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 homologous recombination and 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 to promote efficient DNA synthesis during times of replication stress and 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 ss flaps by increasing DNA polymerase delta (Pol δ) processivity (ROSSI *et al.* 2008; STITH *et al.* 2008). SpPfh1 likewise contributes to the formation of ss 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).

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). However, its exact role(s) remain to be fully elucidated.

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. Such observations suggest that Pif1 may play a subtler role in higher eukaryotes or may have functions redundant with those of 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 of these phenotypes suggest that PIF1 plays an important function during periods of replication stress, such as 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 genetic interaction between PIF1 and BRCA2, which functions both in double-strand break repair and replication fork protection in other organisms. 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. The deletion was confirmed by PCR with oligonucleotides spanning the breakpoint and Sanger sequencing.

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 BRCA2 coding sequence (THOMAS *et al.* 2013), and *brca2*^{KO}, which deletes the first 3321 bp of the 3417 bp BRCA2 coding sequence (KLOVSTAD *et al.* 2008). All double and triple mutants used in this study were created by meiotic recombination and verified by PCR. 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 wildtype 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

About 30 *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.

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

HR repair was monitored after excision of a *P{w^a}* transposable element as described previously (McVey 2010). A third chromosome transposase source (*P{ry$^+$, Δ2-3}99B*, Bloomington stock 406) was used to excise *P{w^a}*. Individual males possessing both *P{w^a}* and the transposase source were mated to females homozygous for *P{w^a}* and repair products were recovered in female progeny. Individual progeny reflecting unique repair events from each vial 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 was recovered 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 information about the *pif1^{167}* mutation and *brca2 pif1* and *brca2 pol32* developmental stage-specific synthetic lethality. File S2 contains 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 created the $pif1^{167}$ null mutant allele 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 (ALEXANDER *et al.* 2016). Flies homozygous for this deletion were viable and apparently healthy, although females displayed greatly reduced fertility.

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 high doses of different mutagens and measured the survival of these larvae into adulthood relative to untreated siblings. The homozygous mutants were not sensitive to 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 oxidative stress response or in mitochondrial maintenance, unlike its yeast orthologs, but is important for survival during conditions of replication stress.

PIF1 is required for rapid nuclear divisions 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 the females were mostly infertile. Because *pif1* mutants are preferentially sensitive to agents that induce replication stress, we hypothesized that the decreased fertility might be due to embryonic lethality resulting from replication defects during early development. Indeed, *pif1* mutant embryos 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.

During *Drosophila* embryogenesis, paternal gene expression of most genes is initiated about two hours after fertilization. To determine 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* embryogenesis, 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). During this period, 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 condensation and 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).

We also employed real-time visualization of developing wild-type and *pif1* embryos to understand how the defects observed with DAPI staining might have emerged. 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.

PIF1 promotes long-range DNA synthesis during homologous recombination repair

S. cerevisiae Pif1 is needed 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). 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). In this system, a 14-kilobase *P* element containing the *white* gene interrupted by a *copia* retrotransposon is inserted into the essential gene *scalloped* on the *X* chromosome (Figure 4A). The excision of $P[w^a\}$ from one sister chromatid by *P*-element transposase generates a 14-kb gap with non-complementary 17-nucleotide overhangs, and the intact sister chromatid serves as a template for repair synthesis. Repair products formed in the male germline are recovered in female progeny and the frequency of the possible repair outcomes are quantified by eye color.

We found that *pif1* mutants had a slight reduction in the number of full homologous recombination (full HR) events compared to wildtype. 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 wildtype, 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 wildtype, 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 4.6 kb (Figure 4D). In contrast, *pif1* mutants had slightly increased repair synthesis at distances of 250 bp-1 kb and no defects at long 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 wildtype 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 SDSA via different mechanisms.

PIF1 and POL32 are essential for adult viability in the absence of BRCA2

The *P{w^a}* assay can also be utilized in a *rad51* or *brca2* mutant background to assess end-joining repair that occurs in the absence of HR (MCVEY *et al.* 2004; KLOVSTAD *et al.* 2008; THOMAS *et al.* 2013). To determine if loss of PIF1 impacts the structure of end-joining repair products, we attempted to create stocks of flies deficient in HR and lacking PIF1. We generated five independent *pif1 brca2* double mutant stocks through meiotic recombination, utilizing two different *brca2* null alleles. Strikingly, we found that *pif1 brca2* double mutants were unable to survive past late larval stages (Figure S2).

