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RESEARCH ARTICLE

Drosophila RecQ5 is required for efficient SSA repair and suppression of LOH *in vivo*

Yixu Chen[1,2*], Wen Dui[1,2,3*], Zhongsheng Yu[1,2], Changqing Li[1], Jun Ma[1,3], Renjie Jiao[1] ✉

[1] State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, China
[2] Graduate School of the Chinese Academy of Sciences, Beijing 100080, China
[3] Divisions of Biomedical Informatics and Developmental Biology, Cincinnati Children's Hospital Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
✉ Correspondence: rjiao@sun5.ibp.ac.cn
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ABSTRACT

RecQ5 in mammalian cells has been suggested to suppress inappropriate homologous recombination. However, the specific pathway(s) in which it is involved and the underlining mechanism(s) remain poorly understood. We took advantage of genetic tools in *Drosophila* to investigate how *Drosophila RecQ5* (*dRecQ5*) functions *in vivo* in homologous recombination-mediated double strand break (DSB) repair. We generated null alleles of *dRecQ5* using the targeted recombination technique. The mutant animals are homozygous viable, but with growth retardation during development. The mutants are sensitive to both exogenous DSB-inducing treatment, such as gamma-irradiation, and endogenously induced double strand breaks (DSBs) by I-Sce I endonuclease. In the absence of *dRecQ5*, single strand annealing (SSA)-mediated DSB repair is compromised with compensatory increases in either inter-homologous gene conversion, or non-homologous end joining (NHEJ) when inter-chromosomal homologous sequence is unavailable. Loss of function of *dRecQ5* also leads to genome instability in loss of heterozygosity (LOH) assays. Together, our data demonstrate that *dRecQ5* functions in SSA-mediated DSB repair to achieve its full efficiency and in suppression of LOH in *Drosophila*.

KEYWORDS *Drosophila RecQ5*, double strand break repair, homologous recombination, nonhomologous end joining, single strand annealing, RecQ helicase

INTRODUCTION

DNA breaks need to be properly repaired to ensure the integrity of an organism's genome. Un-repaired DNA breaks are deleterious to cells during cell divisions leading to cell cycle arrest and eventually lethality of organisms (Johnson-Schlitz and Engels, 2006; Preston et al., 2006). Our understanding of double strand DNA break (DSB) repair in eukaryotes has been greatly facilitated by the use of site-specific endonucleases, such as I-Sce I, a rare yeast cutter that is also active when expressed in other eukaryotic cells such as mammalian and *Drosophila* cells (Plessis et al., 1992; Rouet et al., 1994; Johnson-Schlitz et al., 2007; Wei and Rong, 2007). There are three major DSB repair pathways in *Drosophila* both in mitotic and meiotic cells (Adams et al., 2003; Rong and Golic, 2003; Preston et al., 2006; Wei and Rong, 2007). Single strand annealing (SSA), which has been shown to be the first choice for DSB repair in *Drosophila* (Preston et al., 2006; Wei and Rong, 2007), uses homologous sequences that flank the break. SSA repair always results in a deletion that uncovers the DNA between the two stretches of homologous sequence (Preston et al., 2006). Non-homologous end joining (NHEJ) is usually error prone and often creates small deletions or insertions at the break site, although it can also re-ligate the broken ends perfectly without causing any mutations. Recombinational repair between homologous chromosomes through either synthesis dependent strand annealing (SDSA) or double holiday junction (DHJ) results in gene conversion (GC), while crossovers produce loss of heterozygosity (Rong and Golic, 2003; Preston et al., 2006). These different pathways

*These authors contributed equally to this work

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represent choices for a cell to repair a given DSB with preferences that are dependent on the genomic context of where the DSB occurs, the developmental stages and likely additional factors such as the proximity to an essential gene (Preston et al., 2006; Wei and Rong, 2007). RAD52 and related proteins, such as Rad50, Rad51, Rad54, Rad59 and Mre11, are known to be involved in regulating the gene conversion process (Ghabrial et al., 1998; Wei and Rong, 2007). Furthermore, several conserved proteins have been demonstrated to regulate NHEJ, including the Ku70, Ku80 and the DNA ligase IV (Kusano et al., 2001; Wei and Rong, 2007). However, the factors that participate in SSA in *Drosophila* remain to be elucidated.

RecQ helicase family is a group of ATP-dependent DNA helicases that maintains genome stability by regulating DNA recombination, repair and replication (Bachrati and Hickson, 2008; Chu and Hickson, 2009). The *Drosophila* genome encodes three RecQ helicases, dBLM, dRecQ4 and dRecQ5 (Kusano et al., 1999; Sekelsky et al., 1999; Kusano et al., 2001; Kawasaki et al., 2002; Adams et al., 2003; McVey et al., 2007; Trowbridge et al., 2007; Wu et al., 2008; Nakayama et al., 2009; Xu et al., 2009). In addition, DmWRNexo was recently reported to be homologous to the human WRN exonuclease domain (Saunders et al., 2008; Boubriak et al., 2009). Loss of *dBLM* causes sterility in flies (Kusano et al., 1999; Kusano et al., 2001). *dBLM* mutants also exhibit other defects including impaired DNA synthesis during homologous repair (HR) (Adams et al., 2003), reduced frequency in homologous repair from the homologous chromosome (HR-h) with a concurrent increase in SSA frequency (Johnson-Schlitz and Engels, 2006; Kappeler et al., 2008), and an elevated crossovers due to defects in the dissolution of holiday junctions during DSB repair (Johnson-Schlitz and Engels, 2006). We and others have shown recently that *dRecQ4* is essential for *Drosophila* development and involved in DNA replication and repair (Wu et al., 2008; Xu et al., 2009). In contrast, despite the well characterized biochemical properties of dRecQ5, including strand annealing activities and the unwinding of the substrates of 3' flaps, three-way junctions, forks, and three-strand junctions, some of which are unique to RecQ5 among the RecQ members (Sekelsky et al., 1999; Jeong et al., 2000; Kawasaki et al., 2002; Nakayama et al., 2009), the *in vivo* functions of *dRecQ5* remain largely unknown.

RecQ5 is a conserved gene in all multicellular organisms ranging from worms to mammals. In *C. elegans*, RNAi against *RecQ5* increases the animal's sensitivity to ionizing irradiation (Jeong et al., 2003). In mouse ES cells, RecQ5 is shown to suppress both crossovers and the repair of I-Sce I-induced double-strand breaks by homologous recombination (Hu et al., 2005). Loss of *Drosophila RecQ5* leads to chromosomal aberrations during early embryonic development (Nakayama et al., 2009). RecQ5 in mammalian cells has been shown to suppress inappropriate homologous recombination by disrupting RAD51 recombinase-mediated presynaptic filaments (Hu et al., 2007). It localizes to the sites of double strand breaks by interacting with MRN complex (Zheng et al., 2009). To understand how RecQ5 precisely functions in the DSB repair pathways, we made use of the *Drosophila* DSB systems that were developed in Engels and Rong labs (Preston et al., 2006; Wei and Rong, 2007), which can detect simultaneously the usage of different DSB repair pathways. We examined and compared the changes of induced DSB repair in the presence or absence of *dRecQ5*. We show that *dRecQ5* is specifically required for full efficiency of SSA-mediated DSB repair and *dRecQ5* mutation leads to genome instability as assayed by loss of heterozygosity experiments.

