The *Drosophila melanogaster* PIF1 helicase promotes survival during replication stress and processive DNA synthesis during double-strand gap repair

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Abstract

PIF1 is a 5' to 3' DNA helicase that can unwind double-stranded DNA and disrupt nucleic acid-protein complexes. In *Saccharomyces cerevisiae*, Pif1 plays important roles in mitochondrial and nuclear genome maintenance, telomere length regulation, unwinding of G-quadruplex structures, and DNA synthesis during break-induced replication. Some, but not all, of these functions are shared with other eukaryotes. To gain insight into the evolutionarily conserved functions of PIF1, we created *pif1* null mutants in *Drosophila melanogaster* and assessed their phenotypes throughout development. We found that *pif1* mutant larvae exposed to high concentrations of hydroxyurea, but not other DNA damaging agents, experience reduced survival to adulthood. Embryos lacking PIF1 fail to segregate their chromosomes efficiently during early nuclear divisions, consistent with a defect in DNA replication. Furthermore, loss of the BRCA2 protein, which is required for stabilization of stalled replication forks in metazoans, causes synthetic lethality in third instar larvae lacking either PIF1 or the polymerase delta subunit POL32. Interestingly, *pif1* mutants have a reduced ability to synthesize DNA during repair of a double-stranded gap, but only in the absence of POL32. Together, these results support a model in which Drosophila PIF1 functions with POL32 during times of replication stress but acts independently of POL32 to promote synthesis during double-strand gap repair.

Introduction

Pif1 family helicases are 5' to 3' superfamily 1 helicases that are highly conserved in most eukaryotes and some bacteria and are critical for DNA replication, recombination, and repair (BOCHMAN *et al.* 2010; CHUNG 2014; BYRD AND RANEY 2017). Although Pif1 family helicases possess a conserved single-stranded (ss) DNA-dependent helicase domain, their N- and C-terminal domains differ significantly in size and sequence between organisms (Figure S1) (LAHAYE *et al.* 1991; BOULE AND ZAKIAN 2006). Furthermore, the cellular functions of PIF1 homologs in eukaryotes are variably conserved. Because human PIF1 appears to act as a tumor suppressor but is also required for the survival of cancer cells (GAGOU *et al.* 2014), a better understanding of its contribution to genome stability in diverse cellular and organismal contexts is needed.

Much of what is currently known about Pif1 structure and function comes from studies in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *S. cerevisiae* possesses two Pif1 orthologs, ScPif1 and ScRrm3, which localize to both the mitochondria and nucleus (IVESSA *et al.* 2000; BOCHMAN *et al.* 2010). ScPif1 was first discovered in a genetic screen where its deficiency resulted in reduced mitochondrial (mt) DNA recombination (FOURY AND KOLODYNSKI 1983). Further studies revealed that ScPif1 slows down replication progression in mtDNA to prevent double-stranded breaks (DSBs) and cooperates with base excision repair to resolve oxidative mtDNA damage (O'ROURKE *et al.* 2002; DOUDICAN *et al.* 2005; CHENG *et al.* 2007). Similar to ScPif1, *S. pombe* Pfh1 (PIF1-homolog-1) maintains mtDNA integrity. In addition, both ScPIF1 and SpPfh1 maintain telomeric DNA integrity and normal telomere length (SCHULZ AND ZAKIAN 1994; MCDONALD *et al.* 2014). However, loss of Pfh1 is lethal (ZHOU *et*

al. 2002; PINTER *et al.* 2008), while deletion of *S. cerevisiae* Pif1 is not, for reasons that are still not understood.

Pif1 appears to play multiple roles during DNA replication. ScPif1 promotes Okazaki fragment maturation during lagging strand synthesis by contributing to the displacement of downstream Okazaki fragments and the production of longer single-strand flaps by increasing DNA polymerase delta (Pol δ) processivity (ROSSI *et al.* 2008; STITH *et al.* 2008). SpPfh1 likewise contributes to the formation of single-strand flaps and resolves secondary structures at these flaps to promote their cleavage by the Dna2 nuclease (TANAKA *et al.* 2002; RYU *et al.* 2004). Pfh1 has additional essential roles in DNA replication, as it interacts with multiple replisome core proteins and is required for fork merging at replication termination sites (TANAKA *et al.* 2002; STEINACHER *et al.* 2012). Recently, ScPif1 was also shown to be important for centromere replication in the absence of the Rrm3 protein (CHEN *et al.* 2019)

One of PIF1's most conserved roles is the resolution of G quadruplex (G4) DNA. G4 DNA are stable secondary structures formed by the association of four guanines in G-rich DNA held together by non-canonical Hoogsteen base pairs (MAIZELS AND GRAY 2013). The formation of G4 structures *in vivo* can cause problems for DNA replication due to their high thermal stability (LIPPS AND RHODES 2009; BOCHMAN *et al.* 2012). Both ScPif1 and SpPfh1 unwind intra- and intermolecular G4 structures *in vitro* (PAESCHKE *et al.* 2011; SABOURI *et al.* 2014; WALLGREN *et al.* 2016). In addition, both helicases can remove stably bound protein complexes from DNA to allow for DNA replication and to prevent G4 formation at lagging strand telomeres (SABOURI *et al.* 2012; GALLETTO AND TOMKO 2013; KOC *et al.* 2016). The G4 unwinding activity has been

shown to have *in vivo* relevance, as DNA replication progresses more slowly near G4 motifs in Pif1 deficient cells (PAESCHKE *et al.* 2011; PAESCHKE *et al.* 2013).

In yeasts, Pif1 plays important roles in DNA repair. *S. cerevisiae pif1* mutants show mild sensitivity to methyl methanesulfonate (MMS), a DNA alkylating agent, and hydroxyurea (HU), a ribonucleotide reductase inhibitor that depletes nucleotide pools and stalls replication forks (BUDD *et al.* 2006; WAGNER *et al.* 2006). Interestingly, the sensitivity of *S. pombe pfh1* mutants to these chemicals is much greater (TANAKA *et al.* 2002; PINTER *et al.* 2008). ScPif1 co-localizes with the homologous recombination (HR) protein Rad52 to repair foci after gamma irradiation, and SpPfh1 is recruited to DNA damage foci in camptothecin-treated cells.

Recently, ScPif1 was shown to be required during break-induced replication (BIR), a type of HR repair observed at one-ended DNA breaks caused by replication fork collapse or shortening of telomeres (SAINI *et al.* 2013; BUZOVETSKY *et al.* 2017). During BIR, Rad51-mediated invasion of ssDNA into a homologous template creates a migrating bubble, resulting in conservative synthesis of a nascent DNA duplex (DONNIANNI AND SYMINGTON 2013; KRAMARA *et al.* 2018). Efficient BIR depends upon both Pif1 and Pol32, a non-catalytic subunit of Pol δ (WILSON *et al.* 2013). Currently models suggest that ScPif1 promotes Pol δ-dependent DNA synthesis during the bubble migration and may also unwind the newly-synthesized strand to relieve topological stress (SAKOFSKY AND MALKOVA 2017).

Several recent PIF1 studies have revealed that some of its roles are conserved in metazoans. Human PIF1 localizes to both nuclei and mitochondria. It interacts with the catalytic reverse

transcriptase subunit of telomerase and its overexpression results in decreased telomere length (MATEYAK AND ZAKIAN 2006; ZHANG *et al.* 2006; FUTAMI *et al.* 2007; PAESCHKE *et al.* 2013). Mice lacking PIF1 show a mitochondrial myopathy, suggesting a subtle role in mtDNA maintenance (BANNWARTH *et al.* 2016). The expression of mouse and human PIF1 is limited to highly proliferating embryonic stem cells and peaks in late S/G2 phase, consistent with a role in DNA replication (MATEYAK AND ZAKIAN 2006). In addition, human PIF1 is important for S-phase entry and is thought to allow unperturbed DNA replication by unwinding G4 DNA and stalled replication fork-like substrates (GEORGE *et al.* 2009; SANDERS 2010).

However, the roles and phenotypes of the yeast Pif1 helicases are not always consistent with those of its metazoan orthologs. For example, mice lacking PIF1 show no visible phenotypes, have unaltered telomere length, and display no chromosomal abnormalities. These observations are consistent with a recent study demonstrating substantial evolutionary divergence between yeast and human PIF1 proteins (DEHGHANI-TAFTI *et al.* 2019) and suggest that Pif1 may play a subtler role in higher eukaryotes or may have redundant functions with other helicases (SNOW *et al.* 2007).

To investigate the possible roles of PIF1 in a non-mammalian metazoan, we generated and characterized a *pif1* null mutant in *Drosophila melanogaster*. Here, we show that *pif1* mutants are sensitive to hydroxyurea and exhibit reduced embryo viability associated with nuclear fallout, chromosome clumping, anaphase bridges, and asynchronous mitotic divisions. Both phenotypes suggest that PIF1 plays an important function during periods of replication stress, including that encountered during embryogenesis. In addition, we demonstrate a role for

Drosophila PIF1 in promoting long-range DNA synthesis during HR repair, specifically in the absence of POL32. Finally, we identify a specific genetic interaction between PIF1 and BRCA2, which functions both in double-strand break repair and replication fork protection in mammals. Together, our findings suggest that Drosophila PIF1 shares DNA replication and double-strand break related functions of its yeast counterparts and may play an additional role in the protection of stalled or regressed replication forks.

Materials and Methods

Drosophila stocks and mutants

Drosophila stocks were maintained on standard cornmeal agar medium at 25°C. The *pif1*^{167} null mutant was created via imprecise excision of a *P*-element, (*P{EPgy2}Pif1*^{EY10295}, Bloomington Stock 17658) located in the 5' untranslated region of *CG3238* (ALEXANDER *et al.* 2016). The deletion removes nucleotides 64-1759 (relative to the transcription start site) and inserts the sequence CTGTTATTTCATCATG at the deletion breakpoint.