Since Pol32 acts alongside Pif1 in budding yeast to promote BIR and Okazaki fragment

maturation, we wondered 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 (Figure S2). 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.

Discussion

Drosophila PIF1 is important during early embryo development and in conditions of replication stress

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.

In addition to finding that *pif1* mutants fail to survive exposure to high doses of hydroxyurea, which induces replication stress, we also identified a vital role for PIF1 during early Drosophila

embryogenesis, at a time of extremely rapid genome replication and chromosome segregation. 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 either DNA replication during early embryogenesis. Indeed, unreplicated 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 are subject to nuclear fallout; too many lost nuclei result in gaps in the typically uniform monolayer of nuclei, as we also observed. 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.

We hypothesize that PIF1 might promote efficient replication during embryogenesis and conditions of replicative stress via one or more of three 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.

A third 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 BIR (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.

A role for PIF1 in replication restart?

One of the most surprising results from this study is that *pif1 brca2* mutants exhibit synthetic lethality at late larval stages. In our experiments, homozygous *brca2 pif1* and *brca2 pol32* mutants were created by mating flies heterozygous for both mutations. Thus, maternal deposits of the proteins may have allowed for survival to late larval stages, but not beyond.

The mammalian BRCA2 protein contains eight BRC repeats that enable BRCA2 to bind multiple Rad51 monomers (PELLEGRINI *et al.* 2002; BOULTON 2006). Although, *Drosophila* BRCA2 contains only three of these BRC repeats, fly *brca2* mutants are sensitive to ionizing radiation, HU, and MMS, arguing for conserved functions with its mammalian counterpart (BROUGH *et al.* 2008; KLOVSTAD *et al.* 2008). 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) and DmBrca2 has been shown to have Rad51-independent roles at stalled forks during meiosis (KLOVSTAD *et al.* 2008). Thus, it seems likely that fly BRCA2 might also be important for the stabilization of stalled forks.

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). 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. Although it is still unclear whether BIR is a conserved repair mechanism in *Drosophila*, 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 from our lab 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 that 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 throughout development. Multiple lines of evidence suggest that 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. In the absence of BRCA2, it is needed for development past late larval stages, a function that it shares with POL32. In contrast, 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 counterparts, and lays the groundwork for future investigations into potential roles of PIF1 in replication fork stabilization and restart.

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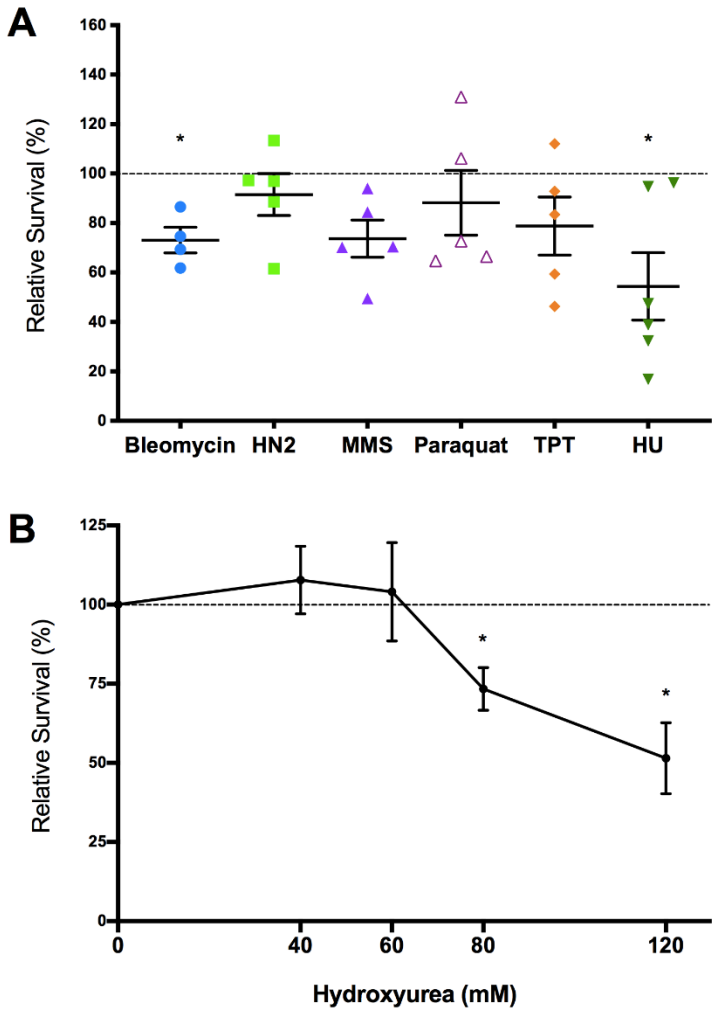