RESULTS

Generation of *dRecQ5* mutants and molecular characterization

To gain a better understanding of the precise molecular functions of *dRecQ5* in *Drosophila*, we set off to knock out the *dRecQ5* gene in flies by the specific ends-in gene targeting technique (Fig. 1A). A donor construct that contains an FRT-flanked ~8 kb *dRecQ5* genomic fragment, which was modified with intended mutations at the ATG site and an artificial I-Sce I cutting site ~2 kb downstream of the ATG, was inserted into the fly genome through P element mediated transformation (see materials and methods for details). After two rounds of homologous recombination, the endogenous wild type copy of *dRecQ5* locus was replaced by the designed mutant copy of *dRecQ5* (Fig. 1A and 1B). Three alleles were obtained after the reduction step of gene targeting, which were confirmed to contain the designed mutations as judged by the introduction of Not I restriction site (Fig. 1C). All three alleles exhibit identical phenotypes of being homozygous viable and fertile. One of these alleles, designated as $dRecQ5^{5}$, was further confirmed by sequencing for the mutation (Fig. 1B) and demonstrating the absence of all three different isoforms of dRecQ5 protein (Fig. 1B and 1D). This allele was used in further studies described in the following sections.

dRecQ5 is required for normal development and mitosis in *Drosophila*

Although *dRecQ5* null mutant flies are homologous viable and fertile without obvious morphological defects, careful examination of the mutant animals revealed that over 80% of the mutants were smaller than the wild type at same developing time points of the larval stages particularly at 72 and 96 h after egg deposition (AED). Images of typical mutant and wild type larvae are shown in pairs for the same developing time points in Fig. 2A. Fig. 2B and 2C show that the mutants develop more slowly than the wild type. At 114 h

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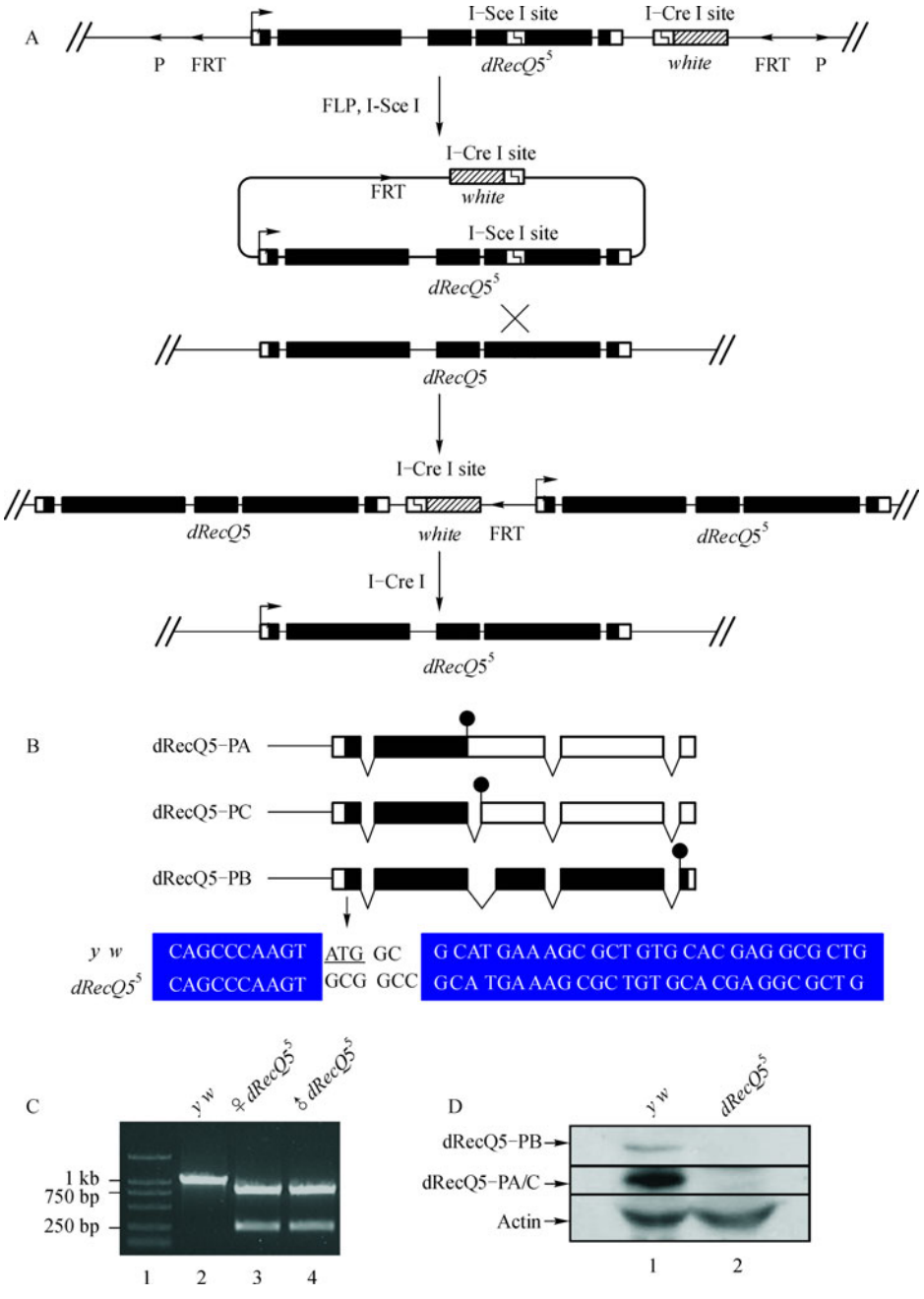


Figure 1. Generation of *dRecQ5* mutant (*dRecQ5*^{5}): strategy and molecular characterization. (A) Schematic diagram of *dRecQ5* genomic locus and gene targeting strategy. A mutant *dRecQ5* (* indicates the mutation site at the start codon) and the marker gene w^+ are circularized from the transgenic genome by FLP recombinase and linearized by the yeast restriction endonuclease I-Sce I before ends-in recombination resulting in a duplication of the *dRecQ5* locus (one wild type and the other mutant). Upon I-Cre I cutting and repairing via homologous recombination, the *dRecQ5* mutant can be selected with the loss of *white* marker gene. (B) Sequence comparison of *dRecQ5*^{5} and the wild type (WT) indicating that the expected ATG (underlined) mutation to GCG (yielding a Not I cutting site) and a frame shift of the coding sequence in *dRecQ5*^{5}. Three mRNA splice isoforms of *dRecQ5* (dRecQ5-PA, -PB and-PC) are shown above the sequence comparison. (C) PCR in combination with Not I digestion (see materials and methods for details) shows that the artificial Not I site in the *dRecQ5*^{5} gives two bands of the PCR products (lane 3 and 4) while the PCR products from wild type flies cannot be cut by Not I (lane 2). Lane 1 is DNA ladders. (D) Western blot showing that the 53 kDa short forms of dRecQ5-PA and dRecQ5-PC, and the 121 KD form of dRecQ5-PB become undetectable in *dRecQ5*^{5} (lane 2). Lane 1 is the wild type control. The 43 kDa actin band is loading control. Molecular weights are indicated on the left of the panel.

