Drosophila ATM and ATR have distinct activities in the regulation of meiotic DNA damage and repair

Eric F. Joyce,[1,2] Michael Pedersen,[3] Stanley Tiong,[3] Sanese K. White-Brown,[1,2] Anshu Paul,[1,2] Shelagh D. Campbell,[3] and Kim S. McKim[1,2]

[1]Waksman Institute of Microbiology and [2]Department of Genetics, Rutgers University, Piscataway, NJ 08854
[3]Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

Ataxia telangiectasia–mutated (ATM) and ataxia telangiectasia–related (ATR) kinases are conserved regulators of cellular responses to double strand breaks (DSBs). During meiosis, however, the functions of these kinases in DSB repair and the deoxyribonucleic acid (DNA) damage checkpoint are unclear. In this paper, we show that ATM and ATR have unique roles in the repair of meiotic DSBs in *Drosophila melanogaster*. ATR mutant analysis indicated that it is required for checkpoint activity, whereas ATM may not be. Both kinases phosphorylate H2AV (γ-H2AV), and, using this as a reporter for ATM/ATR activity, we found that the DSB repair response is surprisingly dynamic at the site of DNA damage. γ-H2AV is continuously exchanged, requiring new phosphorylation at the break site until repair is completed. However, most surprising is that the number of γ-H2AV foci is dramatically increased in the absence of ATM, but not ATR, suggesting that the number of DSBs is increased. Thus, we conclude that ATM is primarily required for the meiotic DSB repair response, which includes functions in DNA damage repair and negative feedback control over the level of programmed DSBs during meiosis.

Introduction

DNA double strand breaks (DSBs) can cause aneuploidy or trigger apoptosis if they are not promptly repaired; consequently, a cell's ability to respond to chromosome DSBs is critical for survival (Wyman and Kanaar, 2006). During meiosis, programmed DSBs initiate meiotic recombination. These breaks are repaired by homologous recombination with a nonsister chromatid as the preferred template. One crucial outcome is crossover recombination, which involves the reciprocal exchange of DNA between homologous parental chromosomes and facilitates accurate chromosome segregation at meiosis I (Hawley, 1988; Youds and Boulton, 2011).

In response to DSB induction, the conserved ataxia telangiectasia–mutated (ATM) and ataxia telangiectasia–related (ATR) kinases are rapidly activated and phosphorylate numerous substrates involved in DNA repair and/or cell cycle checkpoints (Shiloh, 2006). During *Drosophila melanogaster* female meiosis, ATR (MEI-41) is required for DSB repair, crossover formation, and checkpoint activation (Sibon et al., 1999; Laurençon et al., 2003; Jaklevic and Su, 2004; Joyce and McKim, 2009); however, the role of *Drosophila* ATM is not known. The gene encoding *Drosophila* ATM is named *tefu* because of its role in preventing spontaneous telomere fusions (Queiroz-Machado et al., 2001; Bi et al., 2004; Silva et al., 2004; Song et al., 2004). As a result, *tefu* mutant tissues exhibit high levels of chromosome fusions that lead to lethality.

To address the role of ATM in meiosis, we undertook an analysis of DSB formation and repair during *Drosophila* oogenesis. This work was made possible by a temperature-sensitive allele of *tefu* (*tefu*^{8}; Silva et al., 2004; Pedersen et al., 2010), allowing us to bypass the pupal lethality associated with *tefu*-null mutants. Our findings suggest that ATM has unique roles in promoting DSB repair as well as negatively regulating the number of DSBs that are induced during meiotic prophase. Also, we were able to identify H2AV as a substrate of both ATM and ATR after DSB induction, which has revealed surprising features of γ-H2AV dynamics including multiple mechanisms for H2AV clearance. We propose that ATM may help control the level of DNA damage during meiosis as well as the repair response but, in contrast to ATR, is dispensable for the checkpoint.

Correspondence to Kim S. McKim: mckim@rci.rutgers.edu

Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia related; DSB, double strand break.

E.F. Joyce's present address is Dept. of Genetics, Harvard Medical School, Boston, MA 02115.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 195 No. 3 359–367
www.jcb.org/cgi/doi/10.1083/jcb.201104121

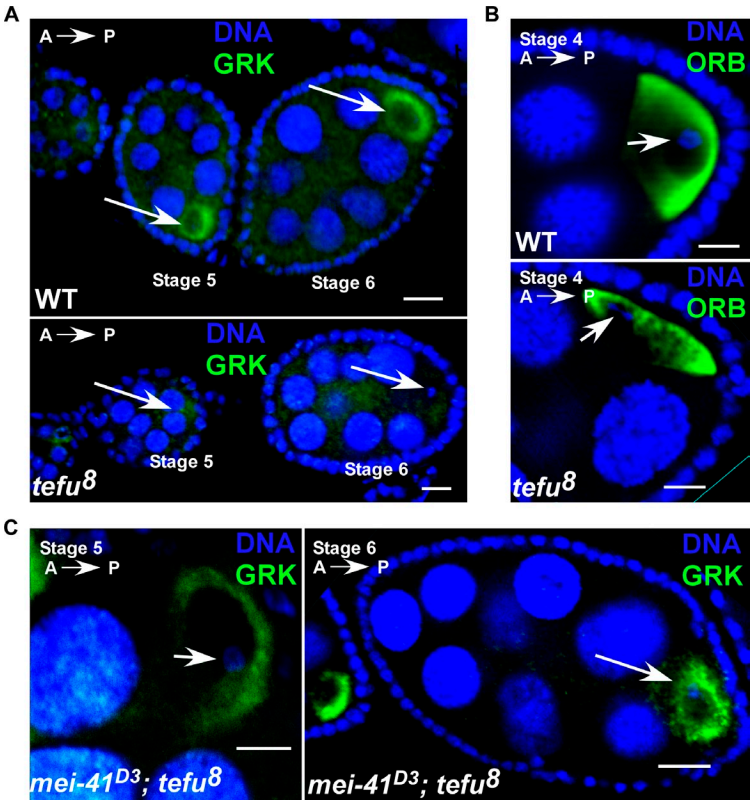

Figure 1. **Loss of ATM activates the *mei-41*–dependent meiotic DSB repair checkpoint.** *Drosophila* ovaries of the indicated genotypes are shown. Each oocyte develops within a 16-cell cyst (Walker and Hawley, 2000; Page and Hawley, 2001). (A) In controls, GRK protein localizes in a ring around the oocyte nucleus (arrows) in stage 5 and 6 egg chambers. In similarly staged *tefu*^{8} mutant egg chambers, GRK staining is much weaker or absent altogether. A, anterior; P, posterior; WT, wild type. (B, top) In control oocytes, chromatin becomes condensed during stage 4 of oogenesis into a spherical structure called the karyosome (arrow). Antibodies to ORB, a protein that localizes to the oocyte cytoplasm, identified the oocyte nucleus. (bottom) Similarly aged ORB-labeled *tefu*^{8} mutant egg chambers contain misshapen or fragmented karyosomes (arrow). (C) The GRK localization and karyosome morphology defects are suppressed in *mei-41*^{D3}*;tefu*^{8} mutants. All cysts are oriented with the anterior end to the left and posterior to the right. Arrows denote the oocytes. Bars, 10 µm.