Figure 1. Drosophila PIF1 mutants are mildly sensitive to hydroxyurea. (A) Flies heterozygous for the *pif*^{167} null mutation were mated in vials for three days and then transferred to new vials for two days. The first set of control vials was treated with water and the second set was treated with 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.

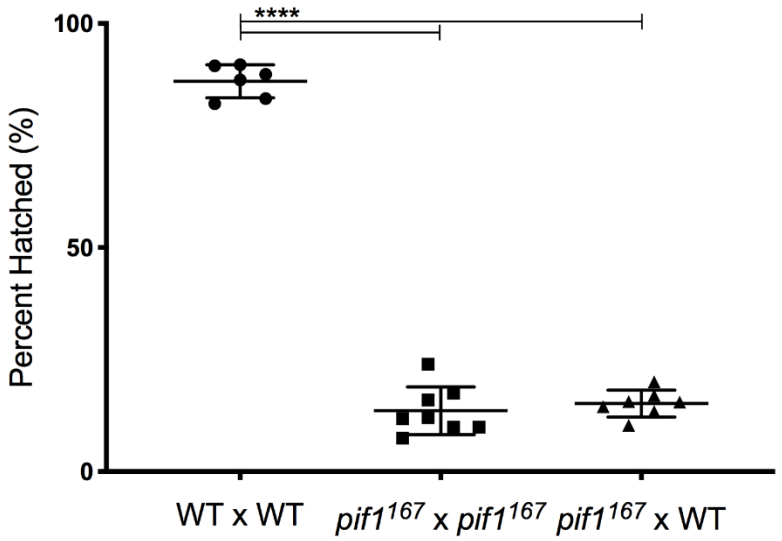


Figure 2. Severely decreased hatching frequency in *pif1* mutant flies is due to an early embryonic function of PIF1. Embryos were collected from wild-type x wild-type, $pif1^{167}$ *x* $pif1^{167}$, and $pif1^{167}$ x w^{1118} matings, maintained at 25°C for 72 hours after collection, and scored for 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.