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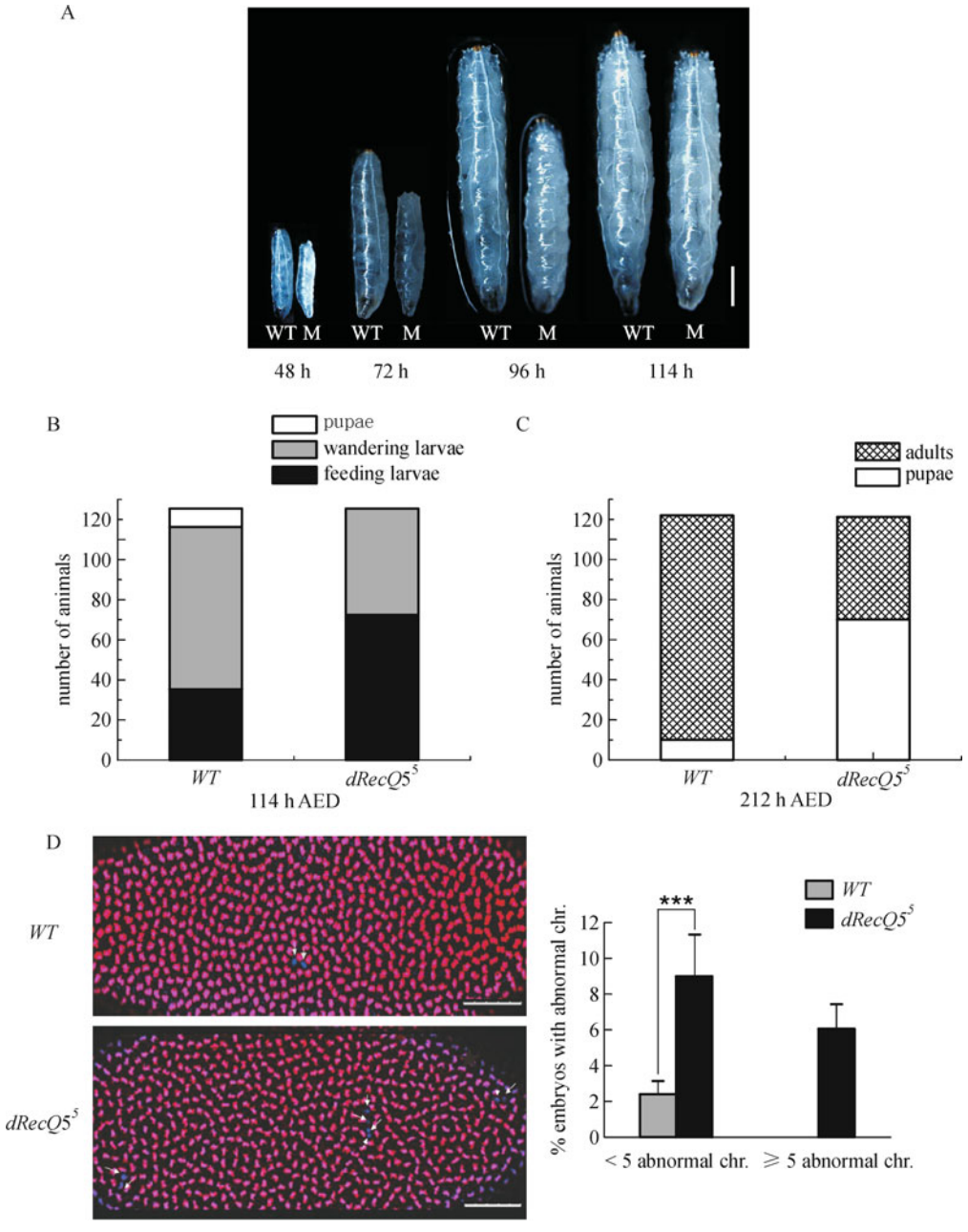


Figure 2. Cellular and growth phenotypes of *dRecQ5* homozygous mutants. (A) Comparison of wild type and *dRecQ5*^{5} animals at indicated time points of larval stages. WT indicates wild type larvae and M indicates mutant larvae. Scale bar, 600 μM. (B) Fractions of feeding larvae, wandering larvae and pupae at 114 h after egg deposition (AED) for both *dRecQ5*^{5} and wild type. (C) Fractions of pupae and adults at 212 h AED for both *dRecQ5*^{5} and wild type. (D) Loss-of-function of *dRecQ5* leads to spontaneous mitotic defects with increased frequency of aberrant chromosomes in early embryos (embryos that contain less than 5 mitotic abnormal chromosomal clusters (abnormal chr.) increased from ~2% to ~9%, while embryos that contain 5 and more mitotic abnormal chromosomal clusters increased from 0 to ~6%). Wild type and mutant embryos (0–2 h) were collected, fixed and stained for DNA (Topro3: blue) and phosphorylated histone H3 (PH3: red). Abnormal wild-type (above) and *dRecQ5* mutant (below) embryos at cycle 13 (prophase/metaphase) are shown on the left. Arrows indicate nuclei with mitotic defects. Scale bar, 50 μM. Statistic analysis is shown on the right. $p < 0.01$, Student's t-test. More than 200 embryos were scored for each category.

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AED, the number of the mutant animals that enter wandering larval stage was about half of that of wild type animals (Fig. 2B). Similarly, the number of adult mutants was also about half of that of the wild type at 212 h AED (Fig. 2C). The observed developmental delay of the *dRecQ5* mutants implies that under the same culture conditions, the mutant animals may encounter more endogenous stress, such as un-repaired DNA damages, than the wild type animals during development. Fig. 2D shows that in early stages of the embryonic development mutant embryos exhibit increased frequency to have abnormal mitotic chromosomes and irregular nuclei as compared with the wild type embryos (see Figure legends for details). These defects are likely reflective of accumulation of endogenous DNA damages as recently reported by Nakayama and colleagues (Nakayama et al., 2009), suggesting that *dRecQ5* mutant animals may be more vulnerable to endogenous DNA damage stress, and consequently exhibit developmental retardation.

dRecQ5 mutants show different sensitivity to double strand breaks (DSBs) generated in different assays

To determine directly whether *dRecQ5* mutants are more sensitive to DNA damages, particularly to double strand breaks, we treated the third instar larvae of wild type and *dRecQ5*^{5} with gamma irradiation at different doses. The treated animals were then allowed to recover and to further develop at normal culture conditions for different periods of time (see the materials and methods and figure legends for details). Our results show that the mutants exhibit a significantly lower survival rate than wild type controls (Fig. 3A), suggesting that *dRecQ5* mutants are more sensitive to DSB-inducing treatment. To further demonstrate this point, we specifically induced endogenous DSBs with I-Sce I cut at the *[wlw]4A* transgene that contains an I-Sce I recognizing sequence (Preston et al., 2006; Wei and Rong, 2007). The expression of I-Sce I enzyme was induced under the control of a ubiquitous promoter (Preston et al., 2006). The ratio of *[wlw]4A Sco/CyO[UIE]* offspring flies (as the DSB-occurring fraction) to offspring *Sp/CyO* flies (as the fraction without DSB) was used as the survival rate (Fig. 3B). In wild type animals, this ratio is almost 100%, indicating that the artificially induced DSBs can be efficiently repaired. However, in the absence of *dRecQ5*, the survival rate dropped to about 60%, demonstrating a compromised DSB repair system in *dRecQ5* mutant animals.

We employed another assay, termed *yellow* reconstitution (Takeuchi et al., 2007), to examine the sensitivity of *dRecQ5* mutant animals to endogenously induced DSBs. As illustrated in Fig. 4A and 4B, a defective *yellow* gene, which consists of only the 5' and 3' segments of the wild type *yellow* gene separated by the I-Sce I cutting sequence, was used for generating DSB. Upon the cut of I-Sce I endonuclease, the broken ends would either find the endogenous *yellow*^{1} homologous sequence to initiate a gap repair, leading to a repaired *yellow* gene with darker (y^{+}) body color (Fig. 4C), or simply be rejoined by non-homologous end joining (NHEJ). The un-repaired broken ends would cause lethality of the flies. Fig. 4E shows that wild type and *dRecQ5* mutants exhibit, surprisingly, similar survival rate (lethality) upon the induction of DSBs (F-R3, 60F5 of the second chromosome) by I-Sce I endonuclease. However, when the DSBs occur on the X chromosome (F1-5, 7E7 of the X chromosome), the survival rate exhibited significant difference in the presence or