Results and discussion

ATM is dispensable for the meiotic DSB repair checkpoint

ATR-dependent checkpoint activity in response to unrepaired DSBs causes oocyte development to proceed abnormally. A previous study (Silva et al., 2004) noted that *tefu* mutants produced embryos with dorsal-ventral polarity defects, a possible indicator of elevated DSB repair checkpoint activity. Another reporter for this effect is Gurken (GRK), a TGF-α–related protein required for establishing dorsal-ventral polarity. When DSBs are not repaired, GRK localization is abnormal (Ghabrial and Schüpbach, 1999; Abdu et al., 2002).

At the restrictive temperature (25°), *tefu*^{8} mutants are recessive lethal. To examine whether the meiotic DSB repair checkpoint was active in *tefu*^{8} mutants, we raised homozygous females at the permissive temperature (18°), shifted them to the restrictive temperature (Silva et al., 2004), and looked for a disruption of GRK localization. GRK is normally concentrated in the cytoplasm of control oocytes (Fig. 1 A). In 87% of similarly staged *tefu*^{8} mutant ovarioles, GRK expression was absent or much weaker than normal and mislocalized (Fig. 1 A and Table I). Another characteristic feature of oocyte development is the assembly of the karyosome, in which the chromatin is condensed into a single round mass within the cell nucleus of stage 4 oocytes (Spradling, 1993b). In control oocytes, the karyosome appeared compact and spherical (Fig. 1 B). However, in 80% of the *tefu*^{8} mutant oocytes, the karyosome appeared abnormally flattened or fragmented (Fig. 1 B and Table I). Abnormal GRK localization and karyosome organization are ATR-dependent phenotypes that are typical of mutants unable to repair DSBs (Ghabrial et al., 1998; Ghabrial and Schüpbach, 1999; Abdu et al., 2002; Staeva-Vieira et al., 2003; McCaffrey et al., 2006). ATM is required for the completion of meiotic recombination but is dispensable for the DSB repair checkpoint.

MEI-W68 is the *Drosophila* homologue of Spo11, a conserved endonuclease that catalyzes meiotic DSB induction in eukaryotes (McKim and Hayashi-Hagihara, 1998). The GRK localization and karyosome morphology defects were suppressed in *mei-W68*^{4572}*;tefu*^{8} double mutants (Table I), indicating that the defects are a result of unrepaired meiotic DSBs. We also tested a double mutant genotype combination with *mei-41*, the *Drosophila* homologue of ATR. The GRK mislocalization and karyosome defects in *tefu*^{8} mutants were suppressed in *mei-41*^{D3}*;tefu*^{8} double mutants (Fig. 1 C and Table I). These results show that loss of ATM function leads to activation of the ATR-dependent checkpoint response to unrepaired meiotic DSBs.

Table I. ***tefu*^{8} activates the DSB repair checkpoint**

Type	Wild type	*tefu*^{8}	*mei-W68*^{4572}*;tefu*^{8}	*mei-41*^{D3}*;tefu*^{8}
GRK defects (%)	0	87 (35)	0	0
Karyosome defects (%)	0	80 (32)	0	0
Total ovaries	38	40	30	30

GRK defects include absent or much weaker expression than normal as well as mislocalized staining. Karyosome defects include abnormally flattened or fragmented morphology. Numbers in parentheses denote the number of defects.