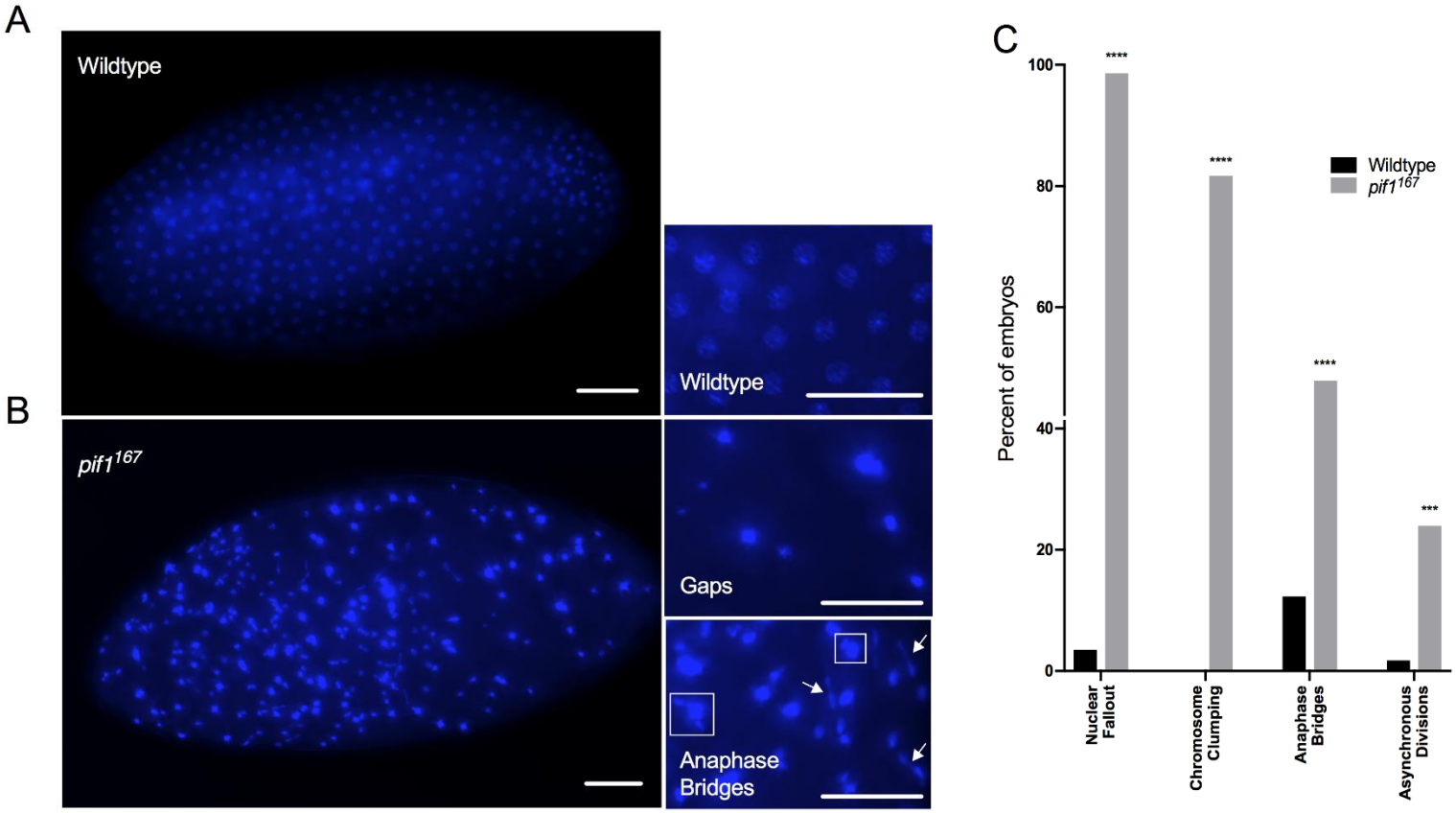


Figure 3. *pif1* mutant embryos exhibit severe chromosome segregation defects. Embryos from both wildtype and *pif1*^{167} mutant flies were collected after 1-2 hours of development at 25C, stained with DAPI, and visualized at 40X magnification using florescence microscopy. (A) Representative wild-type embryo with normal nuclear patterning. Bar = 100 µm (B) Representative *pif1* mutant embryo showing gaps within the normally uniform nuclear monolayer, anaphase bridges, and nuclear clumping. Bar = 100 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.