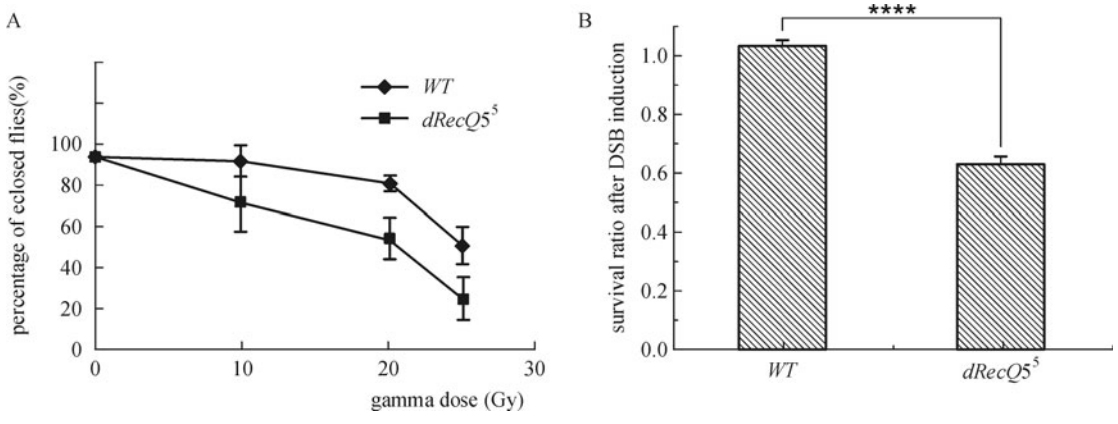


Figure 3. *dRecQ5* mutants are sensitive to agents that cause DSBs. (A) The third instar larvae of wild type and *dRecQ5*^{5} were treated with gamma irradiation at the indicated doses and allowed to recover and further develop at normal culture conditions. The mutants show significantly lower survival rate than the wild type control. (B) The survival rate is significantly reduced for the *dRecQ5*^{5} than the wild type flies upon the endogenous DSBs induction by I-Sce I endonuclease cutting at the *[wlw]4A* transgene (Preston et al., 2006; Wei and Rong, 2007). *[wlw]4A Sco/CyO* females were mated to *Sp/CyO[UIE]* males. *[wlw]4A Sco/CyO[UIE]* flies in the offspring indicate DSB-occurring fraction while *Sp/CyO* flies were scored as the endogenous control. More than 5000 flies in total were scored. Data were analyzed by Student's t test and presented as mean ± SEM with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

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absence of *dRecQ5* (Fig. 4D, columns for total). It is worthwhile to note that, the survival rate difference in males is much more dramatic than in females. A clear difference in the ability to survive the induced DSBs also exists between males and females even in wild type background (Fig. 4D, columns for females and males). Together, the results of

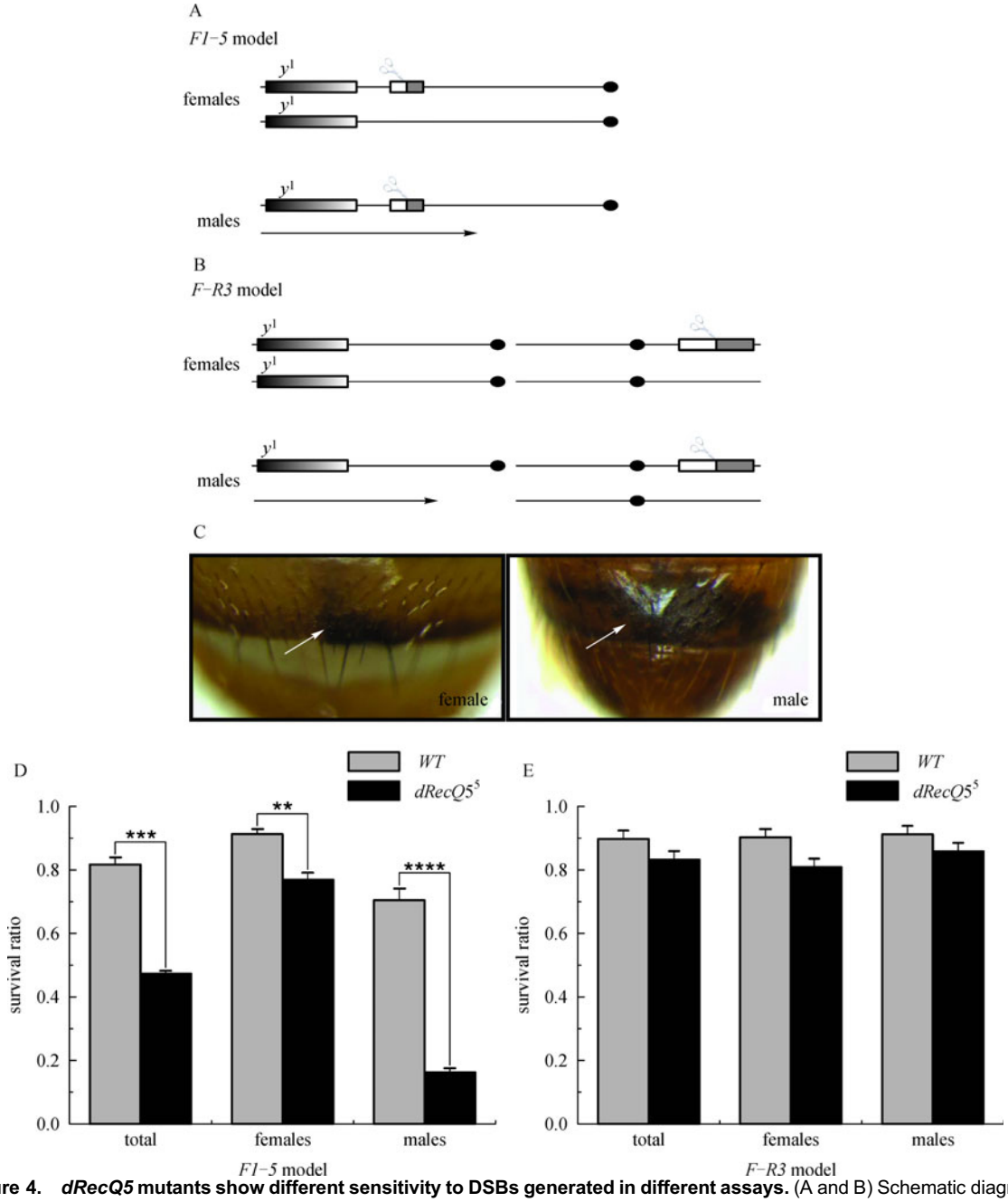


Figure 4. *dRecQ5* mutants show different sensitivity to DSBs generated in different assays. (A and B) Schematic diagrams (not to the scale) of the y^+ reconstitution assays *F1-5* and *F-R3* (Takeuchi et al., 2007) for gap repair. Both *F1-5* and *F-R3* trangenes contain an incomplete yellow gene containing only the 5' and 3' segments that are separated by an I-Sce I cutting site. F1-5 is located at 7E7 on the X chromosome while F-R3 is located at 60F5 on the second chromosome. (C) Reconstituted y^+ somatic clones (arrows) in both females (left) and males (right). (D) Males are more sensitive to I-Sce I endonuclease than females in the absence of *dRecQ5* (*F1-5* assay). (E) Females and males show similar sensitivities to I-Sce I induced DSBs regardless of the presence of *dRecQ5* in *F-R3* assay. *F-R3/CyO* females were mated to *Sp/CyO[UIE]* males in the presence or absence of *dRecQ5*. *F-R3/CyO[UIE]* flies represent the DSB-occurring fraction, and the *Sp/CyO* flies are used as an endogenous control. More than 5000 flies in total were scored. Data were analyzed by Student's t test and presented as mean ± SEM with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

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endogenously induced DSB assays suggest that, depending on where the DSB occurs and what kind of template sequence is available for repair, the induced DSBs may cause different degrees of animal lethality (see discussion).

