Chromosome Healing Is Promoted by the Telomere Cap Component Hiphop in *Drosophila*

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ABSTRACT The addition of a new telomere onto a chromosome break, a process termed healing, has been studied extensively in organisms that utilize telomerase to maintain their telomeres. In comparison, relatively little is known about how new telomeres are constructed on broken chromosomes in organisms that do not use telomerase. Chromosome healing was studied in somatic and germline cells of *Drosophila melanogaster*, a nontelomerase species. We observed, for the first time, that broken chromosomes can be healed in somatic cells. In addition, overexpression of the telomere cap component Hiphop increased the survival of somatic cells with broken chromosomes, while the cap component HP1 did not, and overexpression of the cap protein HOAP decreased their survival. In the male germline, Hiphop overexpression greatly increased the transmission of healed chromosomes. These results indicate that Hiphop can stimulate healing of a chromosome break. We suggest that this reflects a unique function of Hiphop: it is capable of seeding formation of a new telomeric cap on a chromosome end that lacks a telomere.

KEYWORDS telomere; terminin; Drosophila; double strand break; hiphop; HOAP; FLP; dicentric chromosome

CHROMOSOMES that experience double-strand breaks (DSBs) are usually repaired with great efficiency. However, in some cases a cell may have difficulty achieving proper rejoining of two broken ends. If cells are exposed to significant doses of ionizing radiation, the number of DSBs may overwhelm a cell's repair capacity and lead to cell death. Another form of damage that presents difficulty for a cell is the occurrence of a single broken chromosome end. Such damage may arise by breakage of a dicentric chromosome during a mitotic or meiotic division, by division of a cell with an unrepaired DSB, or, in some organisms, by telomere shortening in somatic cells owing to a lack of telomerase expression. A chromosome with a single broken end is unrepairable by normal DNA DSB repair mechanisms, which are geared to rejoin two broken ends.

Dicentric chromosomes, having two centromeres rather than the normal single centromere, can be produced experimentally by using the FLP recombinase to mediate recombination between inverted *FRTs* on sister chromatids (Figure 1) (Falco *et al.* 1982; Golic 1994). The sister centromeres conjoined in this fashion then separate to opposite poles during anaphase. In *Drosophila*, the resulting chromatin bridges typically break in mitosis to produce daughter cells that receive a chromosome with a single broken end (Titen and Golic 2008). The predominant consequence of this event in the soma is cell death (Ahmad and Golic 1999; Titen and Golic 2008). Nonetheless, some cells do escape death and continue to divide and differentiate (Golic 1994; Titen and Golic 2008; Kurzhals *et al.* 2011). In the male germline most cells experiencing dicentric formation are also eliminated, but a small fraction are repaired by *de novo* telomere addition (Ahmad and Golic 1998; Titen and Golic 2010)—a phenomenon known as healing (McClintock 1939, 1941).

Chromosome healing has been observed in many species, from yeast to humans (Gorovsky 1980; Mason *et al.* 1984; Flint *et al.* 1994; Melek and Shippen 1996; Sprung *et al.* 1999; Pennaneach *et al.* 2006). Healing of broken chromosomes is likely to play a significant role in human health. For instance, healing of broken ends has been observed in tumor cells (Fouladi *et al.* 2000), and is

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often associated with constitutional chromosome abnormalities (Fortin *et al.* 2009). During evolution, new telomeres must be generated when a karyotype changes by chromosome fission to produce an increase in chromosome number. Striking examples of this are seen in butterflies, with some species having evolved karyotypes with >200 chromosomes (Lukhtanov 2015; Šíchová *et al.* 2015).

A chromosome that is broken in the germline may be considered healed if it is transmissible through generations without incurring lethality or being subject to continuing chromosome rearrangements. Healing events in the germline can be readily detected in crosses using appropriately marked chromosomes (Levis 1989; Tower *et al.* 1993; Ahmad and Golic 1998; Titen and Golic 2010; Titen *et al.* 2014). Although healing of broken ends clearly occurs in the *Drosophila* germline, it is unknown whether healing can occur in somatic cells that experience dicentric breakage.

In organisms that utilize telomerase, healing requires that new telomeric repeats are added to the broken chromosome end, and that these repeats successfully recruit the full array of capping proteins that recognize these repeats, as well as their critical partners, which then prevent the chromosome end from being perceived as damaged DNA. *Drosophila* are one of a number of organisms that do not use telomerase (Mason *et al.* 2011). In place of the simple sequence repeats that are normally added by telomerase, *Drosophila* extend chromosomal DNA by the mobilization of specific retrotransposons to chromosome ends (Biessmann *et al.* 1990b, 1992; Levis *et al.* 1993). The precise complement of proteins that are needed to form a functional capping complex in *Drosophila* differs from that found in organisms that use telomerase, though there is some overlap. The set of proteins that are found uniquely at *Drosophila* telomeres has been called the terminin complex, in analogy with the shelterin complex found in organisms that use telomerase (de Lange 2005; Palm and de Lange 2008; Raffa *et al.* 2013; Zhang *et al.* 2016).

In *Drosophila*, the end-replication solution and capping functions are not interdependent as they are in organisms that use telomerase. Thus, *Drosophila* may heal a broken chromosome end by the addition of the cap structure without the presence of retrotransposon sequences that are normally found at chromosome ends (Levis 1989; Biessmann *et al.* 1990a). In *Drosophila*, chromosomes with functional caps may terminate at a variety of locations, and within a variety of sequences (Levis 1989; Biessmann *et al.* 1990a; Ahmad and Golic 1998; Titen and Golic 2010; Beaucher *et al.* 2012). Although a fully functional telomere will carry the proteins that form a functional cap, and the retrotransposons that solve the end replication problem, the latter are only necessary in the long term so that vital genes are not lost as chromosomes shorten over many generations. In the short term, possession of a functional cap is sufficient to allow survival, and we consider such a chromosome to be healed.

Flies carrying loss-of-function mutations in terminin complex proteins show end-to-end chromosome fusions and activate the DNA Damage Response (DDR) (Musarò *et al.* 2008; Cipressa and Cenci 2013). The terminin proteins include HOAP (encoded by *cav*), Tea (*tea*), Modigliani (*moi*), Verocchio (*ver*), and the paralogous pair of proteins Hiphop (*hiphop*) and Ms(3)K81 (or simply K81; *ms(3)K81*) (Rong 2008; Raffa *et al.* 2011; Zhang *et al.* 2016). A number of other proteins that are conserved in telomerase and nontelomerase organisms, such as HP1 (*HP1*), ATM (*tefu*), and the MRN complex (*mre11*, *rad50*, *nbs*), function in telomere maintenance in both types of organisms (Fanti *et al.* 1998; Pandita 2002; Rong 2008; Sabourin and Zakian 2008; Lamarche *et al.* 2010; Canudas *et al.* 2011). A common characteristic of members of this latter group is that they also play roles at nontelomeric locations.