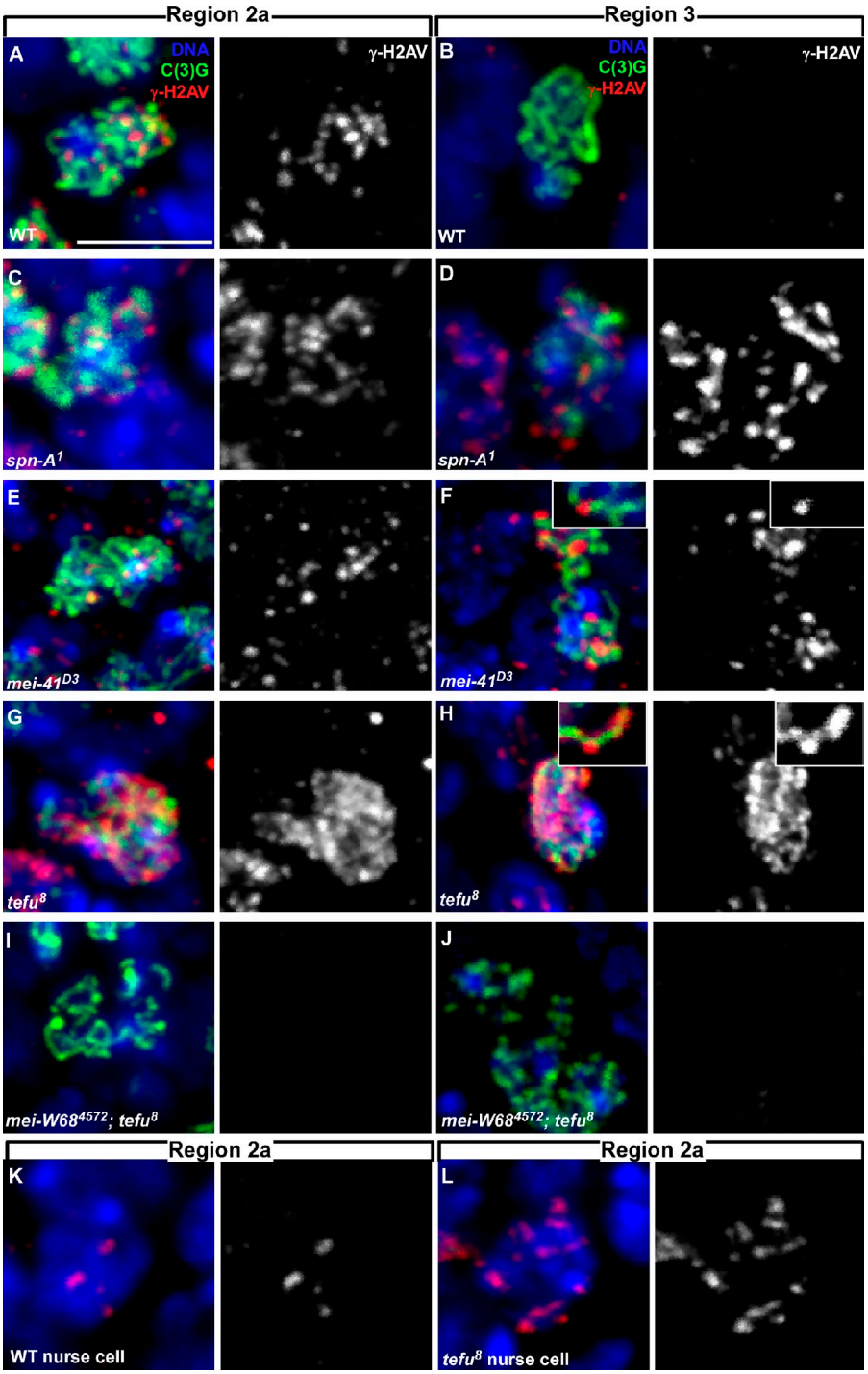

Figure 2. ***tefu* and *mei-41* are required for DSB repair in the oocyte.** Oocytes were identified with an antibody against the synaptonemal complex component C(3)G. (A) γ-H2AV labeling from a wild-type (WT) germarium, showing foci in region 2a pachytene cells in which meiotic DSBs are induced. (B) In region 3 pachytene oocytes, γ-H2AV labeling is absent from wild-type germaria, indicating that DSBs have been repaired. (C and D) In a repair-defective mutant *spn-A*^{1}, γ-H2AV staining persists in region 3 oocytes. (E and F) In a *mei-41*^{D3} mutant, γ-H2AV staining persists in region 3 oocytes as distinct foci (see insets). (G and H) In a *tefu*^{8} mutant, γ-H2AV staining in region 3 oocytes is in threads instead of distinct foci (see insets). (I and J) γ-H2AV is eliminated in *mei-W68*^{4572}*;tefu*^{8} double mutant. (K and L) Region 2a nurse cells in a *tefu*^{8} mutant have more γ-H2AV foci than wild type. Bar, 5 μm.

ATM controls meiotic DSB formation and repair

H2AV is a *Drosophila* H2A variant, like mammalian H2AX, that is phosphorylated at the sites of DNA breaks (Madigan et al., 2002). Antibodies to this phosphorylated protein (γ-H2AV) detect distinctive foci in the nucleus (Jang et al., 2003; Mehrotra and McKim, 2006). To assay for DSB repair defects in *tefu*^{8} mutants, we examined γ-H2AV staining and compared it with wild-type and mutants known to have DSB repair defects. Pachytene oocytes are arranged in order of developmental age within the germarium, which is divided into three regions (2a, 2b, and 3). In wild-type females, a mean of 6.2 γ-H2AV foci was found in region 2a pachytene oocytes (Fig. 2 A and Table II) and was absent in region 3 oocytes (Fig. 2 B). This is consistent with previous results suggesting that meiotic DSBs in wild-type oocytes are induced in region 2a and repaired before region 3 (Jang et al., 2003; Staeva-Vieira et al., 2003; Gorski et al., 2004).

Mutations in DSB repair genes such as *spn-A* (which encodes the *Drosophila* Rad51 homologue) exhibit an accumulation of γ-H2AV foci that persist throughout meiotic prophase, corresponding to unrepaired meiotic DSBs (Fig. 2, C and D;

Table II. **DSB repair defects in *tefu* and *mei-41* mutants**

Genotype	Mean γ-H2AV foci per oocyte	
	Region 2a	Region 3
Wild type	6.2 ± 1.1	0.1 ± 0.3
spn-A^{1}	3.7 ± 1.4[a]	22.8 ± 3.4
		25.2 ± 3.6[b]
tefu^{8}	ND	39.1 ± 8.0[b]
mei-41^{D3}	4.2 ± 0.8[a]	21.0 ± 1.3
Wild-type nurse cells	3.6 ± 1.6	0.0
tefu^{8} nurse cells	9.3 ± 2.8	0.0
mei-W68^{4572}*;tefu*^{8}	0.0	0.0
mei-41^{D3}*;tefu*^{8}, 18°	7.5 ± 1.7	18.2 ± 2.1
mei-41^{D3}*;tefu*^{8}, 25°	0.0	0.0
mei-41^{D3}*;tefu*^{8}, 25–18°	7.3 ± 2.6	17.5 ± 3.3

Foci were manually counted, except as otherwise noted. Means ± SD are shown.

[a]Mutations in DSB repair genes cause a delay in γ-H2AV appearance as a result of the activation of the pachytene checkpoint (Joyce and McKim, 2009).

[b]The foci number is an estimate based on fluorescent intensity (see Materials and methods) in *spn-A*^{1} and *tefu*^{8} mutants in which the foci could not be counted because of nearly ubiquitous threadlike staining.

Mehrotra and McKim, 2006). A mean of 22.8 γ-H2AV foci was present in *spn-A*^{1} region 3 oocytes, which is similar to previous estimates for the total number of DSBs per nucleus (Table II; Mehrotra and McKim, 2006). Similarly, γ-H2AV foci accumulated in region 3 oocytes of *mei-41*^{D3} mutants (Fig. 2 [E and F] and Table II), indicating that ATR is required to repair meiotic DSBs in addition to its role in checkpoint activation. In *tefu*^{8} mutant germaria at the restrictive temperature, γ-H2AV staining persisted into region 3 oocytes, consistent with a DSB repair defect (Fig. 2, G and H). However, in contrast to other repair mutants and wild type, the γ-H2AV staining in *tefu*^{8} mutants exhibited more robust and continuous labeling, colocalizing with most of the chromosomes rather than appearing as foci. All γ-H2AV staining was eliminated in *mei-W86*^{4572}*;tefu*^{8} double mutants (Fig. 2, I and J), indicating that the abundant γ-H2AV staining in the *tefu*^{8} mutant is dependent on the induction of meiotic DSBs.

