Drosophila brca2 Is Required for Mitotic and Meiotic DNA Repair and Efficient Activation of the Meiotic Recombination Checkpoint

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Heterozygous mutations in the tumor suppressor BRCA2 confer a high risk of breast and other cancers in humans. BRCA2 maintains genome stability in part through the regulation of Rad51-dependent homologous recombination. Much about its precise function in the DNA damage responses is, however, not yet known. We have made null mutations in the Drosophila homolog of BRCA2 and measured the levels of homologous recombination, non-homologous end-joining, and single-strand annealing in the pre-meiotic germline of Drosophila males. We show that repair by homologous recombination is dramatically decreased in Drosophila brca2 mutants. Instead, large flanking deletions are formed, and repair by the non-conservative single-strand annealing pathway predominates. We further show that during meiosis, Drosophila Brca2 has a dual role in the repair of meiotic double-stranded breaks and the efficient activation of the meiotic recombination checkpoint. The eggshell patterning defects that result from activation of the meiotic recombination checkpoint in other meiotic DNA repair mutants can be strongly suppressed by mutations in brca2. In addition, Brca2 co-immunoprecipitates with the checkpoint protein Rad9, suggesting a direct role for Brca2 in the transcription of the meiotic recombination checkpoint signal.

Introduction

The genomic stability of eukaryotic cells is constantly challenged by exogenous and endogenous stresses that can lead to the loss or alteration of genetic material. Genomic stability is maintained through robust DNA repair and checkpoint pathways that are tightly coordinated with each other and the developmental cell cycle progression of the organism. For example, mutations in meiotic DNA repair enzymes in Drosophila cause defects in the cell cycle and developmental progression of the egg due to a failure to repair meiotic recombination intermediates [1]. The tumor suppressor and breast cancer susceptibility gene, BRCA2, has been implicated in playing a central role in maintaining genomic stability, but the extent to which BRCA2 is involved in the coordination of DNA repair, checkpoints, and developmental progression remains to be determined.

Murine cells depleted for BRCA2 spontaneously accumulate broken chromosomes and chromatids, triradial and quadriradial structures, and gross chromosomal rearrangements [2,3]. A key function of BRCA2 is the regulation of the Rad51 recombinase during DNA repair by homologous recombination (HR). During HR, Rad51 assembles into a nucleoprotein filament with single-stranded DNA at the site of a double-stranded break (DSB) in order to initiate strand invasion of the homologous chromosome [4]. Recent structural studies have illuminated how BRCA2 regulates Rad51 [5–7]. BRCA2 contains two regions that mediate binding to Rad51: a stretch of 8 repeated short motifs termed the BRC domain of BRCA2. The role of BRCA2 in homologous recombination is likely critical for its role as a tumor suppressor, but BRCA2 is a large protein with many binding partners and it is likely to play multiple roles in safeguarding genomic stability.

Several requirements for BRCA2 outside of homologous recombination have been suggested by protein interaction and cell culture studies, but these functions are far less understood. Most notably, BRCA2 has been implicated in two S-phase checkpoints: the intra-S phase checkpoint and the replication checkpoint. During the intra-S phase checkpoint irradiation-induced lesions outside of the replication fork cause partial depression of replication [8]. The replication...
Author Summary

Breast cancer can arise due to inherited mutations in a few well-defined breast cancer susceptibility genes. BRCA2 is one of two known human genes in which common mutations are associated with high breast cancer risk. A known function of BRCA2 is the repair of damaged DNA using the homologous recombination repair pathway. In the absence of BRCA2, damaged DNA is repaired by error-prone repair. However, as many other genes required for homologous recombination are not known breast cancer susceptibility genes, BRCA2 likely has additional roles in tumor suppression. In this study, we created fruit flies mutant for the homolog of BRCA2. We show that, like in humans, fruit fly Brca2 is required for DNA repair by homologous recombination. Additionally, we use this new animal model to discover a previously unknown role for Brca2 in the production of a signal that tells cells that damaged DNA is present during meiosis. This “checkpoint” signal may allow the cells to coordinate a protective response after DNA damage.

checkpoint stabilizes replicationforks and decreases replication levels in response to lesions at the replication fork. Requirements for BRCA2 in replication fork stabilization after hydroxyurea treatment and in suppressing radiosensitive replication have also been described [9,10]. The G1/S, S/M, and G2/M checkpoints have been found to be largely intact in BRCA2 null mutants. However, these studies have used hypomorphic mutations that preserve half or more of the N-terminal region of BRCA2 due to proliferative defects of BRCA2 null mutants [3,10]. As modest to severe increases in breast cancer susceptibility have been associated with mutations in other checkpoint genes [11], further elucidation of the mechanism of the role of BRCA2 in checkpoints is needed.

Drosophila has emerged as a useful model for studying DNA repair and checkpoint control of genome stability. The DNA repair and checkpoint pathways are remarkably well conserved between flies and higher organisms [12]. Notably, though the function of many of these genes is well conserved, null mutants in Drosophila are sometimes viable in cases in which null mutations in higher organisms result in lethality and complicate mammalian developmental studies [13,14]. We made null mutations in the Drosophila homolog of BRCA2 (CG30169), and show that unlike in mammals, Drosophila brca2 null mutants are viable. We present detailed descriptions of DSB repair pathway balance and irradiation-induced checkpoint function in animals genetically null for brca2. CG30169 represents a functional BRCA2 homolog required for DSB repair in mitotic and meiotic tissues. Additionally, we uncover a novel role for brca2 in the meiotic recombination checkpoint. Finally we show that Brca2 co-immunoprecipitates with the checkpoint protein Rad9, suggesting a mechanism for the role of Brca2 in checkpoint control.