In spite of the identification of many genes required for telomere function, little is known of the mechanisms that mediate chromosome healing in *Drosophila*. Genes involved in the DDR and DNA repair are known to influence the frequency of chromosome healing. Females that carry the *mu-2* mutation allow healing of terminally truncated chromosomes in the female germline (Mason *et al.* 1984), and *mu-2* is homologous to a mammalian DNA damage checkpoint protein (Dronamraju and Mason 2009). The *lok* (encoding Chk2) and *p53* genes also strongly influence the recovery of healed chromosomes (Titen *et al.* 2014). However, it is probable that *mu-2*, *lok* and *p53* all act indirectly, via cell cycle checkpoint delays or control of cell growth and viability, rather than participating directly in the addition of a new telomere to a broken end. DNA repair proteins influence the healing of chromosomes that have been cut by the meganuclease I-*Sce*I, but this effect may also be indirect—when mutants eliminate the normal DSB repair pathways the frequency of healing may increase simply because the preferred repair mechanisms are unavailable (Beaucher *et al.* 2012). The influence of the telomere capping complex proteins on healing has yet to be investigated.

In the work reported here we examined the fate of a broken chromosome in somatic cells and found strong evidence for occasional healing. Additionally, we identified one component of Drosophila telomeres, Hiphop, whose overexpression promotes the survival of cells with a broken chromosome and the transmission of broken-and-healed chromosomes through the male germline. We propose that Hiphop acts to seed telomere cap formation on broken chromosome ends, and that an increased frequency of chromosome healing with *hiphop* overexpression is the basis for these somatic and germline effects.

Materials and Methods

Karyotype examination

DAPI-stained larval brain chromosomes were prepared as described (Fanti and Pimpinelli 2004). To obtain metaphase preparations for the immunostaining of Figure 2, the larval brains were dissected in 0.7% sodium chloride and treated with hypotonic solution (0.5% sodium citrate) for 8 min. Brains were then fixed for 8 min with 2% formaldehyde and 45% acetic acid, and squashed in the same fixative. Slides were frozen in liquid nitrogen, and, after flipping off

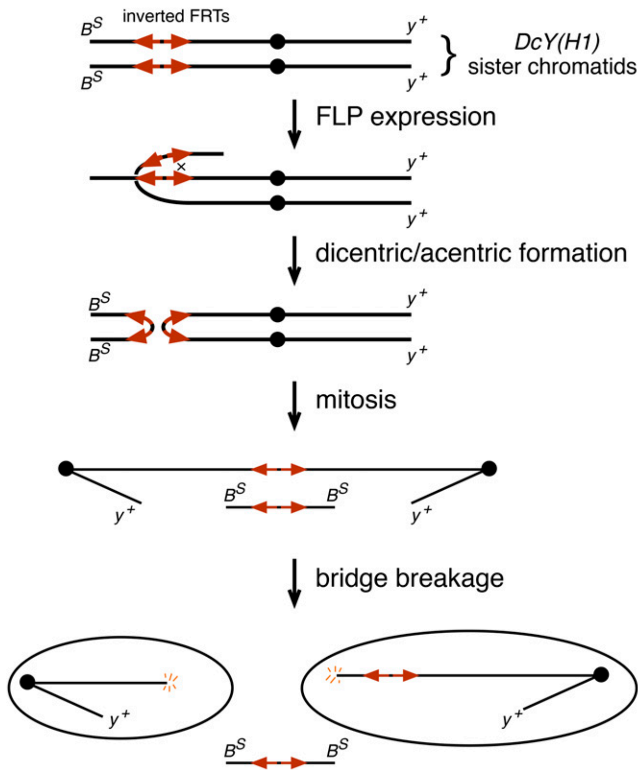

Figure 1 Dicentric chromosome formation mediated by FLP recombinase with the *DcY(H1)* chromosome. The *DcY(H1)* chromosome is a B^S *Y* y^+ chromosome carrying inverted *FRTs* inserted proximal to B^S on *YL*, but distal to all fertility factors. FLP-mediated recombination between inverted *FRT*s on sister chromatids produces a dicentric *Y* chromosome, and a small acentric chromosome carrying both copies of B^S. In mitosis, the dicentric bridge breaks to produce daughter cells, each carrying a chromosome with one broken end. If these chromosomes are healed and transmitted to progeny they may be recognized as y^+ B^+ offspring. Dicentric chromosome formation on other chromosomes uses inverted *FRT*s to similar effect.

the coverslip, were immersed in PBS, washed in PBS containing 1% Triton-X, and incubated in 1× PBS with dried nonfat milk for 30 min (~1 spoonful of milk in 40 ml of 1× PBS). The slides were then incubated with HOAP antibody for 1 hr at room temperature, and then overnight at 4°. The HOAP antibodies, obtained in guinea pig, were diluted 1:100 in PBS 1% bovine serum albumin (BSA). Cy3-conjugated Goat Anti-Guinea Pig IgG (H+L) was used as a secondary antibody (diluted 1:100; Life Technologies). For examination by DAPI staining only, squashes were prepared as described (Gatti and Pimpinelli 1983)