The threadlike γ-H2AV labeling observed in *tefu*^{8} mutant oocytes could be a result of either unrestricted spreading of H2AV phosphorylation from the DSB sites or an increase in the number of programmed DSBs relative to wild type. We investigated these possibilities by examining the nurse cells in the germarium. Each pro-oocyte has 14 neighboring nurse cells that experience on average twofold less DSBs than the oocyte (Mehrotra and McKim, 2006). At the restrictive temperature, *tefu*^{8} mutants exhibited distinct γ-H2AV foci in nurse cells, indicating that ATM-deficient cells can restrict their DSB response to the DSB sites, and the foci could be counted. The *tefu*^{8} mutant nurse cells had a mean of 9.3 γ-H2AV foci, which is >2.5 times greater than the 3.6 γ-H2AV foci per nurse cell nurse in wild type ($P = 0.0042$; Fig. 2 [K and L] and Table II). To estimate the total number of DSBs that occur in *tefu*^{8} mutant oocytes, we used a method that quantitatively measures the intensity of γ-H2AV fluorescence (see Materials and methods). In short, we compared the intensity of a single γ-H2AV focus in adjacent nurse cells with that of total fluorescence in oocytes. Based on this method, we estimate 25.2 γ-H2AV foci in *spn-A* region 3 oocytes, similar to the levels when counted manually. In *tefu*^{8} mutants, we estimate ~39.1 γ-H2AV foci ($P = 0.0152$), a significant increase over *spn-A* that is consistent with the increase in γ-H2AV foci levels observed in nurse cells. Together, these results reveal a novel role for ATM in negatively regulating DSB formation during meiotic prophase.

ATM and ATR are functionally redundant for H2AV phosphorylation

ATM and ATR have been implicated in the phosphorylation of H2AX at sites of chromosomal DSBs in somatic cells of mouse and humans (Burma et al., 2001; Ward and Chen, 2001). To investigate whether *Drosophila* ATM and ATR serve redundant roles in H2AV phosphorylation in response to meiotic DSBs, we examined *mei-41*^{D3}*;tefu*^{8} double mutant germaria. At a permissive temperature (18°), *mei-41*^{D3}*;tefu*^{8} displayed a γ-H2AV staining pattern similar in severity to *mei-41*^{D3} single mutants with a mean of 18.2 foci in region 3 oocytes (Fig. 3 [A and B] and Table II). When shifted to the restrictive temperature (25°) for 24 h, no γ-H2AV staining was observed in the *mei-41*^{D3}*;tefu*^{8} region 2a cysts (Fig. 3 C), indicating that these mutants lost the ability to phosphorylate H2AV near newly generated DSBs. This is the first demonstration that ATM and ATR are redundant for the phosphorylation of H2AV in response to meiotic DSBs and is consistent with a study in somatic cells of other organisms (Stucki and Jackson, 2006).

The absence of γ-H2AV staining from *mei-41*^{D3}*;tefu*^{8} double mutant region 2a oocytes indicated that there was no phosphorylation in response to a DSB. However, γ-H2AV was also absent from older region 3 oocytes (Fig. 3 D), indicating that γ-H2AV was lost from DSB sites after only 24 h at the restrictive temperature. That is, based on previous estimates for the timing of cyst progression (12–24 h per region; unpublished data; King, 1970; Spradling, 1993a), the region 3 oocytes were in region 2b (after DSB formation) at permissive temperature and would have had γ-H2AV staining (Fig. 2 E or Fig. 2 F) before the shift to restrictive temperature. The loss of γ-H2AV staining upon shift to restrictive temperature indicates that there is a rapid turnover of the phosphorylation mark near meiotic DSBs. To confirm that the histone H2AV and DSBs were still present in region 3 nuclei, we transferred the *mei-41*^{D3}*;tefu*^{8} double mutants from the restrictive temperature back to the permissive temperature and analyzed γ-H2AV staining. After only 24 h at the permissive temperature, γ-H2AV staining returned to the double mutant oocytes (Fig. 3 [E and F] and Table II), consistent with the presence of unrepaired DSBs and H2AV in region 3 oocytes. These findings indicate that γ-H2AV at meiotic DSB sites is continuously exchanged or dephosphorylated independent of repair and that rephosphorylation of H2AV is maintained by continuous ATM or ATR activity.

H2AV depends on MRG15 to be incorporated into meiotic chromatin

The aforementioned results suggest that a component of the DSB repair response involves dynamic changes in chromatin structure, which may be important to maintain ATM/ATR