Other mutants used in this study include *pol32*^{L2}, which removes all but the first 42 nucleotides of the POL32 coding sequence (KANE *et al.* 2012); *brca2*^{47}, which deletes the first 2169 bp of the 3417 bp BRCA2 coding sequence (THOMAS *et al.* 2013); *brca2*^{KO}, which deletes the first 3321 bp of the BRCA2 coding sequence (KLOVSTAD *et al.* 2008); *rad51*^{057} (A205V, a null mutation), (STAEVA-VIEIRA *et al.* 2003); and *mus81*^{Nhe}, a 16nt insertion in the coding region (TROWBRIDGE *et al.* 2007). All double and triple mutants used in this study were created by standard genetic crosses and verified by PCR or Sanger sequencing. The H2Av-EGFP stock was obtained from Bloomington Drosophila Stock Center (CLARKSON AND SAINT 1999).

Mutagen sensitivity assays

Sets of five virgin females and three males heterozygous for the *pif1*^{167} mutation were mated in vials containing standard cornmeal agar medium. Females were allowed to lay eggs for 3 days before being transferred into a second vial to lay for an additional 3 days. The first set of vials served as the controls and was treated with 250 µL of water 1 day after the transfer of parents. The second set of vials was treated with 250 µL of the mutagen. Upon eclosion, adults were counted in each vial and the percentage of viable homozygotes in each vial was determined relative to the total number of adult progeny. Relative survival was calculated as (% viable

homozygotes in the mutagen-treated vials)/(% viable homozygotes in control vials) for each trial. Statistical significance was determined using paired t-tests between untreated and treated vials.

Hatching frequency assay

Hatching frequency was measured by mating 30-40 virgin female flies to 15-20 male flies for each genotype. The mating population was placed in a single vial with dry yeast overnight at 25°C to acclimate them for mating and laying. After 24 hours, the flies were transferred to an egg collection chamber consisting of a 100 mL Tri-Stir beaker with small holes in the bottom capped with a yeasted grape juice agar plate. The flies were left to mate and lay eggs for approximately 4-5 hours or until each plate had about 100-150 eggs present on the grape agar. Hatching frequency was visually scored using a stereomicroscope 48 hours after egg collection for wild type or 72 hours after collection for *pif1*^{167} mutants. Three trials of 3 plates for each genotype's progeny were scored. Statistical significance was determined using unpaired t-tests between the three mating groups.

Embryo collection and DAPI staining

Thirty *pif1*^{167} homozygous mutants (3:1 female to male ratio) were collected and placed in a vial with dry yeast overnight at 25°C. The next day, the population was placed in an egg collection chamber and returned to 25°C to lay. Grape agar plates containing newly laid embryos were collected and changed every 2 hours. The embryos were collected from each grape agar plate and dechorionated in 50% bleach for 2 minutes. Embryos were then fixed with a mixture of 1:1 heptane:37% formaldehyde for 20 minutes, stained with 1 μg/mL DAPI in 0.1% Triton-X PBS for 3 minutes, and mounted onto a glass slide with Vectashield mounting media. The embryos were covered with a 18x18mm No 1 coverslip which was sealed with clear nail polish. Embryos were visualized on a Zeiss Axio Z-stepping microscope and fluorescent images were acquired

with a Zeiss Axiocam 503 mono camera. Quantification of the frequency of nuclear defects for DAPI-stained *pif1* and wild-type embryos was performed on deconvolved images. Statistical significance was determined using unpaired t-tests.

Time-lapse microscopy

About 100 wild-type H2Av–EGFP and *pif1*^{167}–H2Av–EGFP homozygous flies (3:1 female to male ratio) were collected and placed in separate bottles with dry yeast overnight at 25°C. After 24 hours, each population was placed in an egg collection chamber and returned to 25°C to lay. Grape agar plates containing newly laid embryos were removed every hour. Embryos were then collected and manually dechorionated. Dechorionated embryos were transferred onto a slide containing Halocarbon Oil 700. The embryos were covered with a 18x18mm No 1 coverslip, while maintaining enough distance between the slide and the coverslip by use of 4 layers of double-sided tape. Embryos were visualized on a Zeiss Axio Z-stepping microscope and fluorescent images were acquired with a Zeiss Axiocam 503 mono camera every 20 seconds.

Nondisjunction assay

Meiotic nondisjunction of the *X* chromosomes was measured by crossing *y w* or *y w; pif1*^{167} females to *y* w^a Ste^1 /*Dp(1;Y)*B^S y^+ males. The duplication on the *Y* chromosome carries a dominant mutation causing bar-shaped eyes. Fertilization of eggs produced through normal meiotic disjunction produces females with non-Bar eyes and yellow bodies and males with Bar eyes and wild-type bodies. Fertilization of diplo-*X* ova created through nondisjunction results in *XXY* female progeny with Bar eyes and normal-colored bodies (and non-viable *XXX* progeny). Fertilization of nullo-*X* ova results in *XO* males with non-Bar eyes and yellow bodies (and non-viable *YO* progeny). *X* nondisjunction was calculated as the percentage of progeny that arose from nondisjunction (Bar females and non-Bar males), after correcting for loss of half of the

diplo-*X* ova and half of the nullo-*X* ova.

$P\{w^a\}$ site-specific gap repair assay

$P\{w^a\}$ is a 14-kilobase *P* element containing the *white* gene, interrupted by a *copia* retrotransposon. In this assay, it is inserted in the essential gene *scalloped* on the *X* chromosome. A third chromosome transposase source ($P\{ry^+, \Delta 2\text{-}3\}99B$, Bloomington stock 406) was used to excise $P\{w^a\}$. Following excision of $P\{w^a\}$, double-strand gap repair events occurring in the male pre-meiotic germline were recovered and analyzed as described previously (McVey 2010). Single males possessing both $P\{w^a\}$ and transposase were mated to females homozygous for $P\{w^a\}$ in individual vials and repair products were recovered in female progeny. Repair events with full HR repair, involving synthesis through the *copia* long terminal repeats (LTRs) are recovered in red-eyed females, while events with aborted HR repair followed by end joining are recovered in yellow-eyed females. Individual progeny were scored for eye color and statistical significance was determined using the Mann-Whitney test, with each vial considered an independent experiment. Genomic DNA from flies possessing independent repair events (one per vial) was recovered (Gloor *et al.* 1993) and PCR with primers tiled across $P\{w^a\}$ were used to estimate the extent of repair synthesis. Statistical significance for repair synthesis tract lengths was determined with Fisher's exact test.

Life stage-specific synthetic lethality

10-15 virgin females and 5 males both heterozygous for the mutation of interest *in trans* to a GFP-bearing balancer chromosome were placed in a vial with dry yeast overnight at 25°C. After 24 hours, the flies were transferred to an egg collection chamber and females were allowed to lay eggs for 5 hours. The resulting progeny were observed daily from the onset of first instar larvae to eclosion. Heterozygotes and homozygotes were scored by presence or absence of GFP,

respectively. Time of synthetic lethality was determined by the life stage at which no homozygotes were observed.

Data and reagent policy

Fly stocks are available upon request. Supplemental files are available at FigShare. File S1 contains supplemental figures. File S2 contains time-lapse videos of embryonic nuclear divisions in wild-type and *pif1* mutant embryos.

Results

pif1 mutants are sensitive to agents that induce replication stress

We identified a *Drosophila melanogaster* PIF1 ortholog encoded by *CG3238*, based on sequence similarity with PIF1 proteins from other eukaryotes (Figure S1). Like other PIF1 family members, Drosophila PIF1 possesses a P-loop containing triphosphate hydrolase, indicative of ATP-dependent helicase activity, and the 21 amino acid Pif1 signature motif (BOCHMAN *et al.* 2010). However, unlike the yeast Pif1 proteins, Drosophila PIF1 does not appear to have a mitochondrial targeting sequence, suggesting that it may not function in mitochondrial maintenance. To investigate the roles of PIF1 in Drosophila, we used the previously generated *pif1*^{167} mutant described in (ALEXANDER *et al.* 2016). This mutant was created via imprecise excision of a *P* element (*EY10295*) located in the 5' untranslated region of *CG3238*, which resulted in the removal of the majority of the helicase domain. Flies homozygous for this deletion were viable and apparently healthy, although females displayed greatly reduced fertility due to maternal effect lethality (see next section).

S. pombe pfh1 mutants are extremely sensitive to compounds that induce replication stress, including the alkylating agent methyl methanesulfonate (MMS) and the ribonucleotide reductase inhibitor hydroxyurea (HU) (TANAKA *et al.* 2002), while *S. cerevisiae pif1* mutants exhibit milder sensitivity to these agents (BUDD *et al.* 2006). To investigate the roles of Drosophila PIF1 in responding to various types of DNA damage, we treated *pif1*^{167} heterozygous and homozygous mutant larvae with increasing concentrations of different mutagens and measured the survival of these larvae into adulthood relative to untreated siblings. The mutagen concentrations were selected based on our previous findings that other DNA repair mutants are sensitive at these

doses (KANE *et al.* 2012; THOMAS *et al.* 2013; BEAGAN *et al.* 2017). Notably, as the dose of each mutagen increased, the total number of flies (both heterozygotes and homozygotes) that survived decreased, indicating the efficacy of the treatments (Figure S2). The homozygous mutants were not sensitive to the highest concentrations of nitrogen mustard (which induces intra- and interstrand DNA crosslinks), topotecan (a topoisomerase I inhibitor that creates one-ended DSBs following DNA replication), or paraquat (induces oxidative damage), and showed only a mild sensitivity to the alkylating agent methyl methanesulfonate and the radiomimetic drug bleomycin (Figure 1A). Interestingly, *pif1* homozygous mutants showed moderate sensitivity when treated with a high dose of hydroxyurea, which can stall replication forks and eventually cause fork collapse (Figure 1A). Further testing showed that loss of PIF1 causes a dose-dependent increase in hydroxyurea sensitivity (Figure 1B). Together, these results suggest that Drosophila PIF1 does not play a major role in responding to oxidative stress or bulky DNA lesions, unlike its yeast orthologs, but is important for survival during conditions of replication stress.