Results

Null Mutations in the Predicted Drosophila BRCA2 Homolog, CG30169, Are Viable

Poor sequence conservation of BRCA2 homologs outside of the BRC repeats and DNA binding domains initially hampered the identification and study of BRCA2 in lower model organisms. However, by using a sequence motif that describes the BRC repeats, Lo et al. (2003) identified putative homologs across many species [15], including some that have since been verified to be functional homologs [16,17]. One gene in Drosophila, CG30169, was predicted to encode BRC repeats. CG30169 encodes a 109 kDa protein containing 3 putative BRC repeats and 4 Nuclear Localization Sequences. Notably, while Lo et al. (2003) predicted DNA binding domains in vertebrate, plant, and some lower eukaryotes, they did not find evidence of a DNA binding domain in CG30169 by sequence analysis. Therefore it was not self-evident that CG30169 encodes a functional BRCA2 homolog, since the expression of isolated repeats is known to dominantly inhibit Rad51-dependent DSB repair, but fusions of a BRCA repeat to Replication Protein A restore DNA binding and are consequently able to rescue the repair defects of BRCA2 mutants [18–20].

In order to determine if CG30169 encodes a functional BRCA2 homolog and examine its role in the DNA damage responses, we made null mutations in CG30169, brca2KO, was created by imprecise excision of the P element P(Supor-P)CG30169KO, which is inserted in the 5’UTR of CG30169, brca2KO removes the majority of the coding sequence of CG30169 and a portion of the 5’UTR of the neighboring gene CG4612, brca2KO was created by ends-out homologous recombination [21], and the resulting chromosome is completely deleted for CG30169 but contains fully intact neighboring genes. We found that homozygous brca2KO, brca2KO, and transheterozygous mutant combinations are viable but recessive female sterile.

The Drosophila BRCA2 Homolog Is Required for Homologous Recombination Repair

The best characterized role of BRCA2 is in the regulation of the recombinase Rad51, which has been shown to be required for DNA repair in Drosophila [14]. In order to determine if CG30169 represented a functional BRCA2 homolog we first tested for a role in somatic DNA repair by assaying sensitivities to the mutagens, methyl methanesulfonate (MMS) and x-rays. X-ray irradiation (IR) results in a range of DNA damage including a large number of DSBs throughout the cell cycle, while MMS leads to replication fork collapse and DSB formation specifically during S phase. We found that similar to rad51spnA and rad54okra mutants (Tables 1 and 2), brca2 mutants are highly sensitive to both MMS and IR. Flies heterozygous for either brca2 allele were crossed together, the larval progeny were exposed to mutagen, and the percent of homozygous mutant flies that survive to adulthood among all surviving adults was determined. brca2 mutants showed complete to near-complete lethality after a 1250 Rad IR exposure and 3–13-fold decrease in survivorship after exposure to 500 Rads of irradiation (Table 1). Exposure to 0.08% MMS resulted in a 5–24-fold decrease in survivorship of brca2 mutants (Table 2). Flies doubly mutant for okra and brca2 showed mutagen sensitivities comparable to the single mutants alone, suggesting the two genes act in the same pathway. The similar mutagen sensitivities between CG30169, okra, and spnA mutants suggest that CG30169 may be a functional BRCA2 homolog involved in DSB repair.

Several pathways are responsible for the repair of DSBs and the choice between these pathways is a highly regulated process influenced by factors such as the nature of the break or the phase of the cell cycle [22,23]. Three major pathways known to repair DSBs are: non-homologous end-joining (NHEJ), homologous recombination (HR), and single-strand annealing (SSA) [4]. In NHEJ, the DNA ends flanking the...
Table 1. Irradiation Sensitivity of brca2 Mutants

| Genotype         | 0 Rads | 500 Rads | 1250 Rads | n  |
|------------------|--------|----------|-----------|----|
| brca2+/+         | 35.5 (3.4) | 2.7 (0.9) | 0 (0) | 540, 295, 676 |
| brca2+/−         | 28.6 (2.1) | 10.1 (2.1) | 0.3 (0.3) | 2215, 1893, 1537 |
| brca2+/−; I-SceI  | 49.1 (0.9) | 48.1 (2.2) | 52.2 (1.0) | 2757, 2156, 2720 |
| okrAA/RR+        | 33.7 (0.6) | 9.3 (2.3) | 0 (0) | 1495, 616, 1339 |
| brca2+/−; okrAA/RR+ | 34.0 (1.7) | 3.1 (1.0) | 0 (0) | 2105, 1521, 598 |

Percent of homozygous mutant progeny among all surviving progeny. Heterozygous parents were mated and the surviving adult progeny were counted. All mutant chromosomes were balanced over CyO. Standard errors between experiments are indicated in parentheses and n indicates the total number of progeny counted. The mutant progeny were expected to constitute 33.3% of the progeny except for the brca2+/−; I-SceI progeny which were expected to comprise 50.0% of the progeny.

We measured two additional parameters of DSB repair. First, we monitored the extent of gene conversion to the left of the break by determining if the repaired chromosome included a 16 bp deletion 156 bp away from the DSB that was specific to the parental EJ1 chromosome. We designate repaired chromosomes containing the 16 bp deletion as being repaired by long-tract HR-h. Long-tract HR-h repair likely represents repair in which there is increased gene conversion due to increased DNA synthesis rates following strand invasion or due to increased resection prior to DNA synthesis. Alternatively, changes in the repair of the heteroduplex DNA could also influence the balance of repair products observed. Second, large deletions to the left of the DSB result in progeny with white eyes due to the loss of the white gene carried on the Rr3 transgene. We performed the Rr3 assay with and without the EJ1 chromosome in order to measure the repair products when repair by HR-h is available and unavailable. All hypothesis testing was done using P-values derived from permutation tests described in [31], which account for potential clustering of outcomes in single males due to multiple sperm produced from a single repair event.