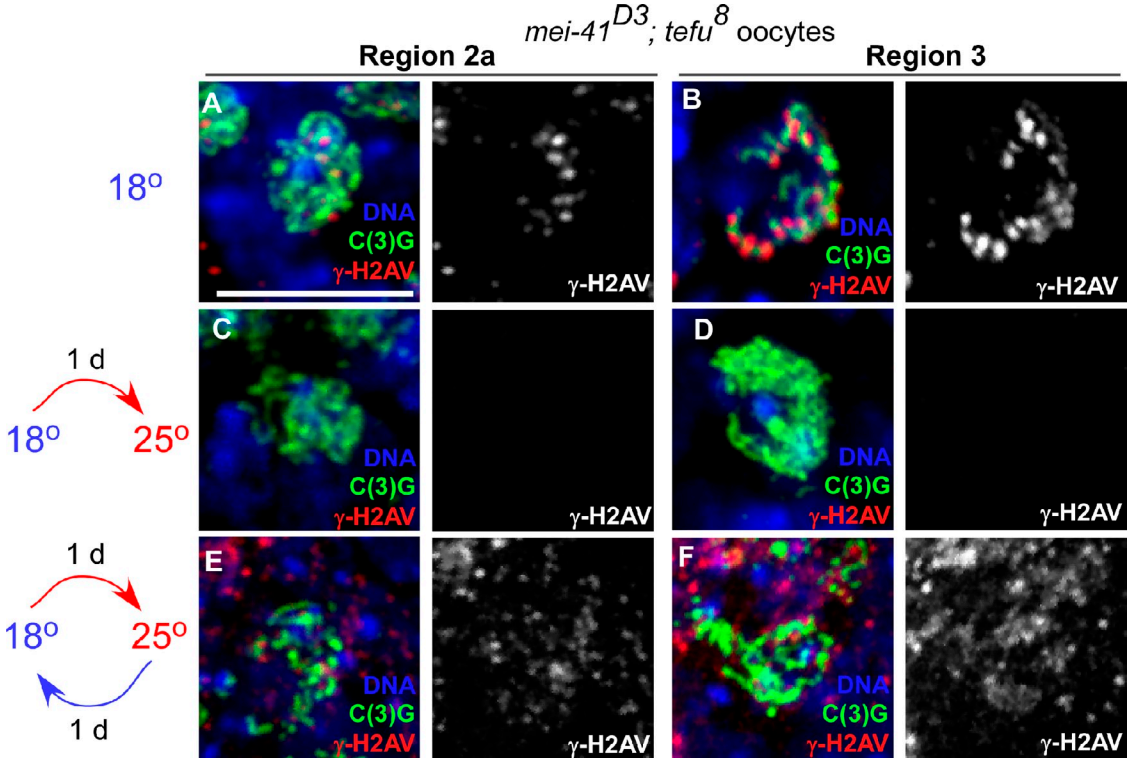

Figure 3. **ATM and ATR are redundant for the phosphorylation of H2AV in response to meiotic DSBs.** (A and B) At the permissive temperature (18°), *mei-41^{D3};tefu^{8}* displayed γ-H2AV foci in region 2a and 3 oocytes, similar to *mei-41^{D3}* single mutants. (C and D) At the restrictive temperature (25°) for 1 d, no γ-H2AV staining was observed in *mei-41^{D3};tefu^{8}* double mutant oocytes. (E and F) When the *mei-41^{D3};tefu^{8}* double mutants were transferred from restrictive temperature back to permissive temperature for 1 d, γ-H2AV staining returned. The staining is less punctate than normal probably because H2AV phosphorylation needs time to accumulate and appear as foci (not depicted; Mehrotra and McKim, 2006). The short time span between the return to permissive conditions and fixation was necessary to accurately stage the oocytes. Bar, 5 µM.

activity until the DSB is repaired. To investigate the mechanism behind the repair-independent constitutive exchange of γ-H2AV, we looked at factors known to regulate H2AV exchange in other cell types. In particular, the exchange of γ-H2AV with unphosphorylated H2AV in somatic cells is preceded by the acetylation of the histone by the Tip60 multiprotein complex (Kusch et al., 2004). We determined whether the Tip60 complex component MRG15 is required for γ-H2AV exchange by creating *MRG15* mutant germline clones (see Materials and methods) and analyzing H2AV levels throughout oogenesis. Strikingly, we observed a complete absence of H2AV, both phosphorylated (not depicted) and unphosphorylated (Fig. 4 A), in *MRG15^{j6A3}* mutant cells throughout oogenesis. Mutant germline clones are generated in the premeiotic stem cells; therefore, these results indicate that MRG15 is required for the incorporation of H2AV into meiotic chromatin. With this function, MRG15 could also be required for a process that promotes γ-H2AV turnover during meiotic prophase by incorporating unphosphorylated H2AV into the nucleosomes after γ-H2AV has been removed (Fig. 4 B).

In addition to the acetyltransferase Tip60, MRG15 has been found in another complex that includes the deacetylase Rpd3 (Lee et al., 2009). We made germline clones of *Rpd3^{04556}* and found that, rather than loss of H2AV, there was abundant γ-H2AV foci and evidence of a repair defect (Fig. S1). These results suggest that the Rpd3 complex is not required for H2AV exchange in the germline. Although the Tip60 complex is a strong candidate for this role, confirmation awaits the analysis of additional Tip60 complex components or the construction of *Tip60* mutants.

DSB-independent removal of H2AV by stage 5 of oogenesis

The aforementioned evidence indicates that γ-H2AV is surprisingly dynamic, being constantly exchanged in a DSB-independent manner. We also confirmed and extended a previous observation (Mehrotra and McKim, 2006) that in mutants with a defect in DSB repair, such as *spn-A^{1}*, *mei-41^{D3}*, and *tefu^{8}*, γ-H2AV labeling persists until stage 5 and yet is never observed in more advanced stages of oogenesis (*spn-A^{1}* shown in Fig. 5 A). We reasoned that this absence of γ-H2AV staining past stage 5 may reflect either a reduction in ATM/ATR activity, use of an alternative repair pathway, or loss of the H2AV substrate from the nucleosomes.

To evaluate the presence of histone H2AV in nucleosomes during oogenesis, we stained ovaries with an H2AV antibody that recognizes both phosphorylated and unphosphorylated versions of the histone variant. As expected, H2AV labeling was abundant throughout the nucleus of all oocytes and nurse cells as well as mitotically dividing follicle cells from the germarium to stage 3 of oogenesis (Fig. 5 B). Strikingly, at stage 4–5 of oogenesis, H2AV staining was drastically reduced in nurse cells and oocytes but not