PIF1 is required for rapid nuclear divisions in early embryogenesis

During our initial characterization of the $pif1^{167}$ allele, we found that we were unable to maintain a stock of homozygous *pif1* mutants, as homozygous mutant females failed to produce viable progeny. Because *pif1* mutants are preferentially sensitive to agents that induce replication stress, we hypothesized that this defect might be due to embryonic lethality resulting from replication defects during early development. Indeed, embryos obtained from *pif1* mutant mothers had a significantly decreased hatching frequency (13.6%) relative to wild-type embryos (87.1%, Figure 2). The small percentage of *pif1* mutant embryos that did hatch developed more slowly than

wild-type embryos, frequently taking up to 48 hours to emerge as first-instar larvae as opposed to 24 hours for wild type.

In Drosophila embryogenesis, paternal gene expression of most genes is initiated about two hours after fertilization. To test whether the contribution of the functional paternal PIF1 could rescue the severe defect in hatching frequency seen in the *pif1* mutant embryos, we mated *pif1* homozygous females with wild-type males. The average hatching frequency of the resultant *pif1* heterozygous embryos was 15.2% and was not significantly different from the *pif1* mutant females mated to *pif1* mutant males. This inability of paternally-derived PIF1 to rescue the *pif1* egg-hatching defects suggests that the crucial function of PIF1 occurs during the first two hours of egg development.

During the early stages of Drosophila embryo development, alternating S and M phases occur every five to six minutes to produce approximately 6000 syncytial nuclei in a common cytoplasm (ZALOKAR AND ERK 1976). Nuclei that fail to complete replication and/or experience aberrant mitotic chromosome segregation are removed through an active process termed nuclear fallout (SULLIVAN AND THEURKAUF 1995). To determine if DNA replication and subsequent nuclear division is affected in the absence of PIF1, we fixed wild-type and *pif1* homozygous mutant embryos and stained them with the fluorescent DNA marker DAPI to visualize the syncytial nuclei within 1-2 hours after fertilization. Wild-type embryos undergoing normal syncytial divisions exhibited an even spatial pattern of nuclei (Figure 3A). In contrast, we observed multiple nuclear defects in *pif1* mutants undergoing embryogenesis, including gaps in the typically uniform monolayer of nuclei, abnormal chromosome clumping, chromosome

fragmentation, anaphase bridges, and asynchronous mitotic divisions (Figure 3B). Quantification of these defects showed that nearly all *pif1* embryos exhibit nuclear fallout and chromosome clumping, while almost half show evidence of anaphase bridges (Figure 3C). These phenotypes were also present in very early nuclear division cycles (Figure S3), suggesting that the requirement for PIF1 begins at the earliest stages of embryogenesis.

To further characterize the developmental defects in *pif1* mutants, we employed real-time visualization of developing wild-type and *pif1* embryos. We introduced constructs encoding the histone H2A variant fused to enhanced green fluorescent protein (H2Av–EGFP) into wild-type and *pif1* mutant backgrounds and followed the nuclei through multiple mitoses. We observed nuclear fallout, anaphase bridging, and chromosome clumping in *pif1*, but not wild-type embryos, consistent with the results from the DAPI-stained fixed embryos (Supplemental Videos 1 and 2). Because failure to complete DNA replication during early embryogenesis can lead to chromosome segregation failures similar to what we observed in the *pif1* mutants (SIBON *et al.* 2000), we conclude that PIF1 is likely acting during DNA replication during early Drosophila development.

In Drosophila, defects in meiotic recombination often cause an increase in chromosome non-disjunction (HUGHES *et al.* 2018). Thus, the maternal effect lethality observed in *pif1* mutants could also be caused by defective meiotic recombination. To test this, we crossed wild type and *pif1* mutants to males possessing a *Y* chromosome with a dominant marker B^S (see materials and methods). While the frequency of nondisjunction for the *pif1* mutants was higher than the wild-type frequency (Figure S4), the increase was not statistically significant due to the small number of adults that we were able to recover from *pif1* mutant mothers. Thus, we conclude the maternal effect lethality observed in *pif1* mutants is largely caused by an inability to

carry out efficient DNA replication, although we cannot rule out the possibility that meiotic recombination defects may also play a role.

PIF1 promotes long-range DNA synthesis during homologous recombination repair

While *S. cerevisiae* Pif1 plays a minimal role during gene conversion via homologous recombination, it is essential for break-induced replication (BIR), a type of homologous recombination repair that requires large amounts of synthesis and proceeds through migration of a mobile D-loop (SAINI *et al.* 2013; WILSON *et al.* 2013). The POL32 subunit of Pol δ is also required for BIR (WILSON *et al.* 2013) and has been shown to promote extensive synthesis during gap repair in Drosophila (KANE *et al.* 2012). To determine whether there is a similar requirement for PIF1 during gap repair via homologous recombination, we used the well-characterized *P{w^a}* assay (MCVEY 2010). This assay uses a *X*-linked *P* element containing an interrupted *white* gene (Figure 4A). The excision of *P{w^a}* from one sister chromatid in the presence of *P*-transposase generates a 14-kb gap that can be repaired via HR using an intact sister chromatid as a template for repair synthesis. Synthesis often terminates prematurely and repair can be completed by end joining.

We found that *pif1* mutants had a slight reduction in the number of full homologous recombination (full HR) events compared to wild type. However, they did not show any changes in the frequency of incomplete HR events that terminated via end joining (HR + EJ; Figures 4B and C). *pol32* mutants, which showed more of a decrease in full HR events than *pif1* mutants, also did not have a significant change in HR + EJ events compared to wild type, consistent with previous findings (KANE *et al.* 2012). Since PIF1 acts with POL32 to promote BIR and long-tract

gene conversion in yeast (LYDEARD *et al.* 2007; WILSON *et al.* 2013), we tested the ability of *pol32 pif1* double mutants to synthesize long stretches of DNA in the *P{w^a}* assay. Strikingly, the percentage of full HR events in *pol32 pif1* mutants was significantly decreased compared to either single mutant or wild type, and the percentage of incomplete HR + EJ events was significantly increased (Figures 4B and C).

To quantify the amount of repair synthesis that took place in the incomplete HR products prior to end joining, we utilized a series of PCRs, focusing on right-side synthesis. Loss of POL32 resulted in decreased repair synthesis, with significant effects at large distances 4.6 kb from the right end of the double-strand gap (Figure 4D). In contrast, *pif1* mutants had slightly increased repair synthesis at distances of 250 bp-1 kb and no defects at large distances. Interestingly, the amount of repair synthesis was significantly decreased in *pol32 pif1* mutants relative to either single mutant at distances greater than 2.5 kb. Together, these results suggest that PIF1 does not play a major role in promoting HR requiring extensive synthesis unless POL32 is also missing, in contrast to what is observed during BIR in budding yeast. However, in the absence of POL32, PIF1 becomes important for long-distance synthesis during HR repair.

We previously observed that in the absence of both POL32 and REV3, the catalytic subunit of translesion synthesis polymerase zeta (Pol ζ), there is a significant decrease in full HR and an increase in incomplete HR + EJ events compared to wild type or either single mutant (KANE *et al.* 2012). These results suggested that both Pol δ and Pol ζ act independently to synthesize DNA during *P{w^a}* gap repair. Given our finding that Drosophila PIF1 becomes important for long-distance synthesis in the absence of POL32, we investigated whether loss of PIF1 would also

result in a further reduction in repair synthesis in a Pol ζ mutant background. Interestingly, we found that *pif1 rev3* double mutants do not show a difference in the frequency of full HR or incomplete HR + EJ events relative to *pif1* single mutants, and repair synthesis is not decreased compared to wild-type gap repair events (Figure 5). This differential requirement for POL32 and PIF1 in the absence of Pol ζ provides further evidence that these two proteins are likely promoting long-distance synthesis during HR via different mechanisms.

PIF1 and POL32 are essential for adult viability in the absence of BRCA2, but not RAD51

The *P{w^a}* assay can also be utilized in a *brca2* mutant background to assess how double-strand break repair proceeds in the absence of HR (McVey *et al.* 2004; Klovstad *et al.* 2008; Thomas *et al.* 2013). To this end, we generated five independent *pif1 brca2* heterozygous double mutant stocks through meiotic recombination, utilizing two different *brca2* null alleles. Strikingly, we found that *pif1 brca2* homozygous double mutants were unable to survive past late larval stages, precluding their analysis using the *P{w^a}* assay (Table 1).

Because Pol32 acts alongside Pif1 in budding yeast to promote BIR and Okazaki fragment maturation, we tested whether removing POL32 in the absence of HR would also prevent survival to adulthood. Indeed, loss of both POL32 and BRCA2 in two independently derived double mutants resulted in third-instar larval synthetic lethality (Table 1). Similarly, *pol32 pif1 brca2* triple mutants died during late larval stages, suggesting that PIF1 and POL32 act together to promote a process which is required for development past late larval stages in the absence of BRCA2.

In mammals, BRCA2 is required to load RAD51 onto single-stranded DNA during homologous recombination repair of double-strand breaks (YANG *et al.* 2005). It also serves to stabilize stalled replication forks, independent of its function in recombination (SCHLACHER *et al.* 2011). To determine which of these roles might relate to the lethality observed in *pif1 brca2* mutants, we attempted to create *pif1 rad51* mutants, which like *brca2* mutants should be unable to initiate recombination. Strikingly, we were able to recover the expected Mendelian numbers of viable *pif1 rad51* mutant adults (Table 1 and Figure S5). Similarly, we obtained the expected numbers of *pol32 rad51* adult flies (Figure S5). Based on these data, we conclude that PIF1 and POL32 are not required for adult viability in the absence of homologous recombination.