Fly husbandry, stocks, and transgenes

All flies were raised at 25° on standard medium. Heat shocks were performed by partial submersion of culture vials in a circulating water bath. The *eyGal4 UASFLP* combination used was *P{Gal4-ey.H}4–8 P{USFLP.D}JD1*; the *eyFLP* transgene was *P{ey-FLP.n}2*; the brain *Gal4* driver was *P{GawB}*6011A. These stocks were obtained from the Bloomington (IN) *Drosophila* stock center. Use of the *eyGal4 UASFLP* and *eyFLP* combinations has been described previously (Kurzhals *et al.* 2011). An *Epgy2* element (*EY09894*, obtained from the Bloomington stock center) was used for overexpression of *hiphop* in Figure 3D. The *UAShiphop* (fused to *mCherry*) and *UASK81* (fused to GFP) transgenes and $hiphop^{L14}$ and $hiphop^{L32}$ mutant alleles have been described elsewhere (Gao *et al.* 2011). Figure 3E used the HRH008-A1 insertion on *2*; Figure 3F used the insertion HRH008-E on chromosome *3*; Figure 3G used the insertion HGK-4B insertion on chromosome *3*. The *UAScav* transgene construct (fused to *GFP*) has been described elsewhere (Raffa *et al.* 2010). We used two different insertions of this element on chromosome *2*, #1 and #3, for Figure 3, H and I, respectively. The *HS-HP1* construct has been described elsewhere (Eissenberg and Hartnett 1993; Fanti *et al.* 1998). The *70FLP*3F line was previously described (Golic *et al.* 1997). For cytological experiments, insertions of the *P{FrTr}* element, carrying inverted *FRT*s and an adjacent mini-*white* gene (Titen and Golic 2010), were used to generate dicentric chromosomes. *P{FrTr}*1D at 72D1 (Figure 2 and Table 1) and *P{FrTr}*1B at 46F3 (Figure 4) were used. The *DcY(H1)* chromosome, carrying y^+ near the tip of the short arm, B^S near the tip of the long arm, and *P{iw}* with inverted *FRT*s proximal to B^S has been described elsewhere, along with its use to assay germline chromosome healing (Kurzhals *et al.* 2011; Titen *et al.* 2014). The *nosGal4* and *UASFLP* transgenes have been described elsewhere (Van Doren *et al.* 1998; Beumer 1997).

BARTL assay

The BARTL assay was performed as previously described (Kurzhals *et al.* 2011).

Transmission of healed chromosomes

To test for the influence of *hiphop* overexpression on healing in the male germline, we generated males of the following genotypes: (1) *y w/DcY(H1)*; *nosGal4 UASFLP/+*; (2) *y w/DcY(H1)*; *UAShiphop/+*; *nosGal4 UASFLP/+* ; (3) *y w/DcY(H1)*; lok^{P6}; *nosGal4 UASFLP/+*; (4) *y w/DcY(H1)*; lok^{P6} *UAShiphop*/lok^{P6}; *nosGal4 UASFLP/+*. Three different *nosGal4 UASFLP* combinations (1, 40, and 95) with different efficiencies were tested for each genotype. The *UAShiphop* insertion HRH008-A1 on *2* was used in these experiments. The males were individually test-crossed to *y w* females, and the progeny were counted through day 18 of the cross.

Antibody staining of Hiphop:mCherry fusion in testis

Testes were dissected from young males (0- to 3-day-old adults) in 1× PBS, fixed for 30 min in 1× PBS + 4% paraformaldehyde, then washed in PBS-T (1× PBS + 0.1% Triton-X) for 60 min at room temperature (RT). The testes were incubated overnight with the primary antibody (1:200 dilution of rabbit polyclonal antibody against mCherry; Thermo-Fisher PA5-34974) at 4° in 1× PBS-T + 3% BSA, then 1 hr at RT. They were then washed three times, 20 min each, in PBS-T at RT, then incubated with the secondary antibody (1:400 dilution of goat anti-rabbit coupled to AlexaFluor 488; Thermo-Fisher A-11008) in PBS-T + 3% BSA at RT for 2 hr. The testes were then washed 3× for 20 min each, in PBS-T at RT. DAPI was added to the final wash at a

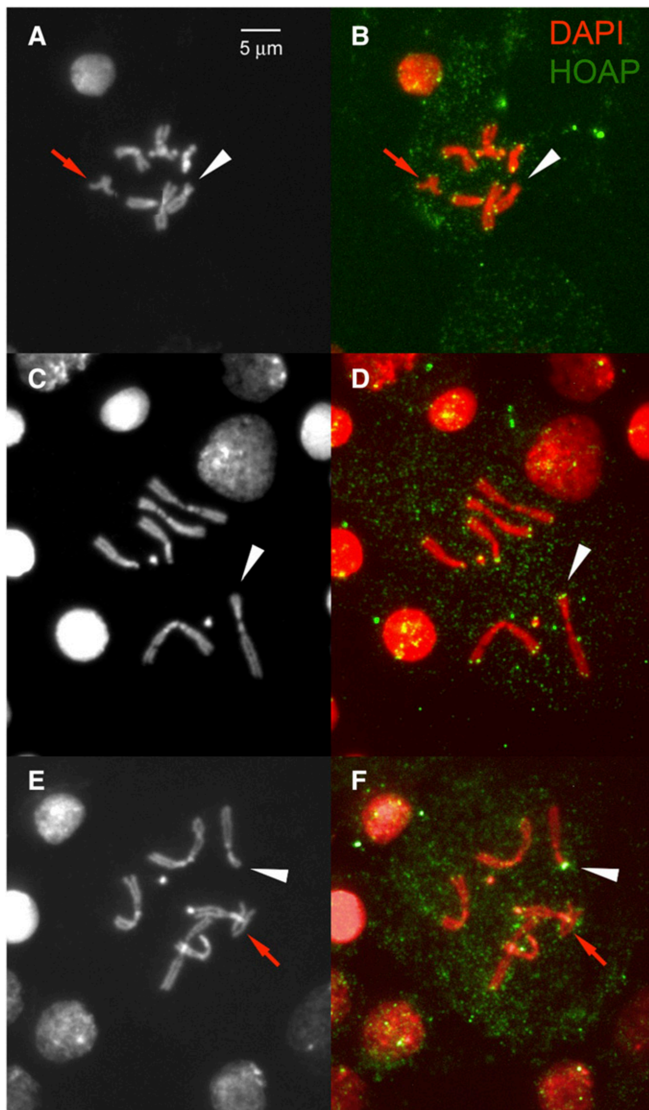

Figure 2 Detection of HOAP on chromosome breaks. Acentric chromosomes are indicated with a red arrow, and are expected to have telomeres on both ends. In each panel the broken end of chromosome *3* is indicated with a white arrowhead. (A, C, E) DAPI staining of chromosomes; (B, D, F) HOAP staining shown in green with chromosomes in red. (A and B) A nucleus with no HOAP staining on the broken end, but HOAP staining clearly visible on the acentric portion and on the remaining chromosomes. (C and D) A nucleus lacking the acentric chromosome, but with clear HOAP staining detected on the broken chromosome end. (E and F) A nucleus with very strong HOAP signal on the broken chromosome end.

concentration of 0.4 μg/ml. Testes were then transferred to fresh 1× PBS, and stored at 4° until examined. Testes were mounted in Vectashield and examined with an Olympus DSU disc scanning microscope using 60× and 100× PlanApo oil immersion objectives.