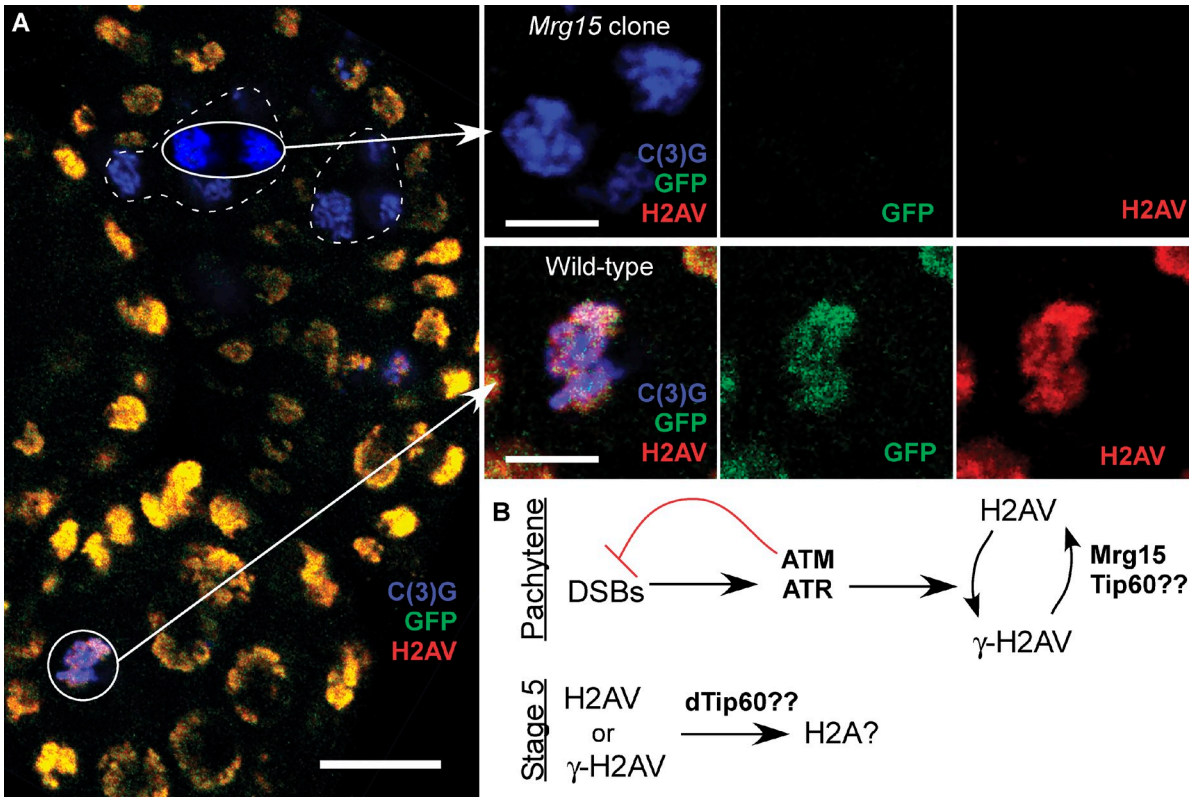

Figure 4. **ATM and ATR in the meiotic DSB response.** (A) *Mrg15* mutant germline clones (dashed circles) were identified by lack of GFP (see Materials and methods). Wild-type oocytes exhibit high levels of H2AV (500,000 fluorescence units), whereas *Mrg15* mutant oocytes lack H2AV (132,000 fluorescence units, similar to background), demonstrating that Mrg15 is required for the incorporation of H2AV into germline chromatin. Bars, 10 µm. (B) A model for the role of ATM and ATR in the regulation of DSB formation and the repair response. Both ATM and ATR are essential for DSB repair (not depicted) and phosphorylate H2AV. Only ATM provides a negative feedback signal to limit the total number of DSBs (red line). The maintenance of γ-H2AV near DSB sites requires continuous ATM or ATR activity as a result of rapid repair-independent H2AV exchange. An Mrg15-containing complex such as Tip60 may be required to incorporate unphosphorylated H2AV into the nucleosomes. It is not clear whether this occurs by direct exchange of γ-H2AV with H2AV as previously described in embryos (Kusch et al., 2004) or via H2A. At stage 5, the attenuation of H2AV incorporation could result in its eventual absence from the nucleosomes.

in follicle cells (Fig. 5 B). This correlates well with the disappearance of γ-H2AV foci in both the oocyte and nurse cells at this stage in repair mutants (Fig. 5 A). Indeed, the absence of H2AV at stage 5 was also found in *spn-A*^{1}, *mei-41*^{D3}, and *tefu*^{8} mutant ovarioles (unpublished data). Therefore, the loss of γ-H2AV signal at stage 5 of oogenesis is a result of the removal of H2AV. Similar results were observed with an H2AV:GFP fusion protein in oocytes, although the signal persisted longer in the nurse cells (Fig. S2). These results have important implications for using γ-H2AV as a DSB reporter late in prophase, as it is impossible to determine whether ATM/ATR responds to DNA damage or whether that damage is repaired before the first meiotic division.

Conclusion

We have shown that the *Drosophila* ATM and ATR kinases have distinct roles in meiotic DSB repair, results that are consistent with the role of ATM in the mouse germline (Xu and Baltimore, 1996; Barchi et al., 2005, 2008; Bellani et al., 2005; Di Giacomo et al., 2005). Unlike ATR, however, ATM is dispensable for the meiotic DSB repair checkpoint, although we cannot rule out a minor role for ATM in the checkpoint because *mei-41* mutants fail to completely suppress the effects of some DSB repair mutants (Ghabrial and Schüpbach, 1999). Interestingly, in *Drosophila* somatic cells, ATM is required for a checkpoint response only at low doses of radiation (Bi et al., 2005). Thus, the amount of damage may be high enough in meiotic cells such that ATR signaling is sufficient for the checkpoint response. An alternative is that the number of breaks is not as significant as how they are processed. DSBs experience rapid resection in meiosis to generate single-stranded DNA, which is necessary for ATR activation (Costanzo et al., 2003; Zou and Elledge, 2003).

ATM and ATR kinases clearly have common targets, such as the phosphorylation of H2AV. Using γ-H2AV as a reporter, we found a surprising dynamic component to this phosphorylation including at least two phases of H2AV clearance in the *Drosophila* female germline (Fig. 4 B). First, γ-H2AV at meiotic DSB sites is rapidly exchanged with unphosphorylated H2AV. Because γ-H2AV is exchanged with H2AV independent of DSB repair, the removal of γ-H2AV from DSB sites after repair may only require the cessation of ATM and ATR activity. Second, most of the H2AV is removed between stages 5 and 6 of oogenesis (after pachytene) and occurs independently of the repair and phosphorylation state.