In cross 2, in which the EJ1 chromosome was present and the HR-h pathway was available, we saw highly significant decreases in HR-h repair in brca2 mutants (P < 1e-5) (Figure 1B). Short-tract HR-h was decreased 64-fold from wild-type values and long-tract HR-h was decreased 9-fold. These values likely underestimate the requirement for brca2 in HR-h. Due to the female sterility of brca2, repair pathway use was measured in the genetically-null offspring of females heterozygous for brca2 and low levels of Brca2 protein may still have been present at the onset of the zygotic transcription when the endonuclease was initially expressed. We also observed a 5-fold increase in flanking deletions in brca2 mutants. We did not see a significant increase in deletions in cross 1 in which HR-h was not available (Figure 1C). Therefore, the deletions most likely resulted from aberrant HR-h repair, as has been previously suggested for spnA and okra mutants [32]. We did not observe a requirement for brca2 in SSA. In fact, there was a significant increase in SSA in brca2 mutants in both cross 1 and 2 (P = 1.4e-7 and P < 1.0e-8 respectively, Figure 1B,C).

We observed significant changes in SSA and NHEJ in cross 1 when HR-h was unavailable (P = 1.4e-7 and P = 4.3e-4)
In cross 1, the use of SSA increased to 111% of wild-type levels and NHEJ decreased to 74% percent of wild-type levels in \textit{brca2} mutants. It is possible to interpret this result as a partial requirement for \textit{brca2} in NHEJ. However, in light of similar results with other HR-h defective mutants \cite{32}, our similar results for \textit{okra} mutants (Figure 1C), and the fact that we did not see a decrease in NHEJ in cross 2 ($P=0.50$, Figure 1B), we favor the explanation that changes in NHEJ and SSA levels in cross 1 were due to defects in HR-s that were indirectly measured by changes in NHEJ and SSA. Deletions are measured by the loss of the $w^+$ marker.

In conclusion we found that \textit{brca2} is required for HR-h and that the loss of HR-h is associated with an increase in the use of SSA repair. In addition, the rate of deletion formation near DSBs increases in \textit{brca2} mutants. These changes in DSB repair represent a shift towards more mutagenic repair of DSBs in \textit{brca2} mutants that are similar to changes seen in \textit{spnA/rad51} and \textit{okra/rad54} mutants \cite{32} and Figure 1). Therefore we do not find evidence for a Rad51-independent role for Drosophila Brca2 in somatic DNA repair.

Failure to Repair Meiotic DSBs in \textit{brca2} Mutants Leads to Partial Activation of the Meiotic Recombination Checkpoint

Mutations in genes required for repair of meiotic DSBs, such as \textit{rad51/spnA}, cause female sterility due to activation of a meiotic checkpoint that affects dorsal-ventral patterning of the eggshell and embryo \cite{1,14}. The DNA endonuclease Mei-W68 catalyzes DSB formation in the germarium of the Drosophila ovary as the initiating step in meiotic recombination \cite{33}. If the DSBs persist, such as in DNA repair mutants, a checkpoint is activated that results in the inefficient translation of Gurken, the ligand for Epidermal Growth Factor (EGF) receptor, which is required for proper eggshell and embryonic development. Failure to repair meiotic DSBs may therefore lead to the activation of the meiotic recombination checkpoint, which can affect dorsal-ventral patterning and lead to female sterility. This checkpoint activation is thought to be a reflection of the failure to repair meiotic DSBs and may be a conserved mechanism to ensure the accurate repair of meiotic DSBs to prevent the transmission of deleterious mutations to the next generation.
Growth Factor receptor responsible for specifying dorsal fates in the Drosophila embryo and eggshell [1,34]. Activation of the meiotic recombination checkpoint results in the ventralization of eggs laid by females mutant for meiotic DSB repair genes. A second characteristic phenotype of checkpoint activation is the failure to properly form the karyosome of the oocyte nucleus. These defects can be suppressed by mutations in the kinases required for checkpoint transduction indicating that they are due to checkpoint activity [1]. Egg chambers from brca2 mutant females had a highly penetrant karyosome defect (Figure 2A). In addition, brca2 mutant females laid weakly ventralized eggs that did not hatch (Figure 2B and 2C). The eggshell patterning and karyosome formation defects can be rescued by a single copy of a genomic rescue construct (Figure 2A and 2C).

Transduction of the meiotic recombination checkpoint signal requires Hus1, a member of the Rad9-Hus1-Rad1 checkpoint complex (9-1-1), and the checkpoint kinases Mei-41/ATR and Chk2 [1,35,36]. Mutations in either mei-P22, a gene required for DSB formation [37], or the checkpoint kinase chk2 suppress the eggshell and karyosome defects of brca2 mutants (Figure 2A and 2C), clearly demonstrating that the ovarian phenotypes observed are due to persistent DSBs that activate the meiotic checkpoint. We observed little or no suppression of either the eggshell or the karyosome defects in mei41D3; brca2KO/56 (Figure 2C) or mei41D3; brca2KO/56 (data not shown) double mutants. This may be due to a possible redundancy between the two upstream checkpoint kinases Mei-41/ATR and ATM. Currently it is not possible to test for a role of atm in the meiotic recombination checkpoint as even the hypomorphic, viable atm mutants available have eggshell patterning and karyosome defects (38 and our unpublished results). Additional explanations for the lack of mei-41 suppression of the brca2 ovarian phenotype are presented in the Discussion. Nevertheless, suppression by chk2 muta-
tions indicate that the ovarian phenotypes of brca2 mutants result from checkpoint activation.