Data availability

Supplemental Material, Table S1 (see File S1 for legend) contain complete progeny counts used for testing the effect of *hiphop* overexpression in the germline. Fly strains are available from the authors upon request.

Results

Experimental system

In these experiments, we used FLP-mediated recombination between inverted *FRT*s on sister chromatids to produce a dicentric and an acentric chromosome. This process is very efficient, and can be induced to occur in nearly 100% of cells. In some experiments, inverted *FRTs* inserted at a medial location on an autosomal arm were used because this generates dicentric and acentric chromosomes that are easily detected in metaphase chromosome preparations. In other experiments we used a $B^S\,Y y^+$ chromosome, carrying the dominant eye shape marker Bar^{Stone} on the long arm distal to the inverted *FRT*s, and the dominant body color marker $yellow^+$ on the other arm (Figure 1). Because the *Y* is not required for cell viability, the generation of dicentric and acentric chromosomes does not produce deleterious aneuploidy as it does on a large autosome. The placement of B^S distal to the inverted *FRT*s makes this system especially useful to assess survival of cells in the eye after dicentric formation (Kurzhals *et al.* 2011). We also used this chromosome to assess breakage and healing in the germline, as described below.

Healing of broken chromosomes in somatic cells

To determine whether chromosome healing may occur in the soma, we generated dicentric chromosomes using inverted *FRT*s at a medial site of the left arm of chromosome *3*. At different times, between 8 and 72 hr following heat shock induction of *FLP* expression, we examined the broken centric fragments of chromosome *3* for the presence of the vital telomere cap component HOAP (Cenci *et al.* 2003) as an indicator of whether a broken end had been healed (Figure 2). We used the frequency of HOAP staining on the acentric fragment (which carries the normal telomeres of 3L sister chromatids) as a measure of staining efficiency (186/260 = 0.715), and normalized the frequency of staining on the broken chromosome accordingly (Table 1). Overall, ~19% (57/413/0.715) of broken chromosome ends exhibited HOAP staining. HOAP is normally only found at telomeres. Its presence at sites of chromosome breakage, far from its normal telomeric location, strongly suggests that healing occurs in a significant, though minor, fraction of somatic cells. Thus, the persistence of somatic cells that have experienced chromosome breakage is likely the result, in part, of healing the broken chromosome end. Curiously, we observed that in a few instances (at the 16 hr time point), the strongest HOAP signal by far was to be found on the broken chromosome end (Figure 2F), though the reason for this is not known.

Most cells did not add a new telomere to the broken end. The persistence of cells with an unhealed chromosome break is not unprecedented. Cells may also survive through other mechanisms such as adaptation, wherein a cell with an unrepaired DSB can survive for an extended period, though it may ultimately succumb to cell death (Sandell and Zakian 1993; Titen and Golic 2008). It is likely that the surviving cells with uncapped broken chromosomes utilize such a mechanism.

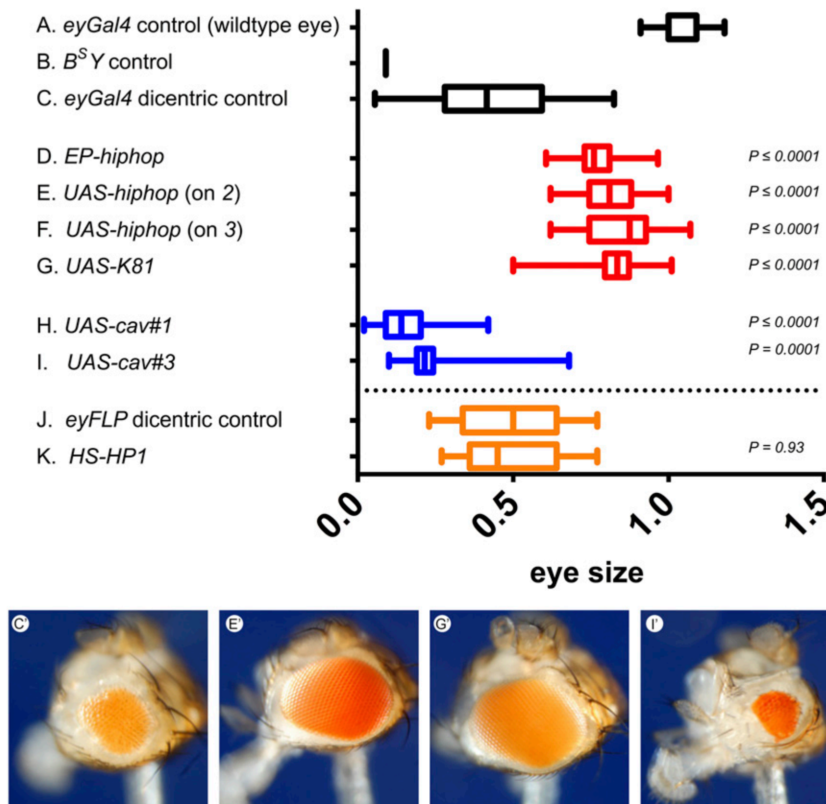

Figure 3 Effects of telomere capping genes in the BARTL assay. Dicentric chromosome formation was induced in the developing eyes of males carrying the *DcY(H1)*, y^{+} B^{S} chromosome, using *eyGal4 UASFLP* (C–I) or *eyFLP* (J and K), and eye sizes were measured for each genotype. (A and B) Wild-type and B^{S} controls, respectively. (C) The effect of *DcY(H1)* dicentric production. The eyes are larger than in B, owing to survival and differentiation of some cells that lost B^{S}. The effect of *hiphop* (D–F), *ms(3)K81* (G), and *cav* (H and I) overexpression. *P* values are for comparison with C. (J and K) Eye size when FLP is expressed from *eyFLP* transgene (J), and when *Su(var)205* (encoding HP1) is also overexpressed with an early developmental heat shock (K). Typical phenotypes are shown below [*i.e.*, (C′) *eyGal4*, (E′) *UAS-hiphop*, (G′) *UAS-K81*, and (I′) *UAS-cav#3*]. The numbers of eyes measured for each genotype (A–K) were 20, 43, 110, 35, 16, 16, 14, 18, 18, 30, and 30, respectively. The plots show 5th, 25th, median, 75th, and 95th percentiles.