SSA repair pathway is impaired in the absence of *dRecQ5*

To further investigate the role of *dRecQ5* in DSB repair pathway(s), we took advantage of the well-established *wIw* repair assay that can measure simultaneously the outcome of different DSB repair pathways (Wei and Rong, 2007). As illustrated in Fig. 5A, flies that harbor the *wIw* reporter construct (a complete *mini-white* and a truncated 3' *mini-white* segment separated by a I-Sce I cutting sequence) and the I-Sce I endonuclease transgene may, in principle, have four different repair pathways for the I-Sce I induced DSBs, which can be detected by crossing again these flies with the ones that contain the I-Sce I endonuclease. Inter-sister chromatid GC and perfect non-homologous end joining (NHEJ without any errors) will reconstitute the intact *wIw* construct that can be re-cut by I-Sce I endonuclease in the somatic cells, leading to mosaic eyes after different DSB repair in different cells (Fig. 5A). Imperfect NHEJ destroys the I-Sce I cutting site making the repaired *wIw* construct uncuttable by I-Sce I in

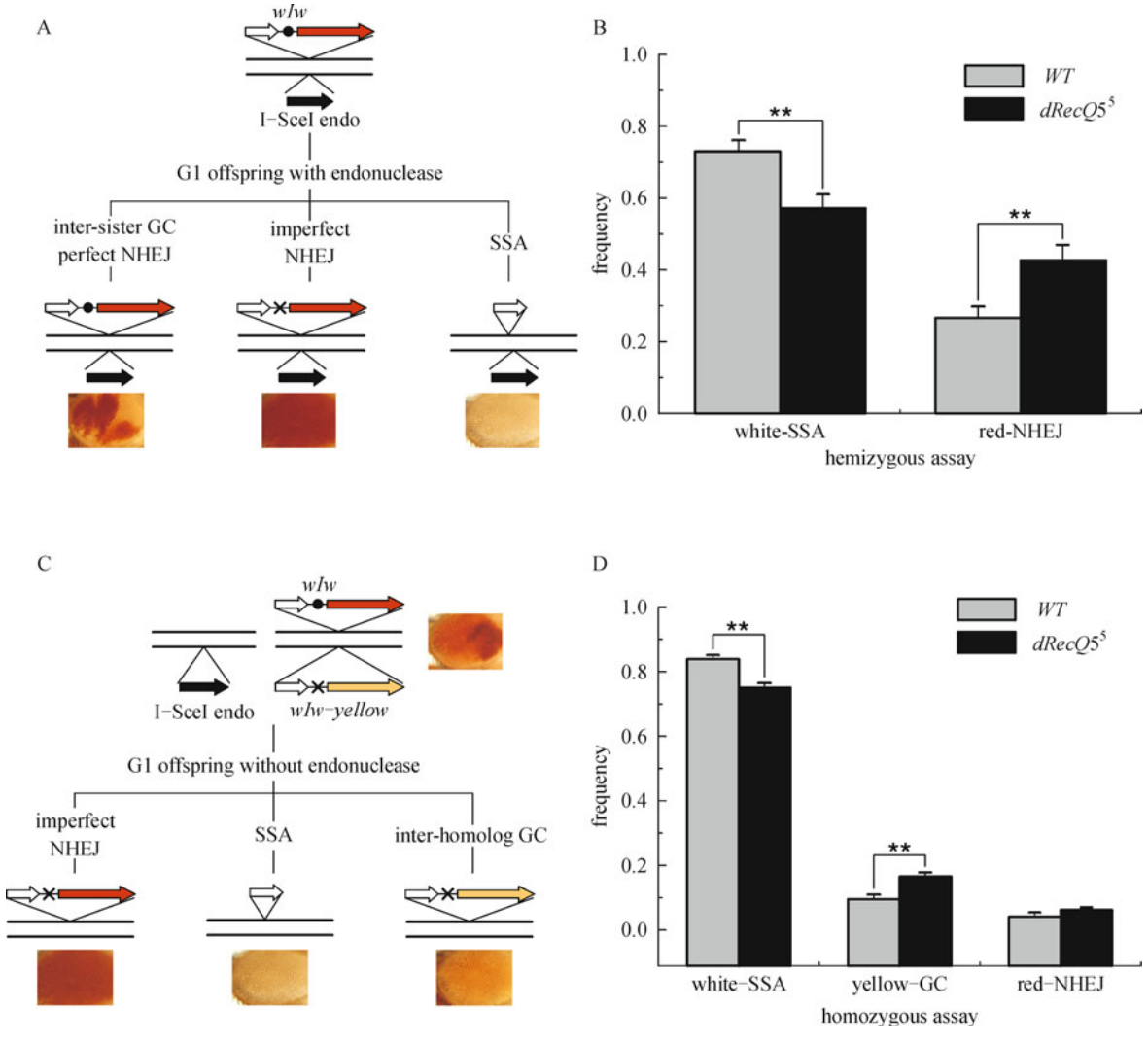


Figure 5. *dRecQ5* mutation impairs SSA mediated DSB repair. (A) Schematic illustration of the hemizygous assay. SSA and NHEJ frequencies in WT and *dRecQ5* mutant backgrounds are shown in B. (C) Schematic illustration of the homozygous assay. The *wIw* insertion contains a part of the 3' portion of w^+ (to the left of the I-Sce I site) and a copy of the functional *mini-white*. G0 generation that carries *wIw* and UIE exhibits mosaic eyes. Among the G1 offspring, three kinds of eyes represent the results of the indicated repair pathways. × represents a mutated I-Sce I site due to imperfect NHEJ. (D) SSA, inter-homolog GC and NHEJ frequencies in WT and *dRecQ5* mutant background are shown. More than 200 flies were scored for each genotype. Data were analyzed by Student's t test and presented as mean ± SEM with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

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somatic cells, thus producing red-eyed flies (Fig. 5A). The direct repeats of the 3' segments of the *mini-white* gene flanking the I-Sce I cutting sequence will lead to SSA repair upon the action of the I-Sce I endonuclease, which results in the loss of the intact *white* gene, producing white-eyed flies (Fig. 5A). Fig. 5B shows that the efficiency of SSA in *dRecQ5* animals was reduced by about 16% when compared with wild type animals, suggesting a role of *dRecQ5* in the SSA repair pathway. Along with the reduction of SSA repair, an increase in imperfect NHEJ was observed (Fig. 5B), suggesting that a compensatory repair is triggered in the *dRecQ5* mutant background.

GC is preferred over non-homologous end joining (NHEJ) for flies to repair DSBs in the absence of *dRecQ5*

In order to examine how GC mediated DSB repair is affected by *dRecQ5* mutation, a modified *wIw* system, termed as *[wIw]yellow*, was employed (Wei and Rong, 2007). As explained in Fig. 5C, in addition to the *wIw* and I-Sce I transgenes, another construct, *[wIw]yellow*, was implanted in the same flies, which can provide a gene conversion template for the broken *wIw* construct. However, there is no I-Sce I cutting sequence in *[wIw]yellow*, in which the *mini-white* gene contains a point mutation that makes the red pigmentation significantly weaker than the wild type *mini-white* gene, thus creating orange-eyed flies when present in the fly genome. The outcome of three major repair pathways after such flies are crossed to *y w* flies is shown in Fig. 5C. In addition to imperfect NHEJ and SSA, which appear also in the *wIw* assay, GC through homologous recombination between homologous chromosomes will give yellow-eyed offspring (Fig. 5C). Fig. 5D shows that in the *[wIw]yellow* assay, similar to the result from *wIw* assay, SSA is reduced in the absence of *dRecQ5* compared with wild type. Unlike in the *wIw* assay, the imperfect NHEJ repair frequency remains unchanged regardless of the presence or absence of *dRecQ5* function. However, the gene conversion between the homologous chromosome-mediated repair increased significantly in the absence of *dRecQ5* (Fig. 5D), suggesting that these flies prefer to use GC than NHEJ to repair DSBs when there is homologous template available.