In mammals, the MUS81 protein acts in a replication fork rescue pathway, cleaving regressed fork that have a single-stranded DNA tail (HANADA *et al.* 2007; LEMACON *et al.* 2017). Following MUS81 cleavage, synthesis is resumed in a POL32-dependent manner. To test whether a similar pathway might operate in Drosophila, we attempted to create flies lacking both MUS81 and PIF1 or POL32. We observed no synthetic lethal interactions between these mutations (Table 1). In addition, we crossed the *mus81* mutation into *pif1 brca2, pol32 brca2,* and *pif1 pol32 brca2* mutant backgrounds. In all three of these experiments, loss of MUS81 failed to rescue the synthetic lethality (Table 1). Together, these results suggest that the role(s) of PIF1 and POL32 in promoting survival in the absence of BRCA2 may be different from their roles in mammals. Alternatively, replication fork restart in Drosophila may be less dependent on MUS81 than in mammals.

Discussion

Pif1 family members have overlapping but distinct functions in different organisms and are differentially required for viability. We anticipated that the identification of phenotypes in Drosophila *pif1* mutants might provide additional insight into this gene's diverse roles in maintaining genomic integrity. Based on our results, it appears that some, but not all, of the functions of PIF1 in other organisms are conserved in Drosophila. For example, Drosophila *pif1* mutant larvae treated with high concentrations of hydroxyurea die prior to adulthood, mimicking the HU sensitivity of both budding and fission yeast. On the other hand, the lack of Drosophila *pif1* mutant sensitivity to many other mutagens suggests that the fly PIF1 does not play a role in the oxidative stress response or in repair of base damage, unlike its yeast orthologs, or that PIF1 has a redundant role in these processes.

A critical role for Drosophila PIF1 during replication stress

In addition to finding that *pif1* mutants fail to survive exposure to high doses of hydroxyurea, we also identified a vital role for PIF1 during early Drosophila embryogenesis, at a time of extremely rapid genome replication and chromosome segregation. We hypothesize that both phenotypes could be due to failure or impairment of the same mechanism. Eggs produced by *pif1* homozygous mutant mothers rarely hatch and this phenotype cannot be rescued by a wild-type paternal copy of PIF1, indicating that the requirement for PIF1 is most acute during the first two hours of embryo development. The nuclear abnormalities seen in *pif1* mutants during this time are consistent with defects in DNA replication during early embryogenesis. Intriguingly, human PIF1 helicase has been shown to support DNA replication and cell growth during oncogenic

stress (GAGOU *et al.* 2014). Thus, one conserved feature of PIF1 in all organisms may be to promote efficient DNA synthesis during replication stress.

Based on our findings and studies done in other organisms, we suggest that PIF1 might promote efficient replication during embryogenesis and conditions of replicative stress via one or more of four non-exclusive mechanisms. First, it might work alongside or in combination with POL32 to promote Pol δ processivity. Flies lacking POL32 are also sensitive to HU (our unpublished data) and eggs lacking POL32 have severe nuclear division defects during embryogenesis and fail to develop to larval stages (TRITTO *et al.* 2015). In addition, *pol32* mutant embryonic defects cannot be rescued by paternal expression of POL32 (TRITTO *et al.* 2015), similar to the *pif1* mutant phenotype.

Second, Drosophila PIF1 might be needed for resolution of G4 or other DNA secondary structures during replication. Although, we have no direct evidence showing that Drosophila PIF1 unwinds G4 DNA, previous studies with *S. cerevisiae* have revealed that DNA replication through G4 motifs is promoted by the PIF1 helicase, where PIF1 unwinds G4 DNA and keeps it unfolded to prevent replication fork stalling and DNA breakage (PAESCHKE *et al.* 2011; ZHOU *et al.* 2014). In addition, *S. pombe* lacking Pfh1 have unresolved G4 structures that lead to increased fork pausing and DNA damage near G4 motifs (SABOURI *et al.* 2014). Strikingly, both yeast and human Pif1 are much more efficient at unwinding G4 DNA than human WRN or budding yeast Sgs1 (PAESCHKE *et al.* 2013). Thus, it is possible that PIF1 is the main helicase performing G4 structure resolution and/or removal of secondary DNA structures during early embryonic stages. In its absence, the formation of DNA secondary structures stalls replication

forks, leading to an accumulation of unresolvable replication intermediates that results in the chromosome segregation defects observed in *pif1* mutant embryos.

Third, PIF1 might be important for completion of replication. Recently, ScPif1 was shown to play a role in replication termination (DEEGAN *et al.* 2019). Under-replicated DNA intermediates might manifest as fine DNA linkages between segregating chromosomes and chromosome clumps, both of which we observed in *pif1* embryos. These damaged nuclei will be subject to nuclear fallout, resulting in gaps in the typically uniform nuclear monolayer.

A fourth possibility is that PIF1 is needed for cellular responses to replication fork stalling. In budding yeast, the restart of stalled replication forks is dependent on several DNA helicases, including BLM, WRN, FANCM, and PIF1, many of which are recruited to forks by RPA or interact with RPA at forks (BACHRATI AND HICKSON 2008; LUKE-GLASER *et al.* 2010; RAMANAGOUDR-BHOJAPPA *et al.* 2014). Additionally, fork restart can be mediated by the cleavage of a regressed fork structure, followed by a BIR-like process (HANADA *et al.* 2007; MAYLE *et al.* 2015). Since BIR is dependent on Pif1 in *S. cerevisiae*, it is possible that *pif1* embryos are unable to rapidly process stalled replication forks, resulting in slowed replication that manifests as improper syncytial nuclear division and embryonic development.

One of the most surprising results from this study is that *pif1 brca2* and *pol32 brca2* mutants, but not the corresponding *rad51* double mutants, exhibit synthetic lethality at late larval stages. While we showed that PIF1 and POL32 are needed prior to this stage of development, we created homozygous double mutants by mating flies heterozygous for both mutations *in cis*.

Thus, maternal deposits of the proteins may have allowed for survival to late larval stages. BRCA2 has been shown to promote replication fork stability in mammalian systems by protecting stalled replication forks from extensive nucleolytic degradation (SCHLACHER *et al.* 2011; LEMACON *et al.* 2017; MIJIC *et al.* 2017). Drosophila BRCA2 has RAD51-independent roles at stalled forks during meiosis (KLOVSTAD *et al.* 2008). Our finding that the *pif1* and *pol32* mutations are lethal in a *brca2*, but not *rad51*, mutant background, suggests that fly BRCA2 may also be important for the stabilization of stalled forks in mitotically dividing cells and supports the idea that it shares a fork protection function with its mammalian counterpart.

We hypothesize that Drosophila PIF1 and POL32 have crucial functions in stabilizing and/or restarting stalled replication forks in the absence of BRCA2. In support of this, human POL32 was shown to mediate the initial DNA synthesis for replication fork restart in the absence of Rad51 (MORIEL-CARRETERO AND AGUILERA 2010; MAYLE *et al.* 2015; LEMACON *et al.* 2017). Specifically, MUS81 cleavage rescues resected forks in BRCA2-deficient cells through a BIR-like mechanism mediated by POL32-dependent DNA synthesis. PIF1 might promote this synthesis, similar to its role in gap repair by HR (see below). However, our failure to observe any rescue of *pif1 brca2* lethality when we removed MUS81 and our successful recovery of viable *pif1 mus81* and *pol32 mus81* mutant adults do not support this model. Alternatively, Pif1 may help with the stabilization of stalled replication forks through pairing of parental ssDNA, migrating HJ structures, and/or directly participating in repair of the lesion (GEORGE *et al.* 2009; RAMANAGOUDR-BHOJAPPA *et al.* 2014).

***Drosophila* PIF1 promotes long-range synthesis during HR**

Using a quantitative assay of double-strand gap repair in the male pre-meiotic germline, we found that lack of POL32, but not PIF1, results in decreased processive repair synthesis and a significant increase in aborted HR repair. However, a requirement for PIF1 was observed in the absence of POL32. In budding yeast, Pif1 is required for efficient BIR, where it promotes D-loop migration during Pol δ-mediated repair synthesis. A recent study demonstrated that PIF1 may also be important during BIR in Drosophila (BHANDARI *et al.* 2019). It is possible that the creation of a 14-kb double-strand gap after *P*-element excision induces a BIR-like mechanism of repair, where the two ends of the break invade homologous templates independently of each other and synthesis proceeds via migrating bubble structures. Supporting this idea, repair of a gap greater than 3 kb depends on POL32-mediated BIR in yeast (JAIN *et al.* 2009).

Previous studies of double-strand gap repair in Drosophila led to the model that repair of large gaps by two-ended synthesis-dependent strand annealing requires multiple cycles of strand invasion, synthesis, and displacement of the nascent strand (MCVEY *et al.* 2004). Genetic evidence suggested that translesion polymerases Pol η and Pol ζ might be utilized during initial synthesis, with a subsequent switch to Pol δ that results in more processive synthesis (KANE *et al.* 2012). This model was supported by the observation that loss of both POL32 and the catalytic subunit of Pol ζ resulted in less repair synthesis than was observed in either single mutant. Here, we have shown that *pif1 rev3* mutants behave differently from *pol32 rev3* mutants, consistent with the idea that PIF1 only becomes important during gap repair when Pol δ is impaired by mutation of POL32.

One speculative model, consistent with all these data, is that POL32 acts in the context of the Pol δ complex to promote processivity, while PIF1 is important for the progression of the mobile D-loop. PIF1 could act in concert with PCNA to promote Pol δ-mediated strand displacement at the front of the replication bubble (BUZOVETSKY *et al.* 2017), and/or behind the D-loop to unwind the newly-synthesized strand to relieve topological hindrance of the nascent strand with the template strand (WILSON *et al.* 2013). If POL32 is present, then Pol δ can engage in strand displacement synthesis and the requirement for PIF1 activity is removed.