Brca2 Is Also Required for Efficient Activation of the Meiotic Recombination Checkpoint

The brca2 eggshell patterning defect is a considerably weaker defect than that of any of the meiotic DNA repair mutants known to activate the checkpoint, spnA/rad51, spnB/xrcc3, spnC/hel308, or okra/rad54 [14,39–41]. We examined the levels of γ-H2AV, a marker for DSBs, in order to determine if the number of DSBs was reduced in brca2 mutants relative to other meiotic DSB repair mutants, or “spindle class” genes. In wild-type flies γ-H2AV foci are seen in pro-oocyte nuclei in regions 2a and occasionally 2b and are absent from region 3 [42]. An average of 14–15 γ-H2AV foci are present in a pro-oocyte nucleus in region 2a before declining in region 2b. Meiotic DNA repair mutants show a delay in γ-H2AV foci formation, but reach a peak number of 20–24 γ-H2AV foci as compared to 21.1 foci in spnA mutants in region 3 (Figure 3A). Therefore it is unlikely that the weak phenotype seen in brca2 mutants is due to decreased accumulation of persistent DSBs.

Surprisingly, when we made double mutants with brca2 and any of the other meiotic DNA repair mutants, brca2 strongly suppressed the eggshell phenotype. For example, spnD mutant females lay 79.3% strongly ventralized eggs (class 3 and class 4 eggs) and 13.8% wild-type-like eggs (class 1). brca2; spnD mutant females lay 18.7% wild-type-like eggs and 79.3% wild-type like eggs. Similar results were seen with okra and spnA mutants (Figure 3B). In order to determine if the suppression of the eggshell phenotype was due to increased DSB repair in the double mutants, we examined the number of γ-H2AV foci present in the double mutants. Figure 3B shows that the double mutants with brca2 all had similar levels of γ-H2AV foci in region 3 of the germarium (Figure 3A). Therefore it is unlikely that increased levels of repair occur in the double mutants, and we propose that Brca2 has a second role in meiosis in transducing the meiotic recombination checkpoint signal.

brca2 and hus1 Are Not Required for Checkpoints That Respond to Irradiation Damage

The DNA damage checkpoints that respond to multiple types of damage and result in diverse damage responses converge upon a common set of related transducer kinases.
and mediator proteins [43]. However, each checkpoint has specific genetic requirements for signal transduction. Therefore, we thought it possible to gain a better understanding of where in the meiotic recombination checkpoint pathway Brca2 acts by examining whether \( \text{brca2} \) was required for other Drosophila checkpoints. We used the well-established assays in the imaginal discs and brain of the Drosophila larvae to monitor the checkpoints that respond to irradiation in animals null for \( \text{brca2} \). Irradiated imaginal discs exhibit a G2 cell cycle arrest within 1 hour of irradiation and induce apoptosis within 4 hours post-irradiation [44,45]. The induction of apoptosis requires \( \text{chk2} \) and \( \text{p53} \), but not \( \text{mei-41} \) or \( \text{grp/chk1} \) [46]. Cell cycle arrest after high dose IR (4,000 Rads) requires \( \text{mei-41} \), to lesser extents \( \text{chk2} \) and \( \text{grp} \), but not \( \text{atm} \) [38,44,46]. Cell cycle arrest after low dose IR (500 Rads) requires \( \text{mei-41} \) as well as \( \text{atm} \) [47]. A \( \text{mei-41} \)-dependent decrease in replication, or an intra-S phase checkpoint, is seen within 2 hours post-irradiation in the larval brain lobes [48].

We did not see any requirement for \( \text{brca2} \) in the checkpoints that respond to irradiation (Figures 4 and S1). After a 4,000 Rads IR exposure, \( \text{brca2} \) mutants demonstrated efficient G2 arrest (Figure 4A) and induction of apoptosis (Figure 4B). Cell cycle arrest after a 500 Rad IR exposure was also not compromised in \( \text{brca2} \) mutants (Figure 4C). We did not see any difference between \( \text{brca2} \) mutants and wild-type larvae in the induction of 5-bromo-2-deoxyuridine (BrdU) staining after a 4,000 Rad exposure (Figure 4D). However, unlike the cell cycle and apoptosis assays in which the difference before and after irradiation is dramatic, the intra-S checkpoint effect is a reduction in replication levels by slightly less than half (Figure 4D). Therefore we do not expect to be able to detect partial requirements for genes involved in the intra-S phase checkpoint. Collectively, the irradiation checkpoints we tested required \( \text{atm} \), \( \text{mei-41/atr} \), \( \text{chk1} \), and \( \text{chk2} \) and did not require \( \text{brca2} \), demonstrating that the activation of the core checkpoint transducers in response to irradiation in Drosophila does not require \( \text{brca2} \).