***dRecQ5* mutation leads to increased loss of heterozygosity (LOH)**

To determine whether *dRecQ5* participates in the formation of crossover between homologous chromosomes, which is one of the driving forces for loss of heterozygosity and genome instability (Saunders et al., 2008) (Fig. 6A and 6D), we took advantage of two assays for LOH detection. The first assay involves mhw^1 mutation, homozygotes of which show extra hairs on the wing (Fig. 6B). Somatic clones of mhw^1 may occur in the heterozygous animals if spontaneous homologous recombination happens between the two homologous chromosomes, leading to the loss of heterozygosity due to crossovers (Fig. 6A). In the absence of *dRecQ5*, the frequency of mhw^1 clone occurrence increased significantly compared with wild type (Fig. 6C). In the second assay of LOH detection, w^+ was used as a marker to monitor the loss of the wild type allele in the presence or absence of *dRecQ5* (Fig. 6D). Fig. 6D shows that in *dRecQ5* mutant background, in all the testing tubes (7 out of 7) mosaic eyed flies were observed while in wild type flies, no mosaic eyed flies (LOH) were found (0 out of 7). These results suggest that *dRecQ5* may be important for suppressing crossovers during homologous recombination, thus maintain the heterozygosity and genome stability.

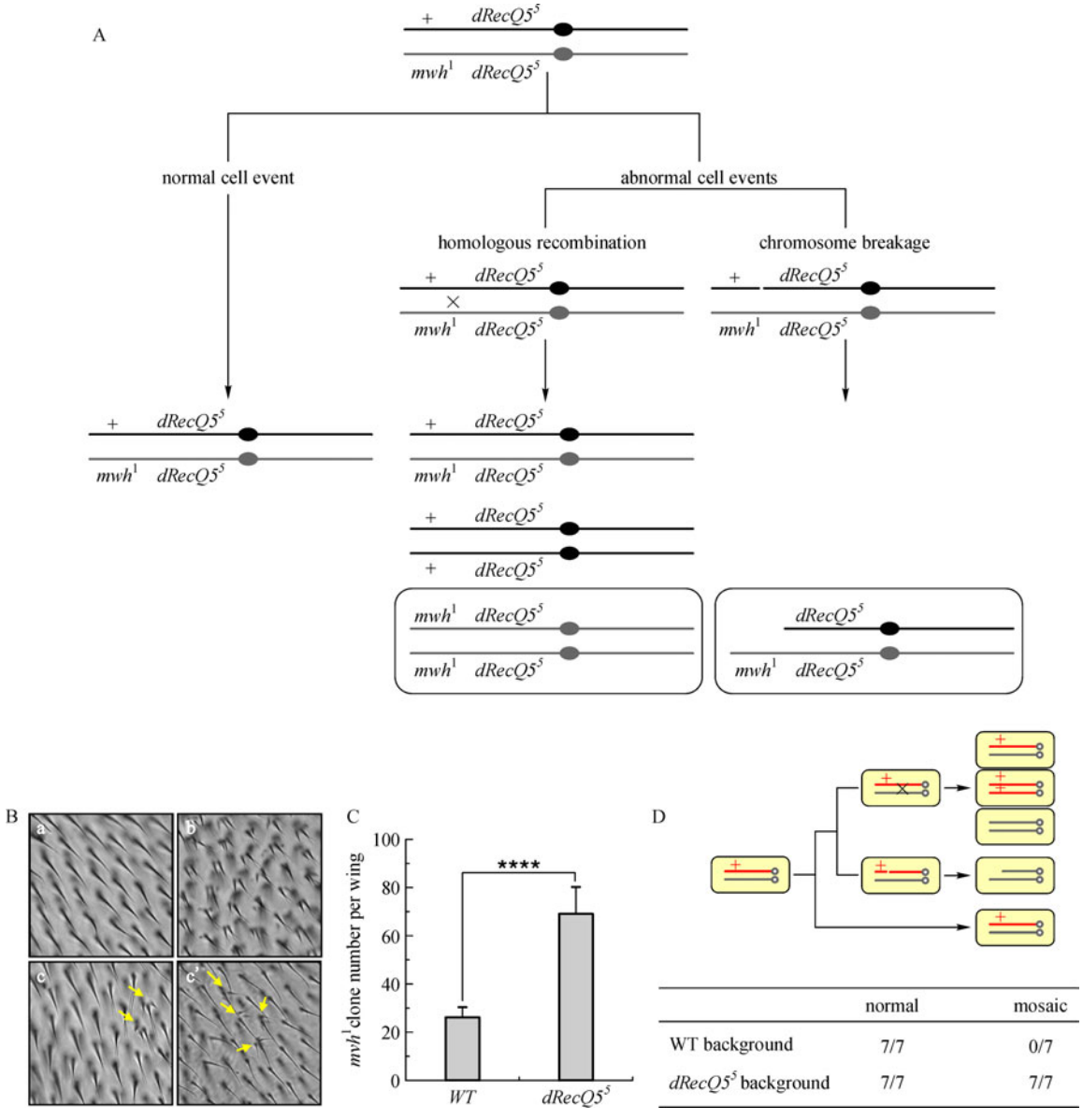
DISCUSSION

In this study we show that *Drosophila RecQ5* has a role in SSA repair upon the stress of induced endogenous DSBs by I-Sce I endonuclease. However, unlike the other *Drosophila* RecQ members, *dRecQ5* is neither essential for the animal development and cell survival as *dRecQ4* is ((Wu et al., 2008; Xu et al., 2009) and this study), nor required for animal fertility as *dBLM* is ((Kusano et al., 2001; Adams et al., 2003; Johnson-Schlitz and Engels, 2006) and this study). Therefore, the *dRecQ5* gene is a functionally divergent RecQ family member in *Drosophila* compared with other RecQ members.

SSA has been shown to be the most frequently used pathway for DSB repair in *Drosophila* (Preston et al., 2006; Wei and Rong, 2007). It is thought that one of the functions of SSA *in vivo* is to reduce any unintentionally duplicated DNA sequence to the original copy in the genome. In *Drosophila* a natural substrate for SSA may come from the hybrid element insertion (HEI) process: a P-element located on one sister chromatid can recombine with a nearby locus on the homologous chromosome, resulting in a deletion and a duplication product. The generated duplication becomes prone to undergoing SSA to preserve the original genome sequence so as to maintain genomic stability. Until now, the factors that are involved in this process have remained unclear in *Drosophila*. Those that are involved in SSA repair in mammalian cells, such as Rad59, Rad52, MSH2 and MSH3, are mostly absent in the *Drosophila* genome (Wei and Rong, 2007; Kappeler et al., 2008); only the MSH2 ortholog, *spel1*, exists in *Drosophila*. In our current study we show that *dRecQ5* mutation affects SSA repair, suggesting that dRecQ5 plays at least a modulator role in the process of repairing artificially induced DSBs. In the absence of *dRecQ5* the SSA repair frequency decreases in both *wIw* and *[wIw]yellow* assays. The *wIw* assay offers no homologous template for gene conversion, in which NHEJ increases but SSA decreases in *dRecQ5* mutants (Fig. 5B). However, once there is homologous sequence available for gene conversion repair, as shown in the *[wIw]yellow* assay, GC increases while

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NHEJ remains unaffected in *dRecQ5* mutants (Fig. 5D). These results demonstrate that: (1) error-free (GC) pathway is a preferred mechanism to repair DSBs in flies over the error-prone (NHEJ) pathway when dRecQ5 function is absent; (2) Gene conversion and non-homologous end joining are not impaired by *dRecQ5* loss-of-function. The mechanism of how



	normal	mosaic
WT background	7/7	0/7
$dRecQ5^5$ background	7/7	7/7

Figure 6. ***dRecQ5* mutants exhibit increased loss of heterozygosity (LOH).** (A) The genetic basis of the LOH assay with mwh^1 as a marker. In the heterozygous *mwh* animals, when the wild type copy of mwh^1 is lost during the proliferation of wing cell precursors, the resulting clone will exhibit the recessive *mwh* phenotype (multiple-wing hair). (B) *dRecQ5* mutation leads to an increase in the number of mwh^1 mutant clones (arrows) resulting from homologous recombination. (a) Individual wing hairs in an animal heterozygous for mwh^1. Each hair follicle has one hair. (b) In an animal homozygous for mwh^1, each hair follicle has two or more hairs. (c and c') In an animal heterozygous for mwh^1 and homozygous for *dRecQ5* mutation, individual cells that lost the wild-type *mwh* gene due to homologous recombination show two or more hairs (arrows). (C) The frequency of mwh^1 clones per wing in $dRecQ5^5$ animals are significantly increased than in wild type. Nine wings for control and seven wings for $dRecQ5^5$ mutants were scored for mwh^1 clones. Data were analyzed by Student's t test and presented as mean ± SEM with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. (D) The genetic basis of the LOH assay with w^+ as a marker (above). The numbers show the frequency of progenies with mosaic eyes. In wild type background, no flies with mosaic eyes were observed while in $dRecQ5^5$ background mosaic flies appeared in all tubes tested.