Summary

Collectively, our data are consistent with multiple roles for Drosophila PIF1. First, it is needed to deal with both endogenous and exogenous replication stress. During early embryogenesis, PIF1 acts to ensure proper DNA replication and subsequent chromosome segregation. In addition, it promotes survival when replication forks are challenged by hydroxyurea. Second, in the absence of BRCA2, PIF1 is needed for development past late larval stages, a function that it shares with POL32. Because HR-defective *pif1 rad51* and *pol32 rad51* mutants survive to adulthood, PIF1 and POL32 are likely acting to protect regressed forks or promote replication restart. Third, PIF1 promotes extensive DNA repair synthesis during homologous recombination repair of double-strand gaps in a POL32-independent manner. Together, these findings suggest that Drosophila PIF1 shares some, but not all, of the roles filled by its yeast and mammalian counterparts and lays the groundwork for future investigations into additional PIF1 functions in metazoans.

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References

Alexander, J. L., K. Beagan, T. L. Orr-Weaver and M. McVey, 2016 Multiple mechanisms contribute to double-strand break repair at rereplication forks in Drosophila follicle cells. Proc Natl Acad Sci U S A 113**:** 13809-13814.

Bachrati, C. Z., and I. D. Hickson, 2008 RecQ helicases: guardian angels of the DNA replication fork. Chromosoma 117**:** 219-233.

Bannwarth, S., L. Berg-Alonso, G. Auge, K. Fragaki, J. E. Kolesar *et al.*, 2016 Inactivation of Pif1 helicase causes a mitochondrial myopathy in mice. Mitochondrion 30**:** 126-137.

Beagan, K., R. L. Armstrong, A. Witsell, U. Roy, N. Renedo *et al.*, 2017 Drosophila DNA polymerase theta utilizes both helicase-like and polymerase domains during microhomology-mediated end joining and interstrand crosslink repair. PLoS Genet 13**:** e1006813.

Bhandari, J., T. Karg and K. G. Golic, 2019 Homolog-Dependent Repair Following Dicentric Chromosome Breakage in Drosophila melanogaster. Genetics 212**:** 615-630.

Bochman, M. L., K. Paeschke and V. A. Zakian, 2012 DNA secondary structures: stability and function of G-quadruplex structures. Nat Rev Genet 13**:** 770-780.

Bochman, M. L., N. Sabouri and V. A. Zakian, 2010 Unwinding the functions of the Pif1 family helicases. DNA Repair (Amst) 9**:** 237-249.

Boule, J. B., and V. A. Zakian, 2006 Roles of Pif1-like helicases in the maintenance of genomic stability. Nucleic Acids Res 34**:** 4147-4153.

Budd, M. E., C. C. Reis, S. Smith, K. Myung and J. L. Campbell, 2006 Evidence suggesting that Pif1 helicase functions in DNA replication with the Dna2 helicase/nuclease and DNA polymerase delta. Mol Cell Biol 26**:** 2490-2500.

Buzovetsky, O., Y. Kwon, N. T. Pham, C. Kim, G. Ira *et al.*, 2017 Role of the Pif1-PCNA Complex in Pol delta-Dependent Strand Displacement DNA Synthesis and Break-Induced Replication. Cell Rep 21**:** 1707-1714.

Byrd, A. K., and K. D. Raney, 2017 Structure and function of Pif1 helicase. Biochem Soc Trans 45**:** 1159-1171.

Chen, C. F., T. J. Pohl, S. Pott and V. A. Zakian, 2019 Two Pif1 Family DNA Helicases Cooperate in Centromere Replication and Segregation in Saccharomyces cerevisiae. Genetics 211**:** 105-119.

Cheng, X., S. Dunaway and A. S. Ivessa, 2007 The role of Pif1p, a DNA helicase in Saccharomyces cerevisiae, in maintaining mitochondrial DNA. Mitochondrion 7**:** 211-222.

Chung, W. H., 2014 To peep into Pif1 helicase: multifaceted all the way from genome stability to repair-associated DNA synthesis. J Microbiol 52**:** 89-98.

Clarkson, M., and R. Saint, 1999 A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for Drosophila chromosome behavior. DNA Cell Biol 18**:** 457-462.

Deegan, T. D., J. Baxter, M. A. Ortiz Bazan, J. T. P. Yeeles and K. P. M. Labib, 2019 Pif1-Family Helicases Support Fork Convergence during DNA Replication Termination in Eukaryotes. Mol Cell.

Dehghani-Tafti, S., V. Levdikov, A. A. Antson, B. Bax and C. M. Sanders, 2019 Structural and functional analysis of the nucleotide and DNA binding activities of the human PIF1 helicase. Nucleic Acids Res 47**:** 3208-3222.

Donnianni, R. A., and L. S. Symington, 2013 Break-induced replication occurs by conservative DNA synthesis. Proc Natl Acad Sci U S A 110**:** 13475-13480.

Doudican, N. A., B. Song, G. S. Shadel and P. W. Doetsch, 2005 Oxidative DNA damage causes mitochondrial genomic instability in Saccharomyces cerevisiae. Mol Cell Biol 25**:** 5196-5204.

Foury, F., and J. Kolodynski, 1983 pif mutation blocks recombination between mitochondrial rho+ and rho- genomes having tandemly arrayed repeat units in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 80**:** 5345-5349.

Futami, K., A. Shimamoto and Y. Furuichi, 2007 Mitochondrial and nuclear localization of human Pif1 helicase. Biol Pharm Bull 30**:** 1685-1692.

Gagou, M. E., A. Ganesh, G. Phear, D. Robinson, E. Petermann *et al.*, 2014 Human PIF1 helicase supports DNA replication and cell growth under oncogenic-stress. Oncotarget 5**:** 11381-11398.

Galletto, R., and E. J. Tomko, 2013 Translocation of Saccharomyces cerevisiae Pif1 helicase monomers on single-stranded DNA. Nucleic Acids Res 41**:** 4613-4627.

George, T., Q. Wen, R. Griffiths, A. Ganesh, M. Meuth *et al.*, 2009 Human Pif1 helicase unwinds synthetic DNA structures resembling stalled DNA replication forks. Nucleic Acids Res 37**:** 6491-6502.

Gloor, G. B., C. R. Preston, D. M. Johnson-Schlitz, N. A. Nassif, R. W. Phillis *et al.*, 1993 Type I repressors of P element mobility. Genetics 135**:** 81-95.

Hanada, K., M. Budzowska, S. L. Davies, E. van Drunen, H. Onizawa *et al.*, 2007 The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. Nat Struct Mol Biol 14**:** 1096-1104.

Hughes, S. E., D. E. Miller, A. L. Miller and R. S. Hawley, 2018 Female Meiosis: Synapsis, Recombination, and Segregation in Drosophila melanogaster. Genetics 208**:** 875-908.

Ivessa, A. S., J. Q. Zhou and V. A. Zakian, 2000 The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA. Cell 100**:** 479-489.

Jain, S., N. Sugawara, J. Lydeard, M. Vaze, N. Tanguy Le Gac *et al.*, 2009 A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair. Genes Dev 23**:** 291-303.

Kane, D. P., M. Shusterman, Y. Rong and M. McVey, 2012 Competition between replicative and translesion polymerases during homologous recombination repair in Drosophila. PLoS Genet 8**:** e1002659.

Klovstad, M., U. Abdu and T. Schupbach, 2008 Drosophila brca2 is required for mitotic and meiotic DNA repair and efficient activation of the meiotic recombination checkpoint. PLoS Genet 4**:** e31.

Koc, K. N., S. P. Singh, J. L. Stodola, P. M. Burgers and R. Galletto, 2016 Pif1 removes a Rap1-dependent barrier to the strand displacement activity of DNA polymerase delta. Nucleic Acids Res 44**:** 3811-3819.

Kramara, J., B. Osia and A. Malkova, 2018 Break-Induced Replication: The Where, The Why, and The How. Trends Genet 34**:** 518-531.

Lahaye, A., H. Stahl, D. Thines-Sempoux and F. Foury, 1991 PIF1: a DNA helicase in yeast mitochondria. EMBO J 10**:** 997-1007.

Lemacon, D., J. Jackson, A. Quinet, J. R. Brickner, S. Li *et al.*, 2017 MRE11 and EXO1 nucleases degrade reversed forks and elicit MUS81-dependent fork rescue in BRCA2-deficient cells. Nat Commun 8**:** 860.

Lipps, H. J., and D. Rhodes, 2009 G-quadruplex structures: in vivo evidence and function. Trends Cell Biol 19**:** 414-422.

Luke-Glaser, S., B. Luke, S. Grossi and A. Constantinou, 2010 FANCM regulates DNA chain elongation and is stabilized by S-phase checkpoint signalling. EMBO J 29**:** 795-805.

Lydeard, J. R., S. Jain, M. Yamaguchi and J. E. Haber, 2007 Break-induced replication and telomerase-independent telomere maintenance require Pol32. Nature 448**:** 820-823.

Maizels, N., and L. T. Gray, 2013 The G4 genome. PLoS Genet 9**:** e1003468.

Mateyak, M. K., and V. A. Zakian, 2006 Human PIF helicase is cell cycle regulated and associates with telomerase. Cell Cycle 5**:** 2796-2804.

Mayle, R., I. M. Campbell, C. R. Beck, Y. Yu, M. Wilson *et al.*, 2015 DNA REPAIR. Mus81 and converging forks limit the mutagenicity of replication fork breakage. Science 349**:** 742-747.

McDonald, K. R., N. Sabouri, C. J. Webb and V. A. Zakian, 2014 The Pif1 family helicase Pfh1 facilitates telomere replication and has an RPA-dependent role during telomere lengthening. DNA Repair (Amst) 24**:** 80-86.