In addition to testing for a requirement for \( \text{brca2} \) in the irradiation checkpoints we also examined \( \text{hus1} \) mutants. \( \text{Hus1} \) is a member of the 9-1-1 complex required for activation of the Drosophila meiotic recombination checkpoint and has been shown to be involved in S-phase checkpoints in mammals [36,49]. Similar to \( \text{brca2} \), \( \text{hus1} \) is not required for the cell cycle arrest or apoptotic induction after high dose IR in Drosophila [36]. In this present study we found that \( \text{hus1} \) is also not required for the G2/M checkpoint after low dose IR or the intra-S checkpoint (Figure 4C and 4D). While we...
were harvested for the immunoprecipitation.

and/or P that the G2/M checkpoint is largely intact in cells after IR as compared to unirradiated discs, indicating Drosophila Brca2 as a Model for Human BRCA2

Brca2 Interacts with Rad9

As brca2 and hus1 mutants are both required for efficient activation of the meiotic recombination checkpoint but are not required for the irradiation checkpoints, we wondered if Brca2 acted in concert with the 9-1-1 complex to enforce checkpoint control. We found that epitope-tagged Rad9, a member of the 9-1-1 complex, and Brca2 expressed in the germline cells of the Drosophila ovary co-immunoprecipitated (Figure 5). Immunoprecipitations for FLAG-tagged Rad9 pulled down HA-tagged Brca2. We roughly estimate from 3 independent experiments that between 1/40 (data not shown) to ~ 1/400 (Figure 5) of the total HA-Brca2 protein was co-immunoprecipitated by FLAG-Rad9. It could be that only a subset of the Rad9 present in all regions of the Drosophila ovary binds Brca2. Alternatively, the interaction between Brca2 and Rad9 may be stage-specific, only occurring in the region of the gerarium in which recombination intermediates are present and in which a transient checkpoint response may occur. The physical interaction of Brca2 and Rad9 and the similarities in genetic requirements for hus1 and brca2 in Drosophila and mammalian checkpoints strongly suggest a common role for Brca2 and the 9-1-1 complex in checkpoint function.

Discussion

Drosophila Brca2 as a Model for Human BRCA2

Our results demonstrate that the predicted gene CG31069 is a functional BRCA2 homolog required for meiotic and mitotic homologous recombination. We have made null mutations in Drosophila brca2 in two distinct genetic backgrounds and rescued the ovarian defects by genomic rescue. Unlike in mammals in which null mutations are early embryonic lethal [50], Drosophila brca2 null mutants are homozygous viable, possibly because of the long period of maternal gene expression during Drosophila embryonic development. It is still uncertain whether Drosophila Brca2 contains a cryptic DNA binding domain similar to mammalian BRCA2 or if this function is encoded in a tightly regulated interacting protein. Further biochemical studies will be necessary to resolve this question, but it is clear from our functional analysis of the role of Brca2 in DNA repair, as well as a recently reported physical interaction with SpnA/ Rad51[51], that Drosophila brca2 represents a functional homolog of the human breast cancer susceptibility gene. Clearly, due to the viability of Drosophila brca2 null mutants and the power of Drosophila genetics, Drosophila offer a promising new opportunity for uncovering novel roles for BRCA2 during development. In this work we presented a thorough characterization of the role of Brca2 in DSB repair and we uncovered a novel function for Brca2 in the meiotic recombination checkpoint.

Drosophila Brca2 and Repair

Using the Rr3 assay [30] we showed that in brca2 mutants, DSB repair is shifted towards repair by potentially mutagenic repair pathways. Repair by homologous recombination is dramatically decreased in brca2 mutants, and repair by single-strand annealing predominates. Repair by SSA always results in the loss of the sequences between annealed repeats. SSA repair is restricted to DSBs flanked by repetitive elements, though due to the highly repetitive nature of higher eukaryotic chromosomes, SSA repair can represent a significant source of mutagenesis in higher eukaryotes. Our results contrast with studies in C. elegans, in which indirect in vivo experiments and in vitro annealing experiments[16,25] have lead to the suggestion that the C. elegans BRCA2 homolog is required for SSA repair. Our results are more similar to the effects seen in mammalian cell culture experiments with hypomorphic BRCA2 mutations, in which decreases in HR repair correlated with increases in SSA repair [26–29]. In addition, Brough et al. (2008) also have recently reported a similar inverse relationship between SSA and HR-h in a Drosophila brca2 mutant using a simplified DSB repair assay [51].

In the Rr3 assay when one pathway is compromised, the sum of the relative pathways usage in these mutants still equals near 100%, even though the percentages are calculated from different populations and are not forced to equal 100% ([32] and our own work). This observation plus the fact that different effects on repair pathway balance have been observed among mutants with decreases in the same pathway suggest that regulated compensation can occur. For example, mutations in mus101 and mei-41 both result in a decrease in SSA, but the former are compensated by increases in NHEJ and HR-h, while the latter is compensated by NHEJ only [32]. In cross 2, we saw a significant increase in the use of the SSA (P <1e-5), but no significant difference in the relative level of NHEJ (P = 0.50) in brca2 mutants (Figure 1B), indicating compensation by the SSA pathway in brca2 mutants.

Compensation of decreases in HR-h by the SSA pathway seems to be a common response to deficiencies in genes required for HR-h. In mammalian cell culture studies, Stark et al. (2004) found a similar inverse relationship between SSA and HR in Brca2 and Rad51 mutants, while Brca1 mutants had
mutations in spnA length. Mutations in
ial for loss of heterozygosity increases with increasing tract
extent of gene conversion during HR-h repair. Gene
HR-h was decreased 64-fold relative to wild-type values and
complex reporter design.
complicated mutants are continuous or discontinuous by using a more
deletion in Drosophila
are thought to have arisen from different mechanisms due to
increased resection prior to strand invasion, increased
mutants may represent increased rate of DNA synthesis,
results suggest that reductions in levels of the enzymes
required for strand invasion can result in increased rates of
loss of heterozygosity during HR-h repair. Gene conversion
tract length during HR-h repair has important implications in maintaining genomic integrity as the potential
for loss of heterozygosity increases with increasing tract
length. Mutations in okra led to a similar shift towards long-
tract HR-h (Figure 1B), and homozygous and heterozygous
mutations in spnA have also been observed to alter the
balance of HR-h in towards long-tract HR-h [32]. These
results suggest that reductions in levels of the enzymes
required for strand invasion can result in increased rates of
loss of heterozygosity during HR-h repair. Increased long-
tract HR-h repair relative to short-tract HR-h repair in brca2
mutants may represent increased rate of DNA synthesis, increased resection prior to strand invasion, increased
stability of recombination intermediates, unequal repair of the
heteroduplex DNA, or a combination of these processes. In
mammalian cells, an increase in the extent of gene
conversion has been observed in both Rad51K137R and Xrc3,
a Rad51 paralog, mutants; though these observed increases
are thought to have arisen from different mechanisms due to
differences seen in gene conversion tract continuity [29,52].
Models explaining the increase in the inclusion of the 16 bp
deletion in Drosophila brca2 and spnA mutants would first
need to determine whether conversion tracts in these
mutants are continuous or discontinuous by using a more
complicated reporter design.