Hiphop increases survival of somatic cells with a broken chromosome

These experiments strongly suggest that chromosome healing can occur in somatic cells. We therefore investigated whether overexpression of telomere cap components might offer additional protection from the lethal effect of a broken chromosome. The BARTL (Bar and Telomere Loss) assay relies on *Y* chromosome dicentric formation (Figure 1) produced by constitutive FLP synthesis during eye development, and uses adult eye size as a metric for survival of cells with broken chromosomes (Kurzhals *et al.* 2011). The broken end activates the DDR, frequently resulting in apoptosis. Flies generated in this screen have eyes that are approximately half the size of wildtype eyes (Figure 3C). Although some cells do survive and differentiate, many clearly do not. An *eyGal4 UASFLP* combination, or *eyFLP*, was used to drive *FLP*. Telomere cap genes were driven simultaneously with Gal4, or by heat shock induction. In this assay we found that overexpression of *hiphop* (Gao *et al.* 2010), or the closely related paralog *ms(3)K81* (Loppin *et al.* 2005; Dubruille *et al.* 2010; Gao *et al.* 2011), produced flies with larger eyes (Figure 3, D–G). However, overexpression of *cav* (encoding HOAP; Figure 3, H and I) or *Su(var)205* [encoding HP1 (Eissenberg *et al.* 1990); Figure 3K] did not produce an increase in eye size. In fact, overexpression of *cav* in this assay had the opposite effect, producing significantly smaller eyes. Overexpression of *hiphop*, *K81*, *cav* or *HP1* had no effect on otherwise wildtype eyes (not shown). These results suggest that the terminin component Hiphop has a unique role in protecting cells from the deleterious effect of a broken chromosome.

To further test whether *hiphop* overexpression ameliorates the effect of a broken chromosome, we examined cells by looking at metaphase chromosome spreads after dicentric chromosome induction, using heat shock to induce *FLP* expression. Previously we showed that, in wildtype flies, cells with broken chromosomes are very frequent shortly after *FLP* induction, but are eliminated over time, so that, after 3 days, few such cells remain (Titen and Golic 2008). When comparing wildtype with *hiphop* overexpressing cells, at the initial assessment 24 hr after heat shock, the majority of cells in each genotype exhibit abnormal karyotypes, characterized by dicentric chromosomes, broken centric fragments, or single or multiple copies of acentric fragments. At this early time point, *hiphop* overexpression had no discernable effect (Figure 4, columns 1, 2). However, when cells were examined 72 hr after heat shock, there was a significant difference between wildtype and *hiphop* overexpressing cells. In wildtype flies, few cells with abnormal karyotypes remain (Figure 4, column 3). In contrast, we found that *hiphop* overexpression rescued cells with abnormal karyotypes, allowing them to persist at high frequency, even 3 days after FLP induction (Figure 4, column 4). This provides further evidence that cells with a broken chromosome can be rescued by *hiphop* overexpression.

Hiphop promotes chromosome healing

The BARTL assay and karyotype examinations both show that *hiphop* overexpression can save cells that would otherwise die following dicentric formation and breakage. The presence of the telomere cap component HOAP on a fraction of broken

Table 1 Frequency of HOAP staining on broken chromosomes

Time after heat shock (hr)	Nuclei with acentric			Nuclei lacking acentric	
	Total	Acentric positive	Broken end positive	Total	Broken end positive
8	28	18	7	3	1
16	138	102	16	74	7
24	58	39	14	30	3
40	23	15	5	19	2
72	13	12	1	27	1
Total	260	186	43	153	14

Nuclei that carried a broken chromosome were scored for HOAP staining on the broken chromosome end, and on the acentric chromosome (if present) as a control for staining efficiency. The larvae that were examined carried *70FLP*3F on their *X* and the inverted *FRT*-bearing element *FrTr*1D (at 72D1 on *3L*). They were heat shocked at 38° for 1 hr, and dissected at various times after heat shock.

chromosome ends in somatic cells is a probable indicator that such chromosomes are healed. It is tempting to speculate that *hiphop* overexpression rescues cells with broken chromosomes by promoting formation of new telomeres on the broken chromosome ends. However, it is also conceivable that *hiphop* promotes cell survival entirely apart from chromosome healing. One may argue whether or not HOAP association to broken ends represents true healing (see *Discussion*), but a definitive test of healing is possible. When a chromosome is transmitted through the germline, and is then recovered in normal viable offspring, by definition, that chromosome must carry a functional telomere. Thus, the transmission and recovery of broken chromosomes through the germline provides a solid and quantifiable test of chromosome healing. Accordingly, we asked whether *hiphop* overexpression in the male germline could increase the transmission of healed chromosomes.

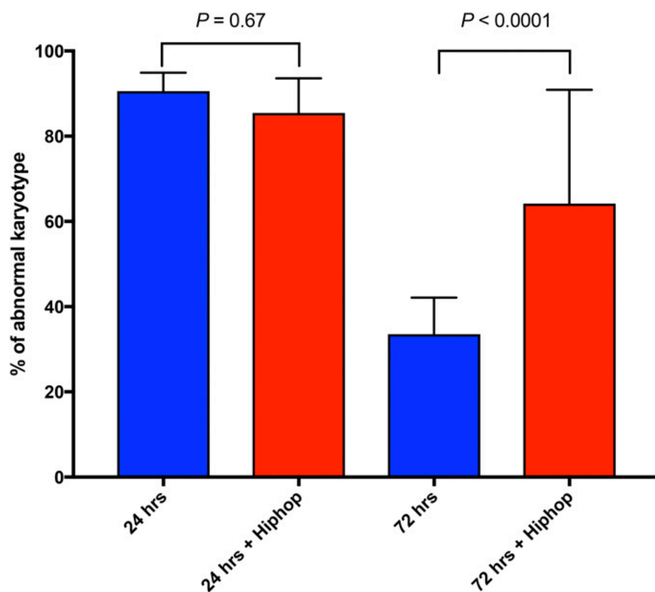

Figure 4 Survival of cells that experienced dicentric formation. Dicentric chromosome formation was induced in larvae of all stages by heat shock (38°, 1 hr) using *70FLP*3F. *P{GawB}6011A* drove expression of *UAShiphop* in the brain. Metaphase nuclei from third instar larval brains were examined at 24 and 72 hr after heat shock. Cells carrying broken chromosomes (visibly shortened) and/or acentric chromosomes were scored as abnormal. The numbers of nuclei examined for each time point and genotype were 115 (5 brains), 99 (4 brains), 133 (4 brains), and 33 (3 brains), respectively. Significance was calculated with a 2 × 2 contingency test. SD are indicated.