McVey, M., 2010 In vivo analysis of Drosophila BLM helicase function during DNA double-strand gap repair. Methods Mol Biol 587**:** 185-194.

McVey, M., M. Adams, E. Staeva-Vieira and J. J. Sekelsky, 2004 Evidence for multiple cycles of strand invasion during repair of double-strand gaps in Drosophila. Genetics 167**:** 699-705.

Mijic, S., R. Zellweger, N. Chappidi, M. Berti, K. Jacobs *et al.*, 2017 Replication fork reversal triggers fork degradation in BRCA2-defective cells. Nat Commun 8**:** 859.

Moriel-Carretero, M., and A. Aguilera, 2010 A postincision-deficient TFIIH causes replication fork breakage and uncovers alternative Rad51- or Pol32-mediated restart mechanisms. Mol Cell 37**:** 690-701.

O'Rourke, T. W., N. A. Doudican, M. D. Mackereth, P. W. Doetsch and G. S. Shadel, 2002 Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins. Mol Cell Biol 22**:** 4086-4093.

Paeschke, K., M. L. Bochman, P. D. Garcia, P. Cejka, K. L. Friedman *et al.*, 2013 Pif1 family helicases suppress genome instability at G-quadruplex motifs. Nature 497**:** 458-462.

Paeschke, K., J. A. Capra and V. A. Zakian, 2011 DNA replication through G-quadruplex motifs is promoted by the Saccharomyces cerevisiae Pif1 DNA helicase. Cell 145**:** 678-691.

Pinter, S. F., S. D. Aubert and V. A. Zakian, 2008 The Schizosaccharomyces pombe Pfh1p DNA helicase is essential for the maintenance of nuclear and mitochondrial DNA. Mol Cell Biol 28**:** 6594-6608.

Ramanagoudr-Bhojappa, R., A. K. Byrd, C. Dahl and K. D. Raney, 2014 Yeast Pif1 accelerates annealing of complementary DNA strands. Biochemistry 53**:** 7659-7669.

Rossi, M. L., J. E. Pike, W. Wang, P. M. Burgers, J. L. Campbell *et al.*, 2008 Pif1 helicase directs eukaryotic Okazaki fragments toward the two-nuclease cleavage pathway for primer removal. J Biol Chem 283**:** 27483-27493.

Ryu, G. H., H. Tanaka, D. H. Kim, J. H. Kim, S. H. Bae *et al.*, 2004 Genetic and biochemical analyses of Pfh1 DNA helicase function in fission yeast. Nucleic Acids Res 32**:** 4205-4216.

Sabouri, N., J. A. Capra and V. A. Zakian, 2014 The essential Schizosaccharomyces pombe Pfh1 DNA helicase promotes fork movement past G-quadruplex motifs to prevent DNA damage. BMC Biol 12**:** 101.

Sabouri, N., K. R. McDonald, C. J. Webb, I. M. Cristea and V. A. Zakian, 2012 DNA replication through hard-to-replicate sites, including both highly transcribed RNA Pol II and Pol III genes, requires the S. pombe Pfh1 helicase. Genes Dev 26**:** 581-593.

Saini, N., S. Ramakrishnan, R. Elango, S. Ayyar, Y. Zhang *et al.*, 2013 Migrating bubble during break-induced replication drives conservative DNA synthesis. Nature 502**:** 389-392.

Sakofsky, C. J., and A. Malkova, 2017 Break induced replication in eukaryotes: mechanisms, functions, and consequences. Crit Rev Biochem Mol Biol 52**:** 395-413.

Sanders, C. M., 2010 Human Pif1 helicase is a G-quadruplex DNA-binding protein with G-quadruplex DNA-unwinding activity. Biochem J 430**:** 119-128.

Schlacher, K., N. Christ, N. Siaud, A. Egashira, H. Wu *et al.*, 2011 Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. Cell 145**:** 529-542.

Schulz, V. P., and V. A. Zakian, 1994 The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. Cell 76**:** 145-155.

Sibon, O. C., A. Kelkar, W. Lemstra and W. E. Theurkauf, 2000 DNA-replication/DNA-damage-dependent centrosome inactivation in Drosophila embryos. Nat Cell Biol 2**:** 90-95.

Snow, B. E., M. Mateyak, J. Paderova, A. Wakeham, C. Iorio *et al.*, 2007 Murine Pif1 interacts with telomerase and is dispensable for telomere function in vivo. Mol Cell Biol 27**:** 1017-1026.

Staeva-Vieira, E., S. Yoo and R. Lehmann, 2003 An essential role of DmRad51/SpnA in DNA repair and meiotic checkpoint control. EMBO J 22**:** 5863-5874.

Steinacher, R., F. Osman, J. Z. Dalgaard, A. Lorenz and M. C. Whitby, 2012 The DNA helicase Pfh1 promotes fork merging at replication termination sites to ensure genome stability. Genes Dev 26**:** 594-602.

Stith, C. M., J. Sterling, M. A. Resnick, D. A. Gordenin and P. M. Burgers, 2008 Flexibility of eukaryotic Okazaki fragment maturation through regulated strand displacement synthesis. J Biol Chem 283**:** 34129-34140.

Sullivan, W., and W. E. Theurkauf, 1995 The cytoskeleton and morphogenesis of the early Drosophila embryo. Curr Opin Cell Biol 7**:** 18-22.

Tanaka, H., G. H. Ryu, Y. S. Seo, K. Tanaka, H. Okayama *et al.*, 2002 The fission yeast pfh1(+) gene encodes an essential 5' to 3' DNA helicase required for the completion of S-phase. Nucleic Acids Res 30**:** 4728-4739.

Thomas, A. M., C. Hui, A. South and M. McVey, 2013 Common variants of Drosophila melanogaster Cyp6d2 cause camptothecin sensitivity and synergize with loss of Brca2. G3 (Bethesda) 3**:** 91-99.

Tritto, P., V. Palumbo, L. Micale, M. Marzulli, M. P. Bozzetti *et al.*, 2015 Loss of Pol32 in Drosophila melanogaster causes chromosome instability and suppresses variegation. PLoS One 10**:** e0120859.

Trowbridge, K., K. McKim, S. J. Brill and J. Sekelsky, 2007 Synthetic lethality of Drosophila in the absence of the MUS81 endonuclease and the DmBlm helicase is associated with elevated apoptosis. Genetics 176**:** 1993-2001.

Wagner, M., G. Price and R. Rothstein, 2006 The absence of Top3 reveals an interaction between the Sgs1 and Pif1 DNA helicases in Saccharomyces cerevisiae. Genetics 174**:** 555-573.

Wallgren, M., J. B. Mohammad, K. P. Yan, P. Pourbozorgi-Langroudi, M. Ebrahimi *et al.*, 2016 G-rich telomeric and ribosomal DNA sequences from the fission yeast genome form stable G-quadruplex DNA structures in vitro and are unwound by the Pfh1 DNA helicase. Nucleic Acids Res 44**:** 6213-6231.

Wilson, M. A., Y. Kwon, Y. Xu, W. H. Chung, P. Chi *et al.*, 2013 Pif1 helicase and Poldelta promote recombination-coupled DNA synthesis via bubble migration. Nature 502**:** 393-396.

Yang, H., Q. Li, J. Fan, W. K. Holloman and N. P. Pavletich, 2005 The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA-ssDNA junction. Nature 433**:** 653-657.

Zalokar, M., and I. Erk, 1976 Division and migration of nuclei during early embryogenesis of Drosophila melanogaster. J. Microscopie Biol. Cell. 25**:** 97-106.

Zhang, D. H., B. Zhou, Y. Huang, L. X. Xu and J. Q. Zhou, 2006 The human Pif1 helicase, a potential Escherichia coli RecD homologue, inhibits telomerase activity. Nucleic Acids Res 34**:** 1393-1404.

Zhou, J. Q., H. Qi, V. P. Schulz, M. K. Mateyak, E. K. Monson *et al.*, 2002 Schizosaccharomyces pombe pfh1+ encodes an essential 5' to 3' DNA helicase that is a member of the PIF1 subfamily of DNA helicases. Mol Biol Cell 13**:** 2180-2191.

Zhou, R., J. Zhang, M. L. Bochman, V. A. Zakian and T. Ha, 2014 Periodic DNA patrolling underlies diverse functions of Pif1 on R-loops and G-rich DNA. Elife 3**:** e02190.