Drosophila Brca2, the 9-1-1 Complex, and Checkpoints

We found a novel requirement for brca2 in transduction of the meiotic recombination checkpoint signal. Initially the
meiotic phenotypes of brca2 mutants were surprisingly
different from the spindle class mutants previously studied.
First, even with the exacerbation of the ventralization defect
by growth at 25 °C, the eggshell ventralization phenotype of
brca2 mutants was significantly weaker in spite of similar
levels of persistent DSBs (Figure 2). Second, the kinetics of the
eggshell phenotype were opposite to the kinetics of classical spindle mutants. In spnA, B, C, D and okra mutants the pheno-
type is weak during the initial days in which the females are
fed yeast, but after 5–7 days on yeast spindle mutants lay
predominately severely ventralized eggs. In brca2 mutants,
ventralized eggs were only reliably laid during the first 1–4
days on yeast (data not shown). Given our results, it is now
clear that the requirement for brca2 in efficient transduction
of the checkpoint signal masks the strong eggshell ventraliza-
tion phenotype that is normally suggestive of a role in meiotic
DSB repair. As an increasing number of proteins with dual
roles in DNA repair and checkpoint function are being
identified, it will be interesting to see if there are additional
dual function proteins functioning in Drosophila meiosis.
While the classical meiotic repair mutants have strong
oogenesis phenotypes, weak or absent eggshell patterning
defects may not preclude a role in meiotic DNA repair if
coupled to a role in checkpoint transduction.

We found that the ovarian phenotypes of brca2 mutants
were suppressed by mutations in chk2, but were not sup-
pressed by mei-41 mutations. This finding is in contrast to the
other spindle class mutants which are suppressed by both mei-
41 and chk2 mutations [1,14,39]. Our model is that in females
with an intact checkpoint response, the checkpoint activation
is dependent upon mei-41 and chk2. However, because Brca2
acts in a similar step in the checkpoint pathway as Mei-41, no
additional suppression is observed in the mei-41; brca2 double
mutant. Since both the eggshell and karyosome defects of
brca2 mutants can be suppressed by chk2 it is probable that
the residual checkpoint activation in brca2 mutants is due to
activation of Chk2 by the upstream checkpoint kinase Atm. It
is not currently possible to test the involvement of Atm in the
checkpoint as even viable, hypomorphic atm single mutants
have eggshell and karyosome defects. There is however
evidence that Mei41-independent checkpoints exist in Dro-
sophila meiosis. Upregulation of transposable elements in the
Drosophila germline, as seen in cutoff mutants, results in
eggshell patterning defects that can be suppressed by chk2 but
not by mei-41 mutations [53]. The checkpoint activated in
cutoff mutants is, however, distinct in at least some aspects
from the checkpoint activated in brca2 mutants. Unlike in
brca2 mutants, the checkpoint activated in cutoff mutants
results in a loss of germline cells as well as eggshell patterning
defects, and these defects cannot be suppressed by mutations
that prevent DSB formation.

According to our model, Mei41-independent checkpoint
activation in brca2 mutants is strong enough, with respect to
either signal strength or signal duration, to result in a
karyosome defect but not strong enough to result in strong
eggshell patterning defects. Clearly Mei-41 is responsible for
the bulk of checkpoint activation in classical spindle mutants
but it remains possible that Atm may play a supporting role.
It is notable that the classical spindle phenotypes are typically
scored after 5–7 days on yeast at ambient temperature and that
at these conditions the brca2 single mutant phenotype
was very weak (data not shown). Therefore it is possible that
under the conditions used in this study mei-41 mutations may
also not completely suppress the classical spindle mutations.
In interpreting the role of brca2 in the transduction of the
meiotic recombination signal we have had to focus on the
eggshell phenotype as brca2 single mutants have karyosome
defects. This phenotype is similar to that seen in has1 mutant
females. has1 mutants are able to suppress the eggshell defects
of spindle mutants but do not suppress the karyosome defects
of the spindle mutants as has1 single mutants have karyosome
defects [36]. Though it has not been tested in this study, it
seems possible that, similar to *brca2* mutants, the *husl* mutant karyosome defect is a result of persistent DSBs and partial activation of the checkpoint.

We also found that Brca2 co-immunoprecipitated with Rad9. The 9-1-1 complex forms a heterotrimeric ring that is loaded onto resected single-stranded DNA flanking a DSB following replication stress [54,55]. The 9-1-1 complex associates with DSBs independent of ATR, and mediates Chk1 phosphorylation by ATR through interaction with TopBP1[56–59]. The 9-1-1 complex may or may not use a similar mechanism to activate Chk2 during the meiotic recombination checkpoint. Although the precise functional relevance of the Rad9-Brc2 interaction remains to be explored, our co-immunoprecipitation results further suggest that the role of Brca2 in checkpoint control is upstream of Chk1/Chk2.