To assay germline breakage and healing we use the same *DcY(H1)* (*Dicentric-forming Y*) chromosome that was used in the BARTL assay. FLP-mediated recombination generates a dicentric chromosome and an acentric chromosome, with B^S on the acentric fragment, which is subsequently lost. Breakage and healing of the dicentric portion generates y^+ B^+ sons (denoted as *FrY*, for *Fragment Y*) in testcrosses to *y w* females. To drive expression of *FLP* in the germline we used chromosomes carrying *nosGal4* and *UASFLP* (indicated as *nGUF*). The transmission of the healed *FrY* chromosomes, as a fraction of all sex chromosomes, varied between 0.2 and 25%, depending on whether a weak or strong *nGUF* combination was used (Figure 5, top row; complete data in Table S1).

We tested whether *hiphop* overexpression would affect the recovery of healed chromosomes by using the same combinations with the addition of *UAShiphop*. The combination of *nosGal4* with *UAShiphop* drives robust expression of *hiphop* in the germline (Figure 6). When this is added to the normal level of Hiphop already present in these $hiphop^{+/+}$ animals, the result is significant overexpression of *hiphop*. With *UAShiphop* the transmission of *FrY* chromosomes increased significantly, ranging from 18% (representing an increase of ~100-fold) to 47% (representing ~99% of all *Y* chromosomes transmitted by these males; Figure 5, second row). The increased transmission of healed chromosomes is consistent with the promotion of new telomere formation by Hiphop.

In this experiment *UASFLP* and *UAShiphop* expression were driven in mitotically dividing cells, at a time when Hiphop normally functions. Although *ms(3)K81* does substitute for *hiphop* late in spermiogenesis (Dubruille *et al.* 2010; Gao *et al.* 2011), that is at a much later stage than the *nos* driven expression used here.

A concern with the proposal that Hiphop promotes telomere cap formation derives from our previous finding that *lok* (encoding Chk2) mutant males also transmit healed *FrY* chromosomes at a higher rate (Titen *et al.* 2014). In principle, Chk2 might act to block chromosome healing, and in *lok* mutants this block would be relieved to allow an increased rate of healing. But with *lok* this is more likely an indirect

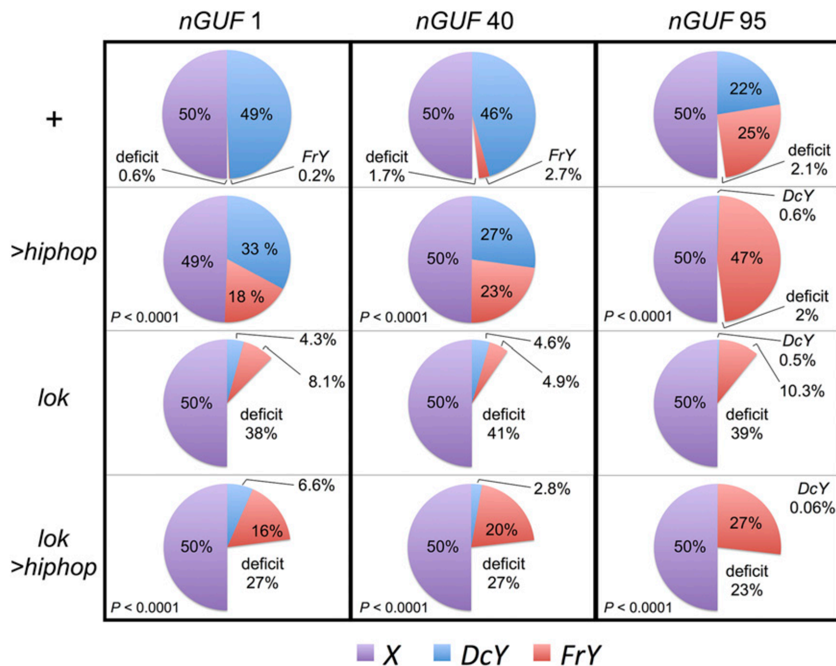

Figure 5 Effect of Hiphop on transmission of healed chromosomes. The transmission of *X*, *DcY*, and *FrY* chromosomes was measured in testcrosses. FLP expression was driven by three combinations of *nosGal4 UASFLP* (*nGUF* 1, 40, or 95) on chromosome *3* in males that were: *y w/DcY(H1)*; *nGUF/+*, or *y w/DcY (H1)*; *UAS-hiphop/+*; *nGUF/+*, or *y w/DcY(H1)*; *lok/lok*; *nGUF/+*, or *y w/DcY(H1)*; *lok UAS-hiphop/lok*; *nGUF/+*. It was assumed that sons should represent half of all progeny. In crosses that produced <50% males, the size of that deficit is indicated. Results were corrected for the slightly reduced viability of the *Y*-bearing progeny. Between 41 and 115 fertile males, with an average of ~80 progeny each, were tested for each combination. *P* values are given for comparisons of *UAShiphop* flies with corresponding genotypes that lack *UAShiphop* (*i.e.*, the group immediately above). *P* values were determined by comparing the *FrY/X* ratios produced by the individual males in each group using the Mann-Whitney test. Complete summary results for each experiment are presented in Table S1.

effect of allowing cells with broken chromosomes to continue to survive and divide, thus providing an extended time to heal, rather than a direct regulation of chromosome healing. In *lok* mutant males, premeiotic germline cells with unhealed chromosomes can continue to divide for at least 9–10 days. When these cells reach meiosis, the unhealed sister chromatids undergo end-to-end fusion and generate Meiosis II dicentric bridges. These bridges lead to the elimination of spermatid nuclei that carry them, resulting in a large deficit of sons when the *Y* chromosome is involved. Thus, if overexpression of *hiphop* increased transmission of healed chromosomes simply by permitting extended survival of cells with broken *Y* chromosomes, rather than by healing broken ends, we would expect to see a deficit of sons. This was not observed, and instead we saw a male: female ratio that was essentially equivalent to the control (Figure 5, second row). In confirmation of our previous findings, when we drove *FLP* expression in *lok* mutant males, we saw an increase in transmission of *FrY* chromosomes relative to intact *DcY* chromosomes, and a large deficit of sons (Figure 5, third row). Moreover, when *hiphop* overexpression was driven in *lok* males, the deficit of sons was partially alleviated, with a concomitant increase in the recovery of *FrY*-bearing sons (Figure 5, fourth row). Very similar results were obtained using all three *nosGal4 UASFLP* chromosomes. In all cases, *UAShiphop* produced a strong increase in *FrY* recovery in lok^{+} *males*, and *UAShiphop* rescued *Y*-bearing sperm in *lok* mutant males, producing *FrY* offspring from sperm that would otherwise have been eliminated. These results argue strongly that Hiphop does not merely promote survival of cells that carry broken chromosomes, but that such cells now survive because Hiphop promotes healing the broken chromosome ends.