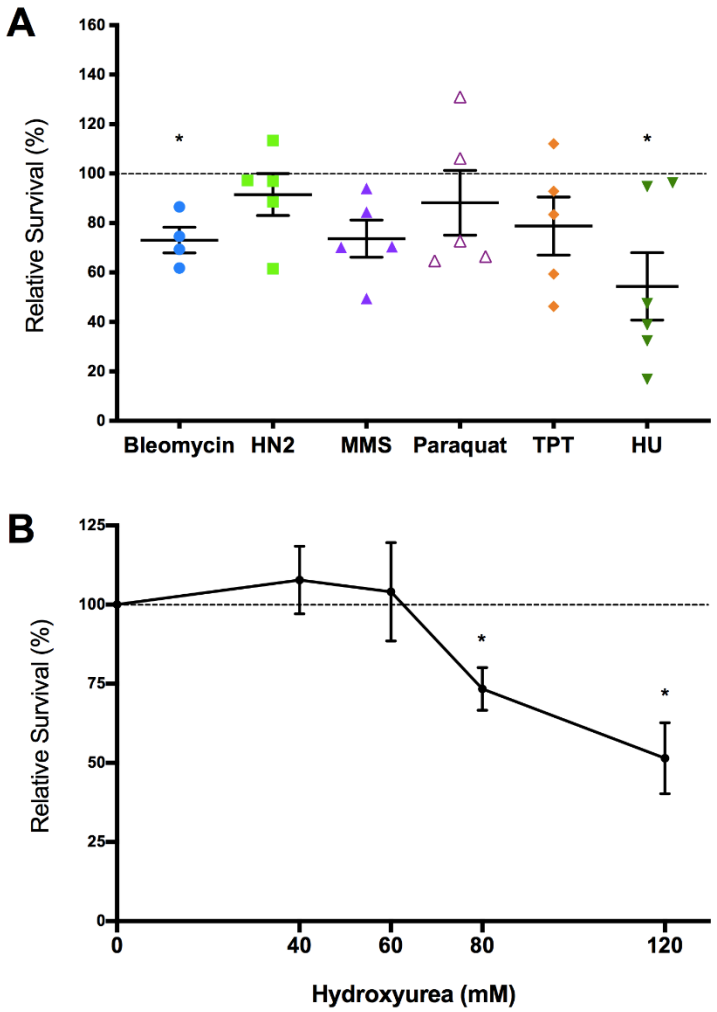

Figure 1. Drosophila PIF1 mutants are mildly sensitive to hydroxyurea. (A) Flies heterozygous for the *pif*^{167} null mutation were mated in vials for three days and then transferred to new vials for two days. The first set of control vials was treated with water and the second set was treated with solutions of 25 uM bleomycin, 0.01% nitrogen mustard (HN2), 0.12% methane methylsulfonate (MMS), 1 mM paraquat, 50 uM topotecan (TPT), or 120 mM hydroxyurea (HU). Relative survival for each pair of vials was calculated as the ratio of the percentages of homozygous mutant flies in treated vs. untreated vials. Each point represents a biological replicate (vial pair), with the mean and SEM shown. The dotted line shows 100% relative survival. * indicates $P < 0.05$ in paired t-tests between control and mutagen treated vials. (B) Flies heterozygous for the *pif1*^{167} mutation were mated in vials and treated with water of increasing concentrations of hydroxyurea. The mean and SEM of at least six biological replicates is shown for each

HU concentration. Dotted line indicates 100% relative survival. * indicates P < 0.05 in paired t-tests between unexposed and exposed vials.

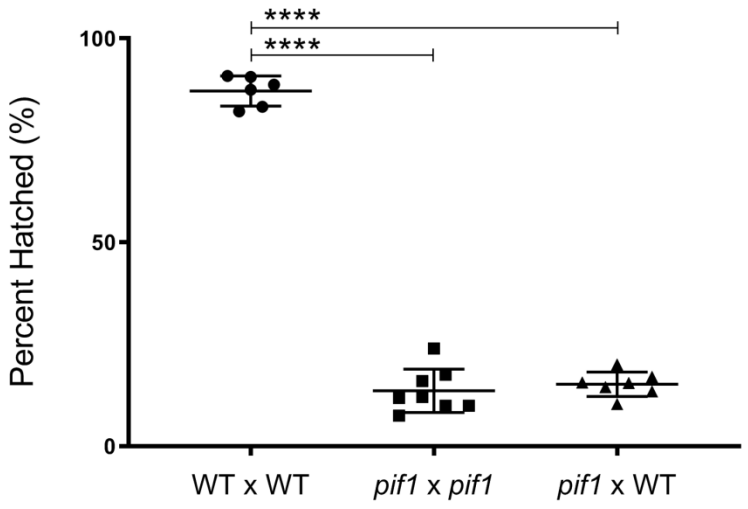

Figure 2. Severely decreased hatching frequency in *pif1* mutant flies is due to an early embryonic function of PIF1. Embryos were collected from wild type x wild type, $pif1^{167}$ x $pif1^{167}$, and $pif1^{167}$(female) x w^{1118} (male) matings, maintained at 25°C for 72 hours after collection, and scored for hatching. For each mating, three independent trials with at least two egg collections per trial were performed, with at least 550 embryos scored for each trial. Each point corresponds to the percentage of eggs hatched per plate. **** corresponds to P <0.0001 in unpaired t-tests between the three mating groups.

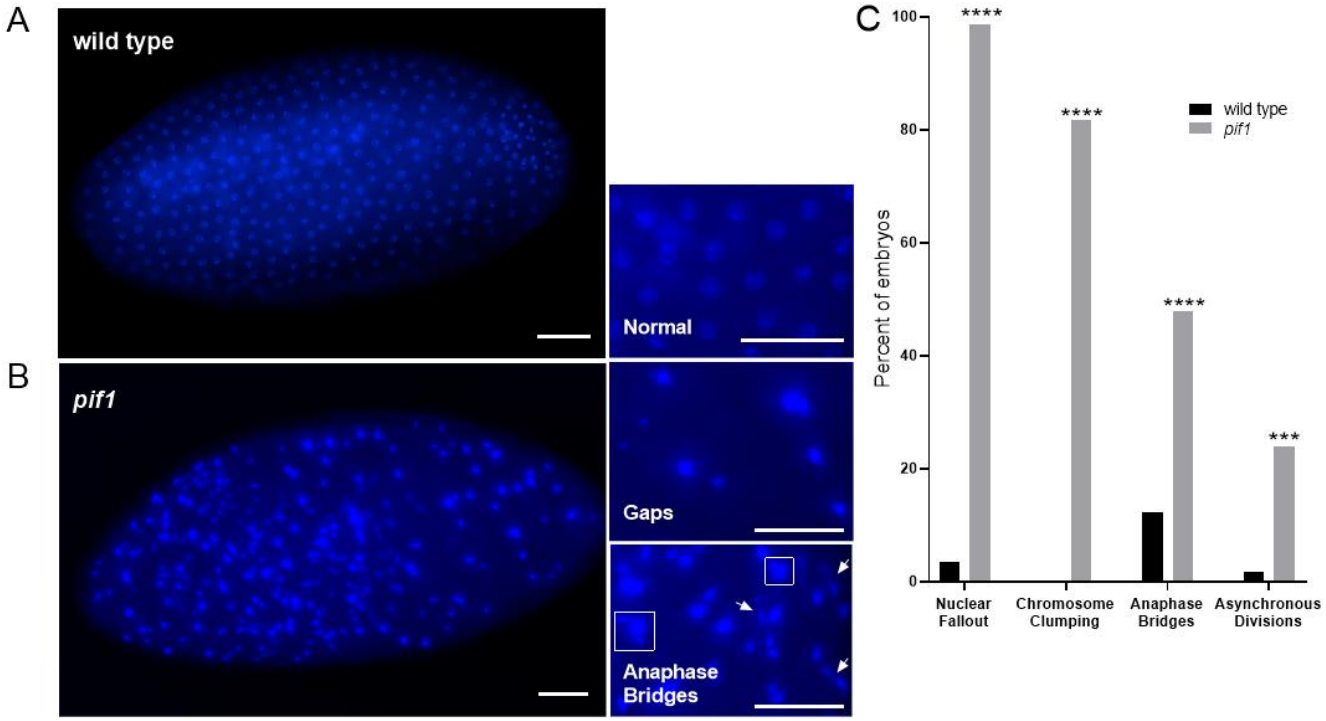

Figure 3. *pif1* mutant embryos exhibit severe chromosome segregation defects. Embryos from both wild-type and *pif1*^{167} mutant flies were collected after 1-2 hours of development at 25C, stained with DAPI, and visualized at 40X magnification using florescence microscopy. (A) Representative wild-type embryo with normal nuclear patterning. Bar = 50 µm (B) Representative *pif1* mutant embryo showing gaps within the normally uniform nuclear monolayer, anaphase bridges, and nuclear clumping. Bar = 50 µm. (C) Quantification of the frequency of nuclear defects seen in DAPI-stained wild-type and *pif1*^{167} embryos. wild type n = 57; *pif1*^{167} n = 71. **** $P < 0.0001$, *** $P < 0.001$ in unpaired t-tests.