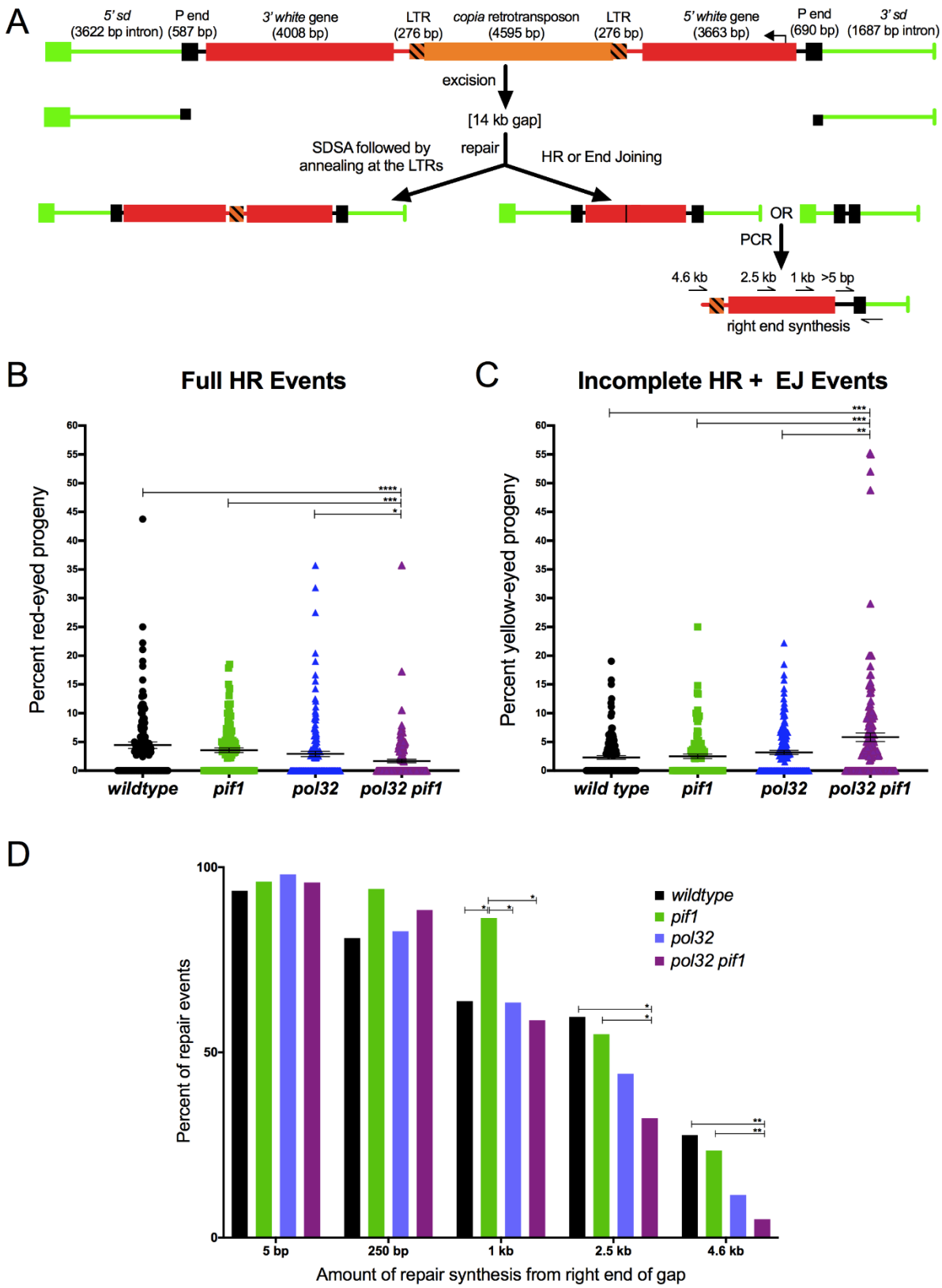

A
5' sd
(3622 bp intron)
P end
(587 bp)
3' white gene
(4008 bp)
LTR
(276 bp)
copia retrotransposon
(4595 bp)
LTR
(276 bp)
5' white gene
(3663 bp)
P end
(690 bp)
3' sd
(1687 bp intron)
excision
[14 kb gap]
repair
SDSA followed by
annealing at the LTRs
HR or End Joining
OR
PCR
4.6 kb
2.5 kb
1 kb
>5 bp
right end synthesis
B
Full HR Events
Percent red-eyed progeny
wildtype
pif1
pol32
pol32 pif1
C
Incomplete HR + EJ Events
Percent yellow-eyed progeny
wild type
pif1
pol32
pol32 pif1
D
Percent of repair events
wildtype
pif1
pol32
pol32 pif1
5 bp
250 bp
1 kb
2.5 kb
4.6 kb
Amount of repair synthesis from right end of gap

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 LTRs, creates female progeny with red eyes. Recovery of repair events involving partial synthesis of *white*, followed by end joining, 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.