We also tested the effect of *hiphop* null mutations. Since *hiphop* is a vital gene it was not possible to test homozygous mutants, but we did test two mutant alleles for their effect in heterozygotes. The rate of fragment transmission was measured as the fragment ratio [FR = $FrY/(FrY + DcY)$]. *FrY* chromosomes were recovered more frequently in the controls of these crosses than in the previous crosses, likely indicating an effect of genetic background. Nonetheless, in every case, the $hiphop^{+/-}$ heterozygotes showed significantly reduced recovery of *FrY* relative to their $hiphop^{+/+}$ siblings with matched genetic background (apart from chromosome *3* where *hiphop* is located; Table 2), supporting the hypothesis that Hiphop is a critical player in the process of *de novo* telomere formation.

Discussion

The ability of organisms with linear chromosomes to heal broken chromosome ends appears to be universal, occurring in organisms that use telomerase, and in those that do not (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; Sprung *et al.* 1999; Fortin *et al.* 2009). Healing in organisms with telomerase most often involves telomerase-mediated addition of telomere repeats to broken ends, and utilizes microhomologies to the normal telomeric repeat (Greider and Blackburn 1985; Mangahas *et al.* 2001; Pennaneach *et al.* 2006; Gao *et al.* 2008; Murnane 2012). It is thought that the newly added repeats are then capable of recruiting the full array of shelterin components and associated proteins. In organisms that do not use telomerase, such as *Drosophila*, among others (Mason *et al.* 2011), the mechanism of healing is unknown.

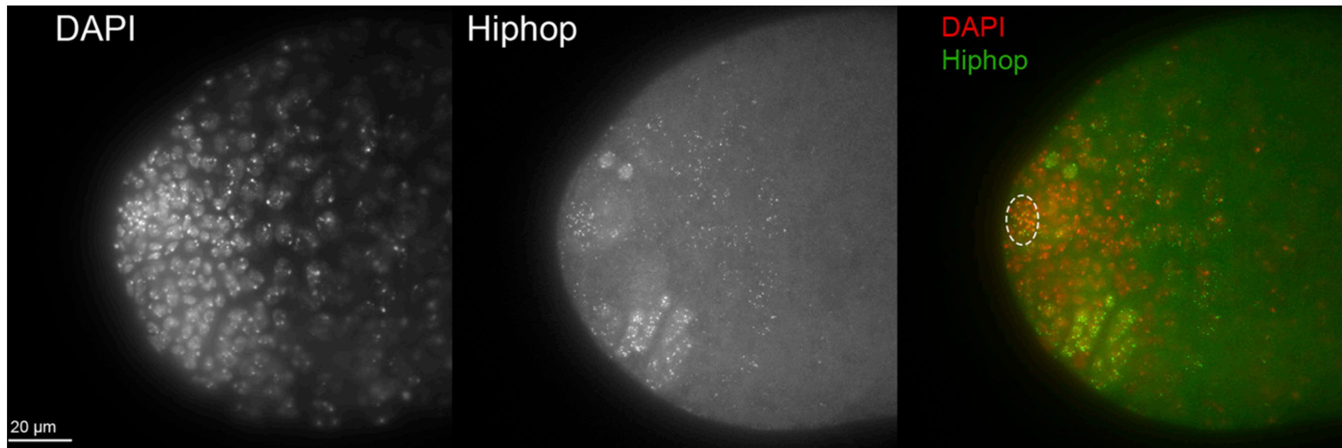

Figure 6 Expression of *UAShiphop* in the male germline. Testes of *y w/Y*; *UAShiphop/+*; *nosGal4 UASFLP/+* males were dissected and stained with DAPI and an antibody to mCherry (to which Hiphop is fused in this construct). The HRH008-A1 insertion and the *nGUF*95 combination were used (the same combination as in Figure 5, third column, second and fourth rows). Seven testes were examined—all showed strong expression in germline stem cells at the hub (circled in panel at right), and variable expression at later stages. Expression was in the form of bright puncta, as expected for telomeric staining, and a diffuse and dimmer cytoplasmic staining.

In most cases, it seems that healing is a repair of last resort, used only after normal repair mechanisms, which conserve the genome, fail. However, there are also well-known examples of developmentally programmed chromosome breakage and healing in particular cells or particular compartments of the cell (Baroin *et al.* 1987; Forney and Blackburn 1988; Spangler *et al.* 1988; Müller *et al.* 1991; Magnenat *et al.* 1999). In some plants, chromosome healing is also differentially regulated between tissues (McClintock 1939). Thus, it is not a foregone conclusion that healing may occur in all tissues of an organism once it has been demonstrated in one.

In previous work, we showed that somatic cells can occasionally survive and differentiate as adult tissue despite the occurrence of a broken chromosome (Golic 1994; Kurzhals *et al.* 2011). Such ends cannot be repaired by normal mechanisms, and, most often, but not always, lead cells into apoptosis (Ahmad and Golic 1999; Titen and Golic 2008). How some cells survive DNA damage that is normally lethal has been an open question. By staining for the unique telomere component HOAP, we find that ~20% of these cells show evidence of chromosome healing. The addition of a new telomere to the broken end would allow these cells to repress the DDR and escape apoptosis. It has been known for some time that mammalian somatic cells may heal broken ends by *de novo* telomere addition (Murnane and Yu 1993). Our results indicate that this is also true for *Drosophila*.

It might be argued that HOAP staining is not an absolute indication of a functional telomere. For example, *moi*, *tefu*, and *tea* mutants have dysfunctional telomeres, yet still show localization of HOAP at chromosome ends (Bi *et al.* 2004; Raffa *et al.* 2009; Zhang *et al.* 2016). However, this appears to result from an epistatic relationship in which *moi*, *tefu*, and *tea* lie downstream of HOAP (discussed further below). Since all telomere cap components were wildtype in the experiment of Figure 2, HOAP association likely reflects an actual healing event. Furthermore, HOAP is normally found exclusively at telomeres, and in our experiments HOAP appeared at sites that are normally found in the middle of a chromosome arm. The occurrence of HOAP at these newly broken ends must represent, at a minimum, an attempt by the cell to heal that end by cap addition.