Research in mammals and our work in Drosophila have shown that the 9-1-1 complex and Brca2 are specifically required for the checkpoints thought to be activated in response to large stretches of single-stranded DNA. In Drosophila we have shown that they are required for the meiotic recombination checkpoint, but not the irradiation checkpoints. In mammals, Brca2 and the 9-1-1 complex are required for several S-phase checkpoints [3,10,49]. Now that a physical connection between Rad9 and Brca2 has been required for several S-phase checkpoints [3,10,49]. Now that a physical connection between Rad9 and Brca2 has been shown that the 9-1-1 complex and BRCA2 are specifically required for mitotic and meiotic homologous recombination. In light of the absence of a functional overlap between Brca2 and the 9-1-1 complex in the checkpoints examined to date, it is clear from mutagen sensitivity assays that the functional overlap between 9-1-1 and Brca2 in DNA repair is not absolute. *husl* mutants are severely sensitive to MMS, but not to IR, while *brca2* mutants are moderately sensitive to MMS and severely sensitive to IR ([36] and this study).

In conclusion we have found that the Drosophila homolog of *BRCA2* is required for mitotic and meiotic homologous recombination and in the absence of *brca2* error-prone repair predominates. *brca2*, similar to the 9-1-1 complex with which it physically interacts, has a second requirement during meiosis in the activation of the meiotic recombination checkpoint but is not required for checkpoints that respond to irradiation induced damage, indicating a specialized role for *brca2* in checkpoint control.

**Methods**

Fly strains and transgenics. Flies were maintained at 25 °C. Additional information about culturing conditions can be found in Text S1. *brca2* was isolated by screening for imprecise excisions after mobilization of P(SU2-P) CG30169GkO03561 which was inserted in the 5′UTR of CG30169. *brca2* removes the majority of the coding sequence of CG30169 and most of the 5′UTR of CG4612; the sequence junction is GTGGTGGCGGCT/AAACTTGCCGGCAA. *brca2* was create by ends-out homologous recombination using the targeting vector pW25 [21]. Flanking homologous sequences were amplified, inserted into the BsoBI/Ascl and Acc65I/SphII sites of pW25, and predicted open reading frames were verified by sequencing. The sequence junction of the deleted region is: TTGTTTGGATACGGGAGGGTCCCCATTAC. The structure of the resulting knock-out was verified by PCR and Southern blot analysis (data not shown). To make P(CG30169) a 5.8 Kb Xhol-NotI fragment containing CG30169 was isolated from the BAC clone BACR11c07 (BACPAC Resource Center) and ligated into pCasper4. To make P(UAS-Brca2-HA) the region containing the coding sequence of Brca2-PA was PCR-amplified using primers that created a 5′ NotI site and a 3′ XbaI site. Three copies of HA followed replication stress [54,55]. The 9-1-1 complex associates with DSBs independent of ATR, and mediates Chk1 phosphorylation by ATR through interaction with TopBP1[56–59]. The 9-1-1 complex may or may not use a similar mechanism to activate Chk2 during the meiotic recombination checkpoint. Although the precise functional relevance of the Rad9-Brc2 interaction remains to be explored, our co-immunoprecipitation results further suggest that the role of Brca2 in checkpoint control is upstream of Chk1/Chk2.

In cross 2 in which both Rr3 and EJ1 were present, SSA repair was unsuccessful reactions. PCR products were run on a 3% MetaPhor gel. The FLAG peptide sequence was added at the 5′ end following the XbaI site. The resulting NotI-KpnI fragment was cloned into pUASp. To make P(UAS-FLAG-Rad9-HA) HA followed by BglII and KpnI sites were placed downstream of the coding sequence of Rad9. The coding sequence of Rad9-PA was amplified using primers to create a 5′ KpnI site and a 3′ XbaI site. The FLAG peptide sequence was added at the 5′ end following the KpnI site. The resulting KpnI-XbaI fragment was cloned into pUASp. The pW25 vector can rescue the meiotic patterning defects of *brca2* mutants (data not shown). P-element-mediated germ-line transformation was carried out according to standard protocols [60]. The following previously-described allele combinations were used: mct42, (except where noted), chkb+, spnf139 su(Hw)00183, spnf139 su(Hw)00183, hsl1. Details about the genotypes can be found at http://flybase.bio.indiana.edu. The P(Rr3)48C, P(Rr3EJ1)48C, and TM3 Sb P(U2)72C transgenic flies were obtained from Bill Engels [30]. The following flies were made by recombination: P(Rr3)48C w1118, L. brca2w1, P(Rr3)48C w1118, L. brca2w1, P(Rr3EJ1)48C brca2w1, P(Rr3EJ1)48C okraA4. Antibody stainings. Ovaries were fixed for 20 minutes in 1:3 4% paraformaldehyde/ PBS:heptane and other tissues were fixed in 4% paraformaldehyde/PBS and blocked in 3% BSA/PBS and further processed according to standard protocols. The following modifications were made: the γ-H2AX stainings were followed by goat serum and P-H3(Ser28) stainings were fixed for 1 hour. Primary antibodies were incubated overnight at 4 °C in PBS + 0.3% Triton (PBST) + 1% BSA or 10% goat serum at the following concentrations: 1:500 rabbit α-γ-H2AX, 1:1000 rat α-Encore, 1:500 mouse α-CTG clone 1A8-1G2, 1:500 rabbit α-Cleaved Caspase-3 (Cell Signaling), 1:1000 mouse α-Brdu clone 3F3 (BD Biosciences), 1:1000 mouse 53BP1 clone 48 (Upstate), 1:1000 Secondary antibodies (Molecular Probes), 1:10,000 Hoechst (Molecular Probes), or 1:500/1000 mouse/wheat germ Agglutinin-488 (Molecular Probes) were incubated 2 hours in PBST.

