber 2014; Vasan *et al.* 2014). But even in *pol32* mutants in yeast, where BIR efficiency is impaired, a broken chromosome can use BIR to copy its homolog completely to the telomere (Deem *et al.* 2008; Vasan *et al.* 2014).

Since BIR can be used to repair and restart stalled or damaged replication forks during normal S-phase replication (Costantino *et al.* 2014; Kramara *et al.* 2018), it is surprising that the commitment to BIR would be irreversible. However, during S phase, a repaired fork would almost always encounter another fork coming from the opposite direction and this could terminate the fork that was restarted by BIR (Mayle *et al.* 2015). In contrast, BIR that is used to restore the end of a chromosome may continue all the way to the end without encountering another replication fork. If BIR only terminates when it encounters another fork, this might account for the rarity of incomplete BIR in our experiments.

Of course our experiments have only addressed the relative frequencies of events that rescue broken chromosomes and allow them to be recovered in viable offspring. In these germline dicentric breakage experiments, the most frequent outcome is almost certainly cell death prior to meiosis (Titen *et al.* 2014; Kurzhals *et al.* 2017). Repair events, or attempted repair events, that fail to rescue a cell with a broken chromosome cannot be seen in these experiments. Furthermore, there is a substantial deficit of the *Sb* progeny, derived from the chromosome with inverted FRTs, relative to Sb^+ progeny. This deficit is almost certainly due to the reduced viability of progeny with healed chromosomes that have lost varying numbers of genes at the end of the chromosome.

Pol32 and Pif1 both participate in HDR, and Pif1 has a role in healing

In yeast, the Pol32 subunit of Polδ and the Pif1 helicase, though not absolutely required for BIR, are important for efficient BIR (Lydeard *et al.* 2007; Deem *et al.* 2008; Donnianni and Symington 2013; Wilson *et al.* 2013; Sakofsky *et al.* 2014; Vasan *et al.* 2014; Vasianovich *et al.* 2014; Buzovetsky *et al.* 2017). Pol32 in *Drosophila* is needed for repair during extensive DNA synthesis and for the maintenance of genomic integrity (Kane *et al.* 2012; Tritto *et al.* 2015). Pif1 in *Drosophila* is needed during the rapid replications of early embryogenesis and in response to replication stress. We tested homozygous *pol32* and *pif1* mutants to see if they play a role in HDR in a higher eukaryote. As has been seen in yeast, there was a significant reduction in HDR in both mutant genotypes, though neither gene was absolutely required for the successful restoration of a chromosome terminus by HDR (Table 1). It has been suggested that, in yeast, Pol32 and Pif1 may have a role in the processivity of DNA synthesis during BIR. If loss of these factors also reduces the processivity of BIR in *Drosophila* and if a chromosome engaged in BIR, even unsuccessful BIR, is precluded from repair by healing, then this would be the expected result.

An unexpected finding was that *pif1* mutants exhibit a reduced rate of chromosome healing. In wild-type flies, healing was by far the most frequent method of repair for a single broken chromosome end, with BIR a distant second. In all four sets of experiments, with markers at different positions, the frequency of healing was significantly reduced in *pif1* mutants (though it still outpaced BIR). In contrast, *pif1* mutants in yeast have an increased rate of healing (Schulz and Zakian 1994). The basis for this difference may lie in the fundamentally distinct mechanisms that these organisms use to establish and maintain telomeres. Yeast utilize telomerase to maintain telomere lengths. Healing typically occurs at sites with microhomology to the telomerase RNA component (Putnam *et al.* 2004). Pif1 inhibits telomerase, disfavoring telomere addition as a mode of DSB repair (Zhou *et al.* 2000; Makovets and Blackburn 2009). *Drosophila* do not use telomerase, but maintain telomere lengths by the addition of retrotransposons to the ends of chromosomes. *Drosophila* require no specific sequence at chromosome ends, and healing may occur at a variety of positions and chromosome sequences. Furthermore, in *Drosophila*, the assembly of the telomere-specific and nonspecific proteins that form the cap, which prevents the cell from perceiving the end as a DSB, is not strictly coupled to length maintenance provided by retrotransposons (Raffa *et al.* 2011; Zhang and Rong 2012). Healing of broken chromosomes by capping is not usually accompanied by retrotransposon addition, though retrotransposons may be added in subsequent generations (Levis 1989; Biessmann *et al.* 1990; Ahmad and Golic 1998). Whether DNA replication and Pif1 play any role in generating ends suitable for capping in *Drosophila*, or whether Pif1 has an entirely different function, is an open question.

HDR at telomeres

Although extensive restoration of broken chromosomes by BIR in higher eukaryotes has not been previously demonstrated, related phenomena are known. In addition to replication fork repair, ALT is a recombinational mechanism that can maintain telomeric repeats in cancer cells that do not express telomerase. Like BIR, ALT appears to elongate ends via conservative DNA synthesis and shows a dependence on DNA Polδ (Roumelioti *et al.* 2016), but it has only been implicated in the extension of telomeric DNA, and not in the restoration of long chromosome segments. In *Drosophila*, chromosome termini can also be extended by copying information from the homolog or sister chromatid, and possibly from homologous sequences on heterologs (Mikhailovsky *et al.* 1999; Kahn *et al.* 2000). This phenomenon, called terminal gene conversion, differs from the BIR that we report here in two key ways. First, this mechanism extends chromosomes that already have a telomere cap, not freshly broken chromosomes. Second, this extension has a very limited range, averaging only 2–3 kb and with an apparent maximum of $<$20 kb. This mechanism is tightly regulated under control of the *E(tc)* gene (*Enhancer of terminal conversion*) (Melnikova *et al.* 2004)

Conclusions

Our results show that a broken dicentric chromosome in *Drosophila* can have its terminus restored using sequence information carried by the homolog. This restoration can be extensive, covering $\geq$1.3 Mb. Several features of our results strongly support the conclusion that this HDR proceeds via BIR, and that the mechanism of BIR is at least partially conserved between yeast and higher eukaryotes. BIR has been implicated in trinucleotide repeat expansion (Kim *et al.* 2017), in rearrangements generated during chromothripsis and similar inherited rearrangements in humans (Nazaryan-Petersen *et al.* 2018; Cleal *et al.* 2019), and in the generation of copy number variants (CNVs) (Hastings *et al.* 2009; Carvalho and Lupski 2016), which account for the majority of human genetic polymorphisms (Stankiewicz and Lupski 2010; Riggs *et al.* 2014). Similar CNVs have also been observed in *Drosophila* (Emerson *et al.* 2008). The importance of examining HDR, particularly BIR, in the germline of a higher eukaryote is clear. Our system provides a means to assess the importance of HDR in generating structural rearrangements and CNVs, and to explore its mechanism and regulation in the germline of a higher eukaryote.

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