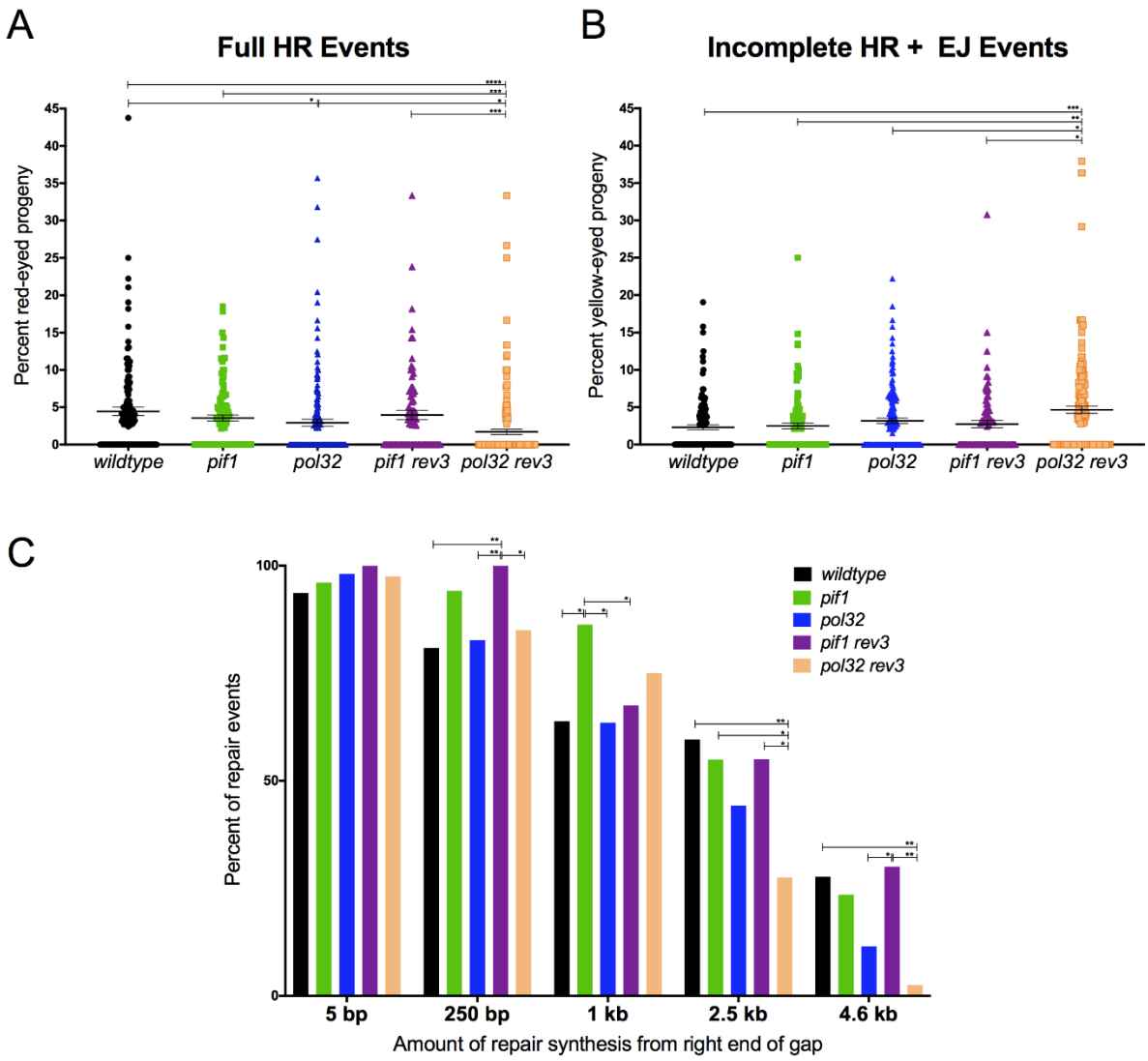
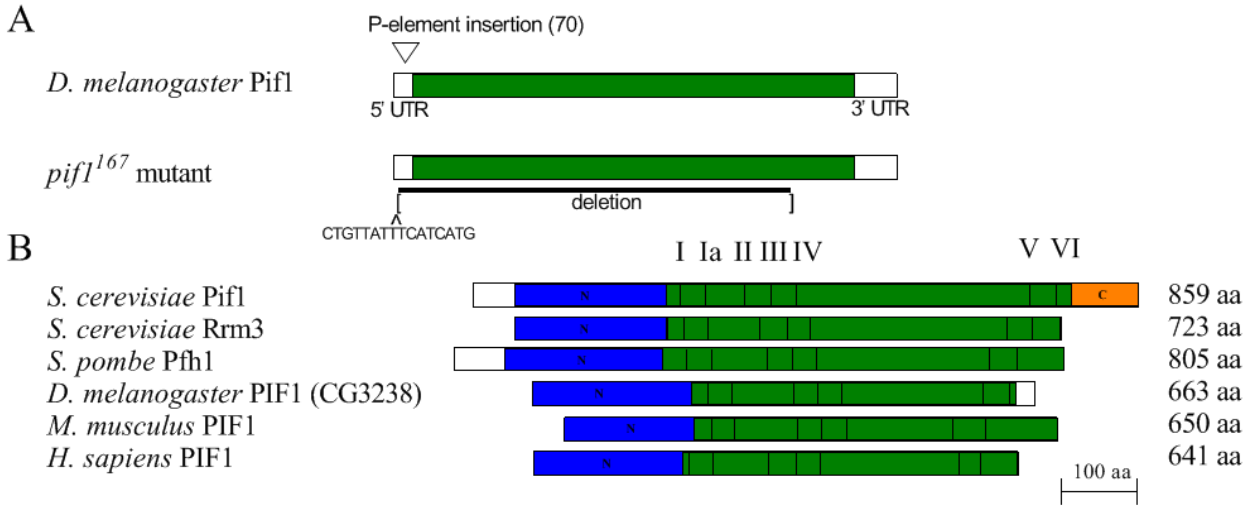
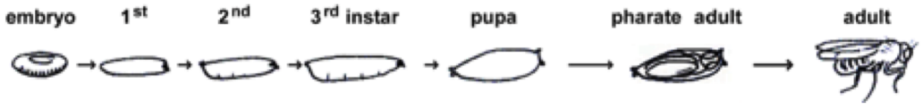