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dRecQ5 may participate in the process of SSA repair will require future investigations. One plausible possibility is that the strand annealing activity of RecQ5 (Ozsoy et al., 2001) may simply facilitate the annealing of the 3' ends of the direct repeats after resection of an induced DSB. However, dBLM is also reported to have such a strand annealing activity, and in the absence of *dBLM* there exists a general shift of homologous recombination (HR-h, homology between homologous chromosomes) to SSA (Kappeler et al., 2008), which might suggest that strand annealing activity is not a key factor that determines the usage of SSA repair. Hu et al. (Hu et al., 2005) reported that RecQ5 and BLM have non-redundant roles in suppressing crossovers in mammalian cells. It remains to be determined how the induced DSBs may be repaired in the absence of both *dBLM* and *dRecQ5*. Unfortunately, we were unable to construct a viable double mutant fly stock of *dBLM* and *dRecQ5* possibly due to a greater accumulation of un-repaired DNA damages.

Developmental defects caused by *dRecQ5* mutation are evident in our study and also in a recent report by Nakayama et al. (Nakayama et al., 2009) in which the authors generated null alleles of *dRecQ5* via P element jump-out, $recq5^{D1}$ and $recq5^{D2}$, both of which show spontaneous mitotic defects and chromosomal aberrations. The chromosomal aberrations and cell cycle changes are likely to be the cause of developmental retardation (Fig. 2). This hypothesis is supported by the observation that induced DSBs by I-Sce I endonuclease cause severe lethality when un-repaired in both the F1-5 assay and *wlw* assay (Fig. 3C and Fig. 4D). Similar to *dRecQ5*, it has been reported that mutations in genes important for DNA damage repair and other related cellular processes can cause developmental defects (Brodsky et al., 2000; Xu et al., 2009).

In the F-R3 assay, the survival rate remains high both in wild type and *dRecQ5* mutant animals, suggesting that the artificially induced DSBs by the cutting of I-Sce I enzyme can be efficiently repaired by either SDSA-mediated gap repair (Takeuchi et al., 2007) or non-homologous end joining regardless of the presence or absence of *dRecQ5* function. However, in similar assays of F1-5, the survival rate (or DSB repair efficiency) is reduced by a loss of *dRecQ5*, with a more pronounced effect in males than in females (Fig. 4D). The F1-5 and F-R3 reporter constructs differ from each other in both the length of the homologous sequences that flank the I-SceI site (Takeuchi et al., 2007) and their chromosomal locations (F1-5 is located at 7E7 of the X chromosome while F-R3 is at 60F5 of the second chromosome). These differences may contribute to the preference of pathways that will be used for the cells to repair the DSBs (Preston et al., 2006; Wei and Rong, 2007). This view is supported by the observation that in the *[wlw]4A* assay, the animal survival rate also drops significantly in the presence of DSB induction (Fig. 3B), because the *[wlw]4A* construct, which is located on the second chromosome, unlike F-R3, offers homologous sequence for SSA repair in addition to NHEJ as shown in Fig. 5A. Furthermore, in F1-5 assay, the survival rate is different in males and females after DSB induction even in wild type background (Fig. 4D), may also suggest that successful DSB repair depends not only on the key repair machinery but also on the availability and type of homologous sequence and the location of the DSB at a given developmental stage (Preston et al., 2006; Wei and Rong, 2007).

In the current study, we used two assays, the *mwh* assay and the loss of w^+ assay, to monitor the loss of LOH in *Drosophila*. Our results show that in the absence of *dRecQ5* leads to a significant increase in LOHs (Fig. 6). First, this result is similar to what has been recently reported for *dWRNexo* (Saunders et al., 2008), suggesting that *dRecQ5* and *dWRNexo* are not functionally redundant in the process of homologous recombination. Secondly, deletion of RecQ5 does not lead to obvious increase of LOH in mammalian cells although it suppresses homologous recombination by interrupting Rad51 presynaptic filaments (Hu et al., 2007). Therefore, our finding may suggest that the functions of RecQ5 are not well conserved between flies and mammals. However, the precise mechanism of how *dRecQ5* controls LOHs remains to be elucidated. We examined Rad51 responses to induced DSBs in the presence (wild type) and absence (*dRecQ5* mutants) of *dRecQ5* function, no significant difference was detected as judged by Rad51 immunostainings (data not shown).

MATERIALS AND METHODS

Fly stocks and genetics

Flies were reared at 25°C on medium containing cornmeal, soybean, yeast, agar, syrup and molasses according to standard protocols. The fly stock of *Sp/CyO P[UIE] 53D* was kindly provided by Dr. William R. Engels (Preston et al., 2006); the transgenic flies of *F1-5* and *F-R3* were kindly provided by Dr. Dieter Egli (Takeuchi et al., 2007); the fly stocks of *[wlw]4A Sco/CyO, Sco/CyO;[wlw]2, [wlw]yellow Sb/TM3, Ser* were generous gifts from Dr. Yikang. Rong (Wei and Rong, 2007). The rest of the fly stocks used in this study were from the Bloomington Drosophila Stock Center except for the *dRecQ5* mutant allele (see below).

Generation of the anti-dRecQ5 antibody

The rabbit polyclonal antisera against dRecQ5 protein were raised using a recombinant polypeptide containing 106 amino acids of dRecQ5, ranging from a.a. 359 to a.a. 464 (YYGREDVRSIRFLLQNDAHRARGRGDKELLTERAIKQFEKITEFCERTTCRHKLFSDFFGDPTPDCSGQCDVCKRPKKAEKALEIFHRLCMDDAFKSHISLQDCAD). The corresponding cDNA fragment was first cloned into pET28b expression vector fused to a hexahistidine tag. Subsequently, the polypeptide expression and purification were carried out using the Ni-NTA resin (Qiagen) method following the manufacturer's instructions. The antibody was affinity-purified with protein A.

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Generation and characterization of *dRecQ5* mutants

A ~4.9 kb *dRecQ5* genomic fragment (coding region of the gene) and a ~3 kb 5' regulatory sequences with intended modifications of ATG site were cloned in the *pTARG* vector (Egli et al., 2006) to make the gene targeting donor construct, *pTARG-dRecQ5*. Mutations of the ATG were introduced by PCR with the following oligos (changed bases are in italic) that were used for genomic DNA amplification. The primers used to amplify the 4.9 kb *dRecQ5* genomic fragment were 5'-*ataataagGCGGCCGCA*TGAAAGCGCTGTGCAC-3' and 5'-*tccCCGCGG*ATATGATCAAGATTGTGGAAC-3'. The primers for amplifying the upstream 3 kb fragment were 5'-*cgACGCGT*G-GAACTCGAGAACTGACCACTC-3' and 5'-*ataagaatGCGGCCG-C*ACTTGGGCTGTTATTTAA-3'. Alteration of ATGGC to GCGGCC generates a Not I restriction site (Fig. 1). Oligos used to introduce the I-Sce I cleavage sequence that are inserted at the Afl II cutting site within the 4.9 kb fragment were 5'-TTAATAGGGATAACAGGGTAAT-3'and 5'-TTAAATTACCCTGTTATCCCTA-3' (the underlined nucleotides indicate cleaved Afl II).