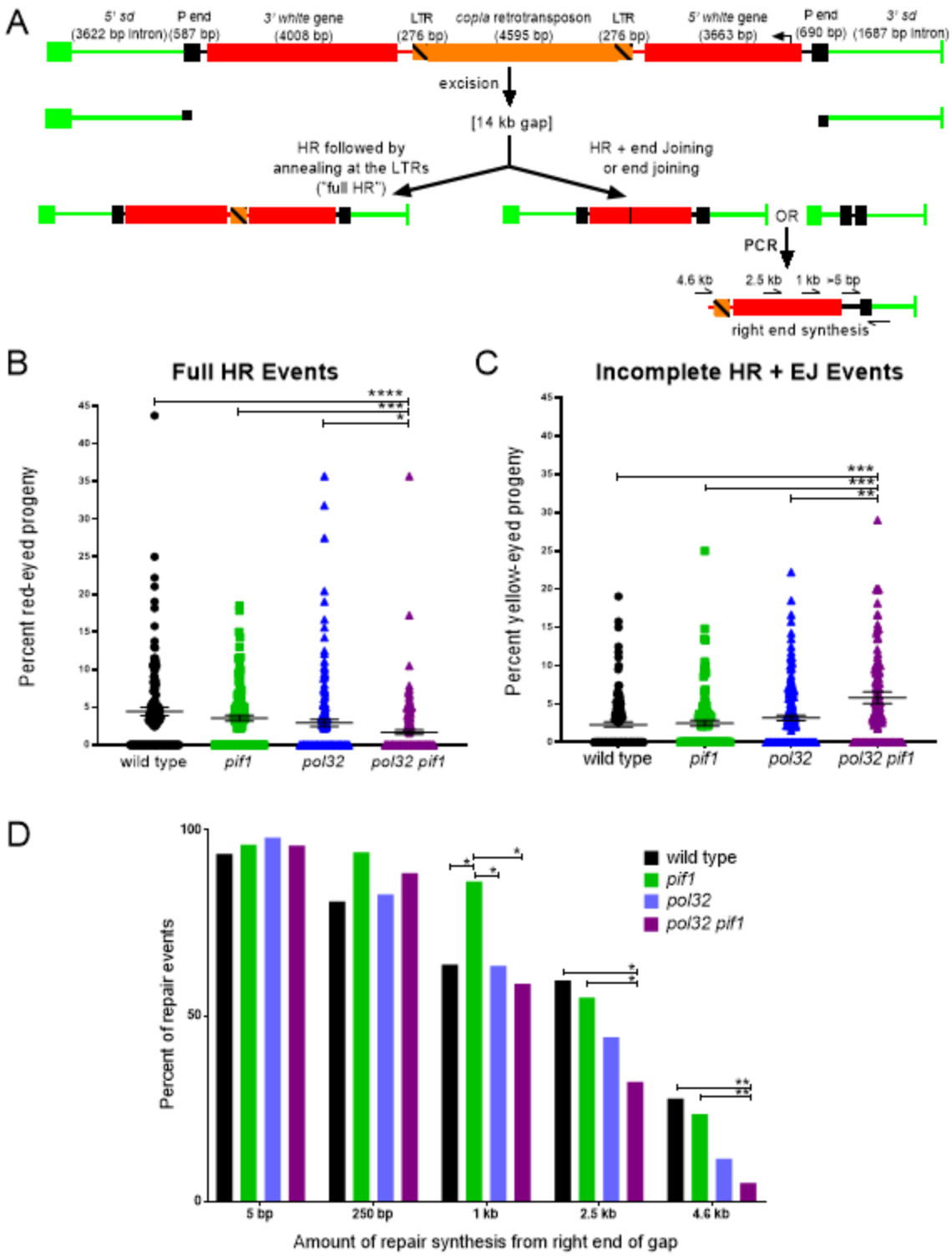

Figure 4. PIF1 and POL32 independently promote DNA synthesis during HR gap repair. (A) The *P{w^a}* site-specific gap repair assay. A *P* element bearing a *copia* retrotransposon in an intron of the *white* gene is inserted in the essential gene *scalloped* (*sd*). Expression of *P* transposase in *P{w^a}*-bearing males results

in a 14-kb gap with 17 nucleotide non-complementary overhangs relative to the uncut sister chromatid. Repair events from the male pre-meiotic germline are recovered in females in the next generation *in trans* to an intact *P{w^a}*. Recovery of repair events involving full synthesis of *white*, followed by annealing at the *copia* LTRs, creates female progeny with red eyes. Recovery of repair events involving partial synthesis of *white* followed by end joining, or just end joining alone, creates female progeny with yellow eyes. The amount of repair synthesis can be estimated by PCR. (B&C) *pol32 pif1* mutants have decreased full HR repair and increased incomplete HR + EJ repair relative to either single mutant. Number of independent vials scored: wild type = 126; *pol32* = 153; *pif1* = 111; *pol32 pif1* = 156. Error bars represent standard error of the mean. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.002$, * $P < 0.05$ in Mann-Whitney tests between the four genotypes. (D) Repair synthesis during HR is significantly decreased at distances ≥ 2.5 kb in *pol32 pif1* mutants relative to wild type and either single mutant. Each bar represents the percentage of events with at least the indicated amount of synthesis, measured by PCR. Number of independent repair events tested: wild type = 47; *pif1* = 51; *pol32* = 52; *pol32 pif1* = 121. * $P < 0.05$, ** $P < 0.01$ in Fisher's exact tests between the four genotypes.

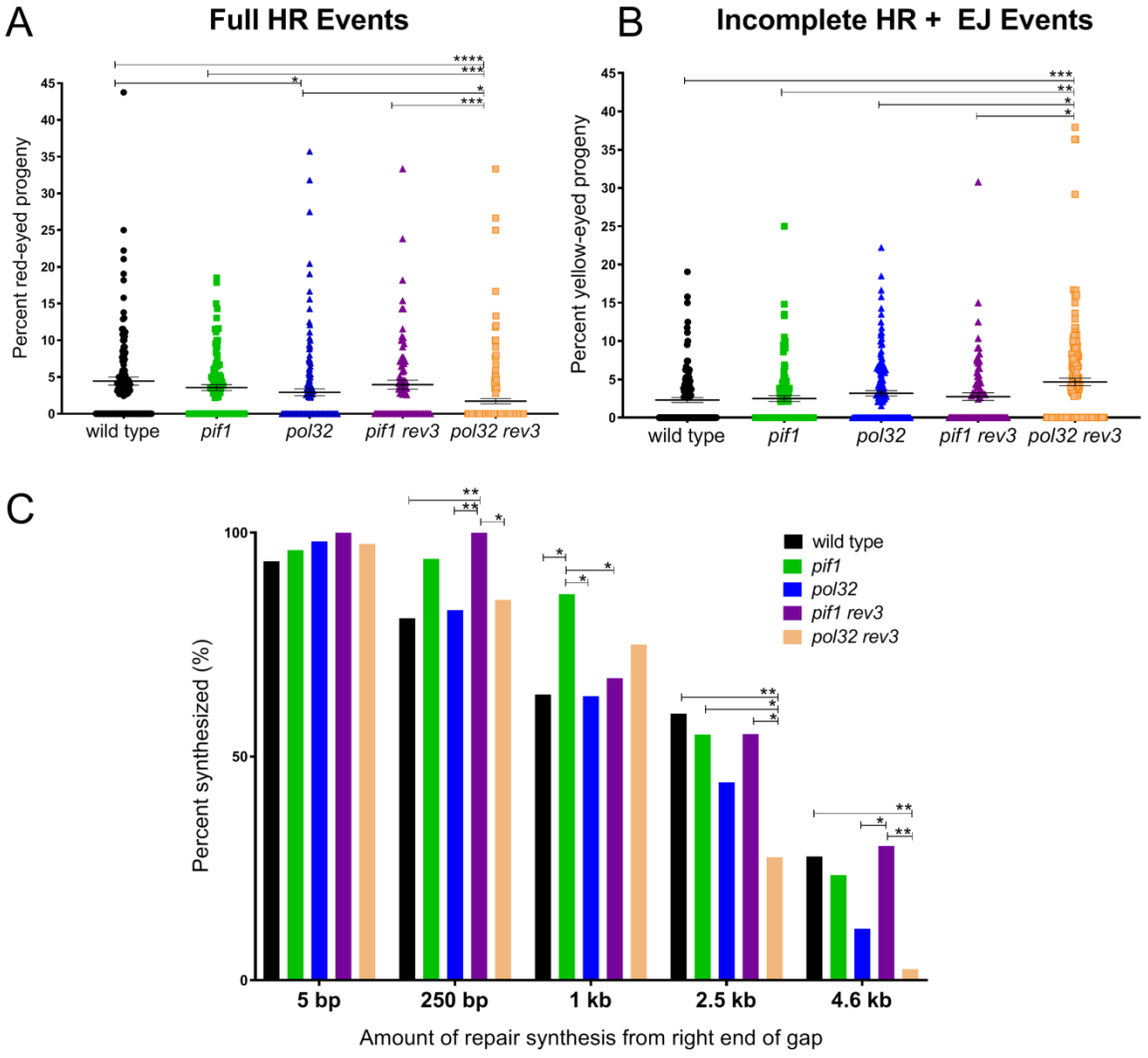

Figure 5. Loss of POL32, but not PIF1, further decreases DNA synthesis during gap repair in *pol zeta* mutants. (A&B) *pol32 rev3*, but not *pif1 rev3* double mutants have decreased full HR and increased incomplete HR + EJ events compared to wild type, *pol32*, or *pif1* single mutants. The wild type, *pif1* and *pol32* data are replicated from Fig. 4. Number of independent vials scored: wild type = 126; *pol32* = 153; *pif1* = 111; *pol32 rev3* = 173; *pif1 rev3* = 86. Error bars represent standard error of the mean. **** P < 0.0001, *** P < 0.001, ** P < 0.002, * P < 0.05 in Mann-Whitney tests between the five genotypes. (C) Repair synthesis is decreased in *pol32 rev3*, but not *pif1 rev3* double mutants relative to wild type, *pif1*, and *pol32* single mutants. Each bar represents the percentage of events with at least the indicated amount of synthesis. Number of independent repair events tested: wild type = 47; *pif1* = 51; *pol32* = 52; *pif1 rev3* = 40; *pol32 rev3* = 40. * P < 0.05, ** P < 0.01 in Fisher's exact tests between the five genotypes.

Table 1: Synthetic lethal relationships for *pif1* and *pol32* mutants

Genotype	**Lethality?**
pif1^{167} *brca2*^{47}	lethal (3rd instar)
pif1^{167} *brca2*^{KO}	lethal (3rd instar)
pol32^{L2} *brca2*^{47}	lethal (3rd instar)
pol32^{L2} *brca2*^{KO}	lethal (3rd instar)
pif1^{167}*pol32*^{L2} *brca2*^{47}	lethal (3rd instar)
pif1^{167}*pol32*^{L2} *brca2*^{KO}	lethal (3rd instar)
pif1^{167} *rad51*^{057}	viable adults
pol32^{L2} *rad51*^{057}	viable adults
pif1^{167} *brca2*^{KO} *rad51*^{057}	lethal (3rd instar)
pol32^{L2} *brca2*^{KO} *rad51*^{057}	lethal (3rd instar)
pif1^{167} *pol32*^{L2} *brca2*^{KO} *rad51*^{057}	lethal (3rd instar)
pif1^{167} *mus81*^{Nhe}	viable adults
pol32^{L2} *mus81*^{Nhe}	viable adults
pif1^{167} *brca2*^{KO} *mus81*^{Nhe}	lethal (3rd instar)
pol32^{L2} *brca2*^{KO} *mus81*^{Nhe}	lethal (3rd instar)
pif1^{167} *pol32*^{L2} *brca2*^{KO} *mus81*^{Nhe}	lethal (3rd instar)