In spite of this strong evidence for chromosome healing in somatic cells, only a minor fraction of the somatic cells with a broken chromosome survive by chromosome healing; the majority show no evidence of healing. Most of these cells must survive by other means. In the soma, the Chk2 and P53-mediated apoptotic response to DNA damage is largely responsible for eliminating cells with damaged genomes. Any mechanisms that interfere with this apoptotic response, such as the P53 negative regulator *corp* (Chakraborty *et al.* 2015), could contribute to continued survival of the remaining cells.

In somatic cells, we tested overexpression of three telomere cap components: HOAP, HP1, and Hiphop/K81. Of these, only Hiphop and its paralog K81 [which is capable of substituting for Hiphop in somatic cells (Gao *et al.* 2011)] increased the survival of cells with a broken chromosome. Moreover, in germline cells, *hiphop* overexpression increased the transmission of broken chromosomes by healing chromosomes that would otherwise have been eliminated. This result indicates that Hiphop is capable of promoting chromosome healing, and this likely represents the mechanism of its action in somatic cells as well. We propose that this reflects a unique role for Hiphop at broken ends: Hiphop "seeds" the formation of a new cap structure.

It was surprising to find that overexpression of *cav* (Raffa *et al.* 2010) in somatic cells had a negative effect on survival of cells with a broken chromosome, leading to smaller eyes in

Table 2 The recovery of healed chromosomes from males carrying a *hiphop* mutant allele

	Fathers		Progeny				
Genotype	Fertile	Sterile	*DcY*	*FrY*	*X*	FR	*P* value
nGUF40/TM6	20	31	523	99	686	0.16	
nGUF40/hiphop^{L14}	18	12	705	16	796	0.02	<0.0001
nGUF40/ hiphop^{L32}	38	22	1487	21	1583	0.01	<0.0001
nGUF95/TM6	11	20	30	283	386	0.90	
nGUF95/ hiphop^{L14}	7	20	174	95	341	0.35	<0.0001
nGUF95/ hiphop^{L32}	15	16	113	369	505	0.77	<0.0001

y w/DcY males carrying *nosGal4 UASFLP* (40 or 95) and homozygous for *hiphop*$^+$ (one copy on the *nGUF* chromosome *3* and one on *TM6*), or heterozygous for *hiphop*$^+$ and a mutant allele (either *hiphop*^{L14} or *hiphop*^{L32}) were testcrossed to *y w* females. Progeny were scored as *DcY* (B^S y^+ sons), *FrY* (B^+ y^+ sons) or *X* (*y w* daughters). Fragment ratio is calculated as *FrY/total Y* progeny. Heterozygous mutants were compared with their respective +/+ controls using a 2 × 2 contingency test comparing *DcY and FrY* progeny from each, with *P* values shown.

the BARTL assay. This may reflect a real effect of excess HOAP, and possible interference with the stoichiometry needed for *de novo* telomere capping. Alternatively, since HOAP physically interacts with Hiphop (Gao *et al.* 2010), excess HOAP may prevent Hiphop from reaching chromosome ends and seeding telomere formation. Finally, it is possible that this reflects a deleterious effect that is unique to this HOAP-GFP fusion construct.

It is interesting to note that, in a few nuclei, the HOAP signal on the broken chromosome end was far stronger than HOAP signals at normal chromosome ends. We speculate this may reflect an early stage in the healing process, with either a quantity or a configuration of HOAP that differs from that found at normal "mature" telomeres. Either possibility suggests that chromosome healing occurs in stages, with the cap progressing from an immature to mature structure. Zhang *et al.* (2016) examined telomeric localization of the known terminin components in mutants that lacked one of these components, and elucidated a hierarchy of telomere associations. Hiphop and HOAP lie at the top of this hierarchy, followed by Tea, which is followed by Ver and Moi. Altogether, these results suggest a model for healing in which Hiphop is the first terminin component to recognize a nontelomeric chromosome end, perhaps in cooperation with components of the DDR, such as ATM or the MRN complex, which are also required for functioning telomeres and for the normal localization of Hiphop to telomeres (Ciapponi *et al.* 2004; Ciapponi 2006; Gao *et al.* 2009, 2010). Hiphop then recruits HOAP and HP1, the Hiphop-HOAP-HP1 complex recruits Tea, and this assembly then recruits Ver and Moi.

Such a role for Hiphop fits perfectly with what is known about the Hiphop and K81 proteins. K81 is required during spermiogenesis when histones that package chromosomal DNA are replaced with protamines (Jayaramaiah-Raja and Renkawitz-Pohl 2005). The telomeric cap is also remodeled at this time, with K81 replacing Hiphop and HOAP at chromosome ends (Dubruille *et al.* 2010; Gao *et al.* 2011). This replacement is vital, because sperm without K81 fail to construct functional telomeres in the male pronucleus after fertilization, leading to chromosome fusions and zygotic lethality. Gao *et al.* (2011) suggested that a role for K81 might be to recruit other cap components to chromosome ends in the male pronucleus. Our findings, that *hiphop* or *ms(3)K81* overexpression promotes survival of cells with broken chromosomes, and, more critically, that *hiphop* overexpression promotes healing of broken chromosome ends in the male germline, provide strong support for the model that one role of Hiphop (and K81) is to seed telomere formation. The hypothesis that Hiphop plays a critical role in recruiting other telomere capping proteins is further supported by recent results showing that overexpression of Hiphop in the male germline is accompanied by increased localization of HOAP and HP1 to telomeres (Dubruille and Loppin 2015).

One striking aspect of the germline results is the revelation that the mechanism which preserves a functional germline with undamaged chromosomes is extremely robust. This is apparent when considering the results with *lok*; *nGUF*1 males. Sons with an intact *DcY* chromosome accounted for only ~4% of all their progeny, indicating that dicentric chromosome formation must occur in at least ~90% of germline cells with this least effective driver combination. Nevertheless, nearly all of the sons of *nGUF*1 wildtype males carry the intact *DcY* chromosome. When using the *lok*; *nGUF*95 combination, sons with an intact *DcY* were nearly nonexistent, indicating that dicentric formation must occur in nearly 100% of germ cells, but the intact chromosome still accounted for almost half of the sons of *nGUF*95 wildtype males. The fact that these males are reasonably fertile (~80%, Table S1) points to the existence of an extremely effective mechanism to preserve the germline, even in the presence of pervasive DNA damage. Repair of broken chromosomes by d*e novo* telomere addition must be one part of this mechanism, but is clearly not preferred. The favored mechanism must involve removing the cells with a broken chromosome and replacing them with undamaged cells. Chk2 and P53 are involved in the former and latter aspects, respectively (Titen *et al.* 2014; Wylie *et al.* 2014; Ma *et al.* 2016).

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