**Repair reporter 3 assay.** Details on the Rr3 strain can be found in [30–32]. Briefly, individually males bearing the parternally inherited endonuclease TM3 Sb P(U2)72C and the reporter constructs, P(Rr3)48C and P(Rr3EJ1)48C, were backcrossed to w1118vir. All matings occurred within two weeks of eclosion to minimize age-related effects. L w1118, −P-H3(Ser28) progeny were scored for eye color, presence of the endonuclease, and BrdU fluorescence. In cross 1, in which the EJ1 chromosome was not present, SSA repair was scored as the percent of DsRed positive nonendonuclease-bearing (endo−) progeny among all endo− progeny. NHEJ was scored as the percent of DsRed-negative, endonuclease-bearing (endo+ progeny) among all endo− progeny. In cross 2 in which both Rr3 and EJ1 were present, SSA repair was again scored as DsRed-positive endo−/all endo−. The DsRed-negative endo+ flies represented repair by either HR-h or NHEJ and were distinguished by PCR. We used primers to specifically amplify the region containing the mutated I-SceI site on the EJ1 chromosome [30] and template a band HR-h reaction used for control primers in the same PCR reaction to exclude rare unsuccessful reactions. PCR products were run on a 3% MetaPhor agarose gel (Lonza) to simultaneously distinguish between repair by short and long tract HR-h. Ten Dark endo+ progeny per male or as many as were available used for PCR. Deletions were scored as percent of white-eyed males among total Rr3 progeny; the means and standard errors were multiplied by 2 to allow comparisons with previously reported values. The deletion class was not isolated prior to DsRed and PCR scoring and therefore also represents a small portion of the NHEJ class. Permutation tests were used to compute two-tailed P-values as described in [31].

**Mutagen sensitivities.** Heterozygous parents were mated and eggs were collected for 24 hours at room temperature in vials for chemical mutagenesis or apple juice plates for irradiation. 48 hours later the larvae were treated with 250 μl of 0.025% or 0.08% methyl methanesulfonate (Sigma) or irradiated with 500 or 1,250 Rads in a Faxitron X-ray cabinet. Control flies were treated with 250 μl water or not irradiated. After eclosion the percent of homzygous mutontype flies was determined, and the sensitivity was expressed as the percent of mutant flies total progeny in the treated vial as compared to the relative percent of mutant flies in untreated control vials.

**Checkpoint assays.** Climbing third instar larva were irradiated at 500 or 4,000 Rads in a Faxitron X-ray cabinet. For analysis of cell cycle progression and apoptosis, larva were inverted in PBS and fixed after 1 hour. For analysis of cell cycle progression, larva were inverted in Schneider’s media 1.5 hours after irradiation. Larvae were incubated with Schneider’s media + 10 μM BrdU (Sigma) for 30 minutes, washed 2 times 3 minutes and fixed in 4% paraformalde-
hyde/PBS for 20 minutes. Larvae were washed and then incubated in 2N HCl for 30 minutes, 100 mM borax for 2 minutes, washed and then blocked and stained as described above. Confocal stacks of 0.5 μm intervals were analyzed using Velocity 3DM software (Improvis) for cell cycle and apoptosis assays and ImageJ for the replication assay. To quantitate the BrdU stainings, the mean grayscale value of three evenly spaced regions of 1,400 square pixels in the wide nuclei-dense bands of the brain lobes were averaged for each brain lobe.

**Co-immunoprecipitation and Western blots.** Epitope-tagged Rad9 and Brca2 were expressed using the germline Gal4 driver nanosVP16 [61]. 10 ovary pairs were ground in 150 μL IP buffer (50 mM Tris pH 8.150 mM NaCl, 1mM CaCl2, 0.1% Tween-20) supplemented with complete mini protease inhibitor EDTA-free (Roche) and type I and II phosphatase inhibitors (Sigma). Cleared lysate was incubated at 4°C for 2 hours with mouse αFLAG M2 antibody (Sigma) bound to 20 μL Protein A/G Plus Agarose slurry (Santa Cruz Biotechnology). A 30 minute preincubation with an equal volume of Protein A/G beads without antibody. Fusion proteins were detected using mouse αFLAG-M2-HRP conjugate (Sigma) at 1:2000 and rat αHA-HRP conjugate (Roche) at 1:3000 for Western analysis.

**Supporitng Information**

**Figure S1. brca2 Is Not Required for the Checkpoints That Respond to Irradiation Damage in the Drosophila Larval Wing Discs and Brain Lobes**

Climbing third instar larvae were irradiated and then dissected prior to fixation. Representative images are projections of 0.5 micron interval stacks of control tissues (A-R) or tissues that were irradiated to fixation. Representative images are projections of 0.5 micron interval stacks of control tissues (A-R) or tissues that were irradiated to fixation. (A-E, F-M) Larvae were fixed 1 hour after a 1,000 Rad IR exposure and stained for the mitotic marker P-H3. (B,F,J,N B,F,J,N) Larvae were fixed 4 hours after a 4,000 Rad IR exposure and stained for activated Caspase-3. (C,G,K,O,Q,C,G,G, K,O,Q,G). Larvae were fixed 1 hour after a 500 Rad IR exposure and stained for the mitotic marker P-H3. (D,H,P,R,D,H,P,R) Larvae were inverted 1.5 hours after a 4,000 Rad IR exposure and incubated in Schneider’s media with BrdU for 30 minutes. Genotypes tested were: (A-D) OreR, (E-H) brca2Δ956 (I-L) brca2Δ956, (M,O-P*,O) mei41, (N,N*) chk2, and (Q-R*) basl.

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**Text S1. Supplemental Methods**

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