Our most surprising result is that ATM negatively regulates meiotic DSB formation. Induction of DSBs is essential to generate crossovers. Approximately 20 DSBs occur per meiosis

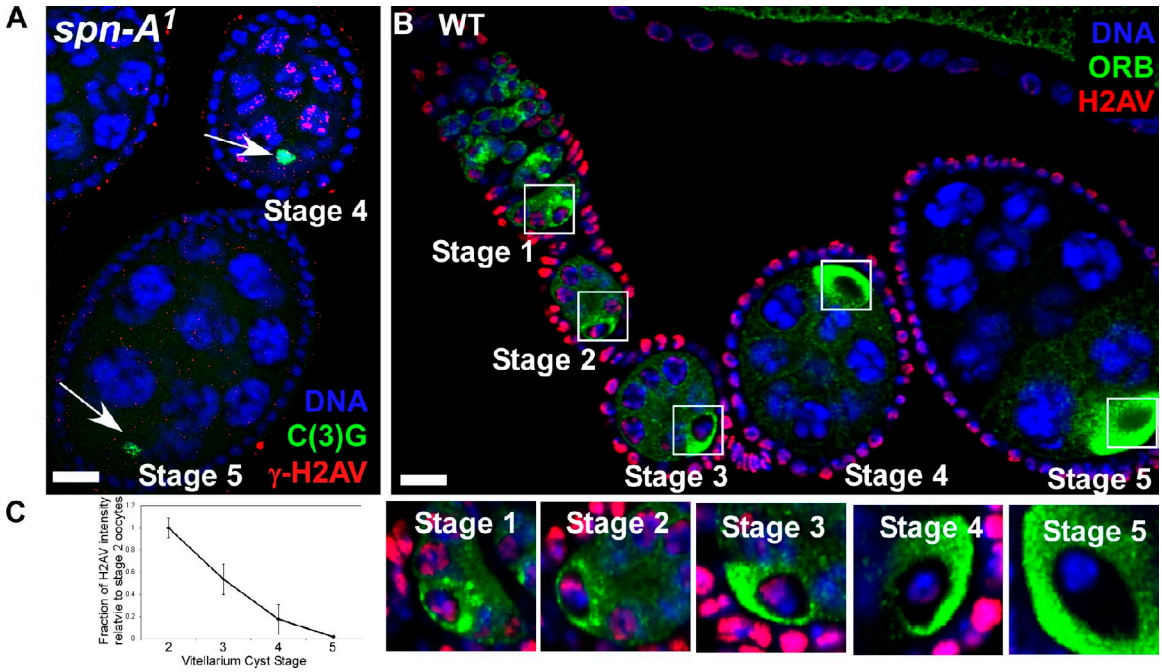

Figure 5. **H2AV is removed by stage 5 of oogenesis.** (A) In *spn-A*^{1} mutants, γ-H2AV foci are not observed past stage 4 of oogenesis. Arrows point to the oocytes. (B) In wild type (WT), an antibody that recognizes both phosphorylated and unphosphorylated versions of H2AV showed abundant staining in germarium cells until stage 3 of oogenesis and was then drastically reduced in egg chambers at stages 4 and 5. The somatic-derived follicle cells surrounding the egg chambers show strong H2AV labeling at all stages. Bars, 5 µm. (C) A graph presenting the relative H2AV fluorescence intensity of wild-type oocytes through oogenesis. The mean fluorescence intensity of H2AV was calculated for oocytes from each stage of two complete ovarioles. The error bars denote the SD.

in *Drosophila*, but only six or seven become crossovers (Mehrotra and McKim, 2006). Similarly, in yeast and mice, a surplus of DSBs is generated to produce crossovers (Keeney, 2001). What remain unknown are the mechanisms that limit the number of DSBs to prevent excessive genomic damage. We suggest that ATM is part of a negative feedback mechanism to limit the total number of DSBs (Fig. 4 B). This mechanism of DSB regulation appears to be conserved, as DSB levels are also increased in mouse spermatocytes lacking ATM (J. Lange, M. Jasin, and S. Keeney, personal communication), which may explain circumstances in which crossovers are increased in the absence of ATM (Barchi et al., 2008).

Materials and methods

Drosophila genetics

The genotype of the temperature-sensitive *tefu*^{8} mutant referred to in this study was *p*^{o} *tefu*^{8} *e* (Silva et al., 2004). *tefu*^{8} mutant progeny were raised at the permissive temperature of 18°C. Once the flies reached adulthood, the *tefu*^{8} mutants were shifted to the restrictive temperature of 25°C. This regimen was based on temperature shift experiments that had previously defined temperature-sensitive phases for specific developmental defects (including lethality or female sterility) in *tefu*^{8} mutants (Silva et al., 2004). After 4 d at the restrictive temperature, *tefu*^{8} mutant germaria failed to produce new cysts, indicating premeiotic cell death. Other alleles analyzed in this study include the following: *mei-41*^{D3} (Laurençon et al., 2003), *mei-W68*^{4572} (Jang et al., 2003), *spn-A*^{1} (Staeva-Vieira et al., 2003), and the H2AV:GFP fusion protein (no. 1719; Clarkson and Saint, 1999). A *P(neoFRT)82B MRG15*^{i6A3} chromosome was made to generate germline mutant clones using the FLP recombination target/FLP system (Chou and Perrimon, 1992). Mutant cells in the germlines of *P(neoFRT)82B MRG15*^{i6A3}*/P(neoFRT)82B P(Ubi-GFP(S65T)nls)3R* females that were expressing FLPase were identified by the lack of GFP expression. A similar strategy was used to analyze *Rpd3* germline clones using an *Rpd3*^{04556} *P(FRT(w*^{hs}*))2A* chromosome.

Cytology and immunofluorescence

For immunolocalization experiments, females were aged at room temperature for ∼16 h (unless otherwise noted as in *tefu*^{8} mutants), and ovaries were dissected and fixed using the buffer A protocol (McKim et al., 2009). In brief, the ovaries from 15–20 flies were dissected in 1× Robb's media and moved to a clean well containing fresh media. A tungsten needle was used to remove the ovariolar sheath and to tease the ovaries apart. After no more than 20 min, the separated ovaries were moved to the cap of a graduated 1.5-ml Eppendorf tube containing 500 µl of buffer A fixative solution for 10 min at room temperature. After several washes, the primary antibodies were diluted into a volume of 300 µl. The antibody to γ-H2AV (Mehrotra and McKim, 2006) was used at a 1:500 dilution. An unpurified version of the antibody that recognized all H2AV was used at 1:500. Additional primary antibodies included mouse anti-C(3)G antibody used at 1:500 (Page and Hawley, 2001), a combination of two mouse anti-Orb antibodies (4H8 and 6H4) used at 1:100 (Lantz et al., 1994), and a mouse anti-GRK used at 1:10 (Queenan et al., 1999). The secondary antibodies were Cy3-labeled goat anti–rabbit (Jackson ImmunoResearch Laboratories, Inc.) used at 1:250 and Alexa Fluor 488 goat anti–mouse (Invitrogen) used at 1:100. Chromosomes were stained with Hoechst at 1:50,000 (10 mg/ml solution) for 7 min at room temperature and mounted in SlowFade (Invitrogen). Images were collected using a true confocal scanning microscope (SP2; Leica) with a 63× 1.3 NA lens in a room maintained at 20–22°C. In most cases, whole germaria were imaged by collecting optical sections through the entire tissue. These datasets are shown as maximum projections generated by the Leica confocal software and then cropped in Photoshop (Adobe). However, the analysis of the images was performed by examining one section at a time. The γ-H2AV foci were counted manually by examining each section in a full series of optical sections containing complete pro-oocyte nucleus (Joyce and McKim, 2009). C(3)G staining was used to identify oocytes.