For generation of the *dRecQ5* mutant, we used the ends-in gene targeting method (Rong et al., 2002; Egli et al., 2003). The flow chart of the targeting process is shown in Fig. 1A. Donor transgenic flies that bear the targeting construct on the second chromosome were crossed to flies that contain *hs-I-Sce I* and *hs-FLP* transgenes. Three rounds of heat shock (38°C, 1 h each) were applied on days 2, 3 and 4 after egg-laying. Heat-shocked virgins were singly crossed to *y w; ey-FLP; MKRS/TM2,* y^+ males, and females were screened for targeted integration of targeting construct indicated by the w^+ marker. Reduction of two *dRecQ5* copies (one wild type and one mutant copy) by I-Cre I was performed by crossing the targeted alleles to w^{1118}; *hs-I-Cre I, Sb/TM6*. The offspring were given a single heat shock (36°C, 1 h) at the third instar larval stage. w^- males were crossed individually to *y w*; *Sp/CyO; MKRS/TM2,* y^+ to establish stocks. A specific pair of primers that was used to check mutations at the ATG site by Not I digestion of the PCR products is as follows: 5'-CGCTTATAGGCGAGATGAATG-3' and 5'-TATACGATTCCG-CAGCCTCT-3'. Not I digestion of the PCR products yields two fragments of 245 bp and 846 bp when the designed mutations appear, and one band of 1091 bp for wild type flies. The allele, we designated $dRecQ5^5$, was further confirmed by DNA sequencing for the designed mutations, and further analyzed throughout this study.

For dRecQ5 protein detection in both wild type and mutant flies, 0–4 h embryos of each genotype were collected and homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, 0.5% Sodium deoxycholate, 1 mM PMSF) in the presence of a protease inhibitor cocktail. 2×SDS buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 2% SDS, 0.1% bromophenol blue, 20% 2-mercaptoethanol) was added to the extracts. Then the samples were boiled for 5 min, and spun at max-speed at room temperature for 5 min. The supernatants were applied for SDS-PAGE before transferred to PVDF membrane. The membranes were blocked for 1 h at room temperature and incubated with polyclonal dRecQ5 antibody (1:1000) overnight at 4°C. Horse-radish peroxidase-linked anti-rabbit IgG (1:3000) and Super Signal West Pico Trial Kit were used for signal detection according to standard manufacturer's instructions (Thermo Scientific, ECL-Kit).

Physiological measurement assays

The adult flies of *y w* and $dRecQ5^5$ were used to collect embryos for 30 min, and embryos were raised on agar plate containing agar/sucrose/apple juice/H_2O (2.3g/2.5g/25mL/77mL). After 24 h, the hatched first instar larvae were transferred to the standard food containing cornmeal, soybean, yeast, agar, syrup and molasses. Subsequently, 125 first instar larvae of each genotype were selected as a group to assay for the developmental stages.

DSB sensitivity assays

For gamma irradiation sensitivity tests, third instar larvae of both wild type and *dRecQ5* mutants were treated with different doses of γ-irradiation (0, 10, 20, 25 Gy) at a rate of 0.5 Gy/min using ^{60}Co source. The irradiated larvae were transferred to a new food bottle. 100 irradiated larvae were placed in one bottle, and four parallel bottles were set out for each genotype. The number of eclosed flies of each bottle was scored and analyzed. For detection of the sensitivity of $dRecQ5^5$ to endogenously induced DSB damage by the endonuclease I-Sce I, 40 females of *y w; Sp/CyO[UIE]* in the presence or absence of *dRecQ5* mutation were mated to 10 *w*; *[wIw]4A Sco/CyO* males with or without *dRecQ5* mutation respectively, and the progenies of different crosses were scored and analyzed for endogenous DSB sensitivity. For the F1-5 and F-R3 assays, females of *y w F1-5;* $dRecQ5^-$ (or *y w F1-5; +/+*) and *y w; F-R3/CyO;* $dRecQ5^-$ (or *y w; F-R3/CyO; +/+*) were mated to *y w; Sp/CyO[UIE];* $dRecQ5^-$ (or *y w; Sp/CyO[UIE]; +/+*) males respectively. *F1-5/+; +/CyO[UIE]* and *F-R3/CyO[UIE]* flies represent the DSB-occurring fractions, and those flies without UIE were scored as the endogenous control independently. Survival rate was calculated as the ration of DSB-occurring fraction to the endogenous control fraction. For each experiment, six repeats were carried out. Data were analyzed by Student's t test and presented as mean ± SEM with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Genome instability assay

The *mwh (multiple wing hairs)* assay (Saunders et al., 2008) was used to examine the genome stability of *dRecQ5* mutant flies. See Fig. 6A for the principle of this experiment. For the detection of *mwh* loss, fly wings with correct genotypes were dehydrated with isopropanol and mounted in media containing 1:1 methylsalicilate and Canada balsam (Sigma). All intervein wing hair cells were examined for the mwh^1 phenotype. Five to ten wings were examined for each genotype. 15 Gy of γ-irradiation was applied to make a genomic instability stress.

***In vivo* DSB repair assays**

The *wIw* reporter system for assaying *in vivo* DSB repair has been described previously (Rong and Golic, 2003; Wei and Rong, 2007). The flies used in this study were *[wIw]4A* on chromosome 2 and *[wIw]2* on chromosome 3 respectively. The line *[wIw]yellow* were derived from *[wIw]2* by imperfect NHEJ (Wei and Rong, 2007) and used in combination with *[wIw]2* in the homozygous assays (see Fig. 5E for schematic presentation). Another transgenic line *y w; Sp/CyO[UIE]* was used to produce I-Sce I endonuclease to generated specific DSBs, *[UIE]* is the short-form for *Ubiq::I-Sce I* (Preston et al., 2006). Details of crossing procedure are as follows: for the hemizygous assay, *y w; Sp/CyO[UIE] (;*$dRecQ5^5$*)* virgins were crossed to *w; [wIw]4A Sco/CyO (;*$dRecQ5^5$*)* males, followed by selecting *y w/Y; [wIw]4A Sco/CyO[UIE] (;*$dRecQ5^5$*)* males to cross

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with *y w; Sp/CyO[UIE] (;dRecQ5^5)* virgins. *Sco, Sp* progeny were scored for different repair products. For the homozygous assay, virgins of *y w; Sp/CyO[UIE] (;dRecQ5^5)* were crossed to *w; [wIw]yellow Sb/TM, Ser (dRecQ5^5)* males, followed by selecting *y w/Y; +/CyO[UIE]; [wIw]yellow Sb/+ (dRecQ5^5)* males to cross with *w; Sco/CyO; [wIw]2 (dRecQ5^5)* virgins, from the offspring of which *w/Y; Sco/CyO[UIE]; [wIw]yellow Sb/[wIw]2 (dRecQ5^5)* males were picked up to cross with y w virgins. *Sco, CyO$^+$, Sb$^+$* progeny were scored for different repair products.

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ABBREVIATIONS

AED, after egg deposition; DSB, double strand break; GC, gene conversion; HEI, hybrid element insertion; HR, homologous repair; HR-h, homologous recombination between homologous chromosomes; DHJ, double holiday junction; LOH, loss of heterozygosity; NHEJ, non-homologous end joining; SDSA, synthesis dependent strand annealing; SSA, single strand annealing

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