Figure 5. Loss of POL32, but not PIF1, further decreases DNA synthesis during gap repair in *pol zeta* mutants. (A&B) *pol32 rev3*, but not *pif1 rev3* double mutants have decreased full HR and increased incomplete HR + EJ events compared to wild type, *pol32*, or *pif1* single mutants. Number of independent 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.



Supplemental Figure 1 – Creation of a Drosophila pif1 mutant. (A) A large deletion that removes most of the PIF1 coding sequence was created through imprecise excision of a P element inserted in the 5' UTR. The extent of the deletion is indicated by brackets. A carat indicates an accompanying insertion at the deletion site. (B) Alignment of PIF1 orthologs from multiple species. The conserved helicase domain is shown in green, the conserved N-terminus in shown in blue, and a non-conserved C-terminal extension found in S. cerevisiae Pif1 is shown in orange.



Isolate	Stage of Lethality
$brca2^{47}$pif1^{167} #9	3rd instar larvae
$brca2^{47}$pif1^{167} #16	3rd instar larvae
$brca2^{47}$pif1^{167} #22	3rd instar larvae
$brca2^{KO}$pif1^{167} #1-4	2nd instar larvae
$brca2^{KO}$pif1^{167} #3-2	3rd instar larvae
$brca2^{47}$pol32^{L2} #1	3rd instar larvae
$brca2^{KO}$pol32^{L2} #36-2	3rd instar larvae
$brca2^{KO}$pol32^{L2} #24-3	3rd instar larvae
$brca2^{47}$pol32^{L2}pif1^{167} #8	3rd instar larvae
$brca2^{KO}$pol32^{L2}pif1^{167} #1-6	3rd instar larvae
$brca2^{47}$pif1^{167} #9 x $brca2^{KO}$pif1^{167} #1-4	3rd instar larvae
$brca2^{47}$pif1^{167} #16 x $brca2^{KO}$pif1^{167} #1-4	3rd instar larvae
$brca2^{47}$pol32^{L2} #1 x $brca2^{KO}$pol32^{L2} #36-2	3rd instar larvae
$brca2^{47}$pol32^{L2}pif1^{167} #8 x $brca2^{KO}$pol32^{L2}pif1^{167} #1-6	3rd instar larvae

Supplemental Figure 2. Loss of BRCA2 and PIF1 or POL32 results in synthetic lethality at late-larval stages. Double and triple mutants were created via standard meiotic recombination. # signs signify different recombinant isolates. Two different *brca2* allele were used: *brca2*^{47} (Thomas *et al*., 2013) and *brca2*^{KO} (Klovstad *et al*., 2008). The *pol32*^{L2} allele used is from Kane *et al*., 2012.