Estimating γ-H2AV foci numbers by quantitative measurement of fluorescence intensity

The method used consists of scanning several individual γ-H2AV foci as well as γ-H2AV staining within an adjacent oocyte in serial sections and displaying each as a suitable projection. We then divided the fluorescence intensity in such projection from a single oocyte by that of a mean of single foci in an adjacent cell of the same image. For *tefu*^{8} mutants, we averaged

the results of 12 oocytes taken from 6 different germaria, which provided an estimate of the total number of foci present. We tested this method in wild-type germaria in which γ-H2AV foci could be counted. 8 wild-type oocytes with 10 γ-H2AV foci each were estimated to have a mean of 11 (±1) foci when measured by fluorescence intensity. We concluded that this method is accurate and suitable to estimate foci levels within meiotic cells. P-values were calculated using an unpaired *t* test.

Online supplemental material

Fig. S1 shows H2AV staining in *Rpd3* mutant germline clones. Fig. S2 shows H2AV:GFP localization to the chromatin within stage 2 and 5 egg chambers. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201104121/DC1.

We are grateful to Janet Jang and Li Nguyen for technical assistance and Scott Keeney for insightful discussions. Some stocks used in this study were received from the Bloomington Drosophila Stock Center at Indiana University, and some antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, developed under the auspices of the National Institute of Child Health and Human Development.

This work was supported by fellowships from the Busch foundation to E.F. Joyce, the National Science Foundation to K.S. McKim, and the Natural Sciences and Engineering Research Council and A-T Children's Project to S.D. Campbell.

Submitted: 22 April 2011
Accepted: 22 September 2011

References

Abdu, U., M. Brodsky, and T. Schüpbach. 2002. Activation of a meiotic checkpoint during *Drosophila* oogenesis regulates the translation of Gurken through Chk2/Mnk. *Curr. Biol.* 12:1645–1651. http://dx.doi.org/10.1016/S0960-9822(02)01165-X

Barchi, M., S. Mahadevaiah, M. Di Giacomo, F. Baudat, D.G. de Rooij, P.S. Burgoyne, M. Jasin, and S. Keeney. 2005. Surveillance of different recombination defects in mouse spermatocytes yields distinct responses despite elimination at an identical developmental stage. *Mol. Cell. Biol.* 25:7203–7215. http://dx.doi.org/10.1128/MCB.25.16.7203-7215.2005

Barchi, M., I. Roig, M. Di Giacomo, D.G. de Rooij, S. Keeney, and M. Jasin. 2008. ATM promotes the obligate XY crossover and both crossover control and chromosome axis integrity on autosomes. *PLoS Genet.* 4:e1000076. http://dx.doi.org/10.1371/journal.pgen.1000076

Bellani, M.A., P.J. Romanienko, D.A. Cairatti, and R.D. Camerini-Otero. 2005. SPO11 is required for sex-body formation, and Spo11 heterozygosity rescues the prophase arrest of $Atm^{-/-}$ spermatocytes. *J. Cell Sci.* 118:3233–3245. http://dx.doi.org/10.1242/jcs.02466

Bi, X., S.C. Wei, and Y.S. Rong. 2004. Telomere protection without a telomerase; the role of ATM and Mre11 in *Drosophila* telomere maintenance. *Curr. Biol.* 14:1348–1353. http://dx.doi.org/10.1016/j.cub.2004.06.063

Bi, X., M. Gong, D. Srikanta, and Y.S. Rong. 2005. *Drosophila* ATM and Mre11 are essential for the G2/M checkpoint induced by low-dose irradiation. *Genetics*. 171:845–847. http://dx.doi.org/10.1534/genetics.105.047720

Burma, S., B.P. Chen, M. Murphy, A. Kurimasa, and D.J. Chen. 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J. Biol. Chem.* 276:42462–42467. http://dx.doi.org/10.1074/jbc.C100466200

Chou, T.B., and N. Perrimon. 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics*. 131:643–653.

Clarkson, M., and R. Saint. 1999. A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for *Drosophila* chromosome behavior. *DNA Cell Biol.* 18:457–462. http://dx.doi.org/10.1089/104454999315178

Costanzo, V., D. Shechter, P.J. Lupardus, K.A. Cimprich, M. Gottesman, and J. Gautier. 2003. An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. *Mol. Cell*. 11:203–213. http://dx.doi.org/10.1016/S1097-2765(02)00799-2

Di Giacomo, M., M. Barchi, F. Baudat, W. Edelmann, S. Keeney, and M. Jasin. 2005. Distinct DNA-damage-dependent and -independent responses drive the loss of oocytes in recombination-defective mouse mutants. *Proc. Natl. Acad. Sci. USA*. 102:737–742. http://dx.doi.org/10.1073/pnas.0406212102

Ghabrial, A., and T. Schüpbach. 1999. Activation of a meiotic checkpoint regulates translation of Gurken during *Drosophila* oogenesis. *Nat. Cell Biol.* 1:354–357. http://dx.doi.org/10.1038/14046

Ghabrial, A., R.P. Ray, and T. Schüpbach. 1998. *okra* and *spindle-B* encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in *Drosophila* oogenesis. *Genes Dev.* 12:2711–2723. http://dx.doi.org/10.1101/gad.12.17.2711

Gorski, M.M., R.J. Romeijn, J.C. Eeken, A.W. de Jong, B.L. van Veen, K. Szuhai, L.H. Mullenders, W. Ferro, and A. Pastink. 2004. Disruption of *Drosophila* Rad50 causes pupal lethality, the accumulation of DNA double-strand breaks and the induction of apoptosis in third instar larvae. *DNA Repair (Amst.)*. 3:603–615. http://dx.doi.org/10.1016/j.dnarep.2004.02.001

Hawley, R.S. 1988. Exchange and Chromosome Segregation in Eukaryotes. In Genetic Recombination. American Society for Microbiology, Washington, D.C. 743 pp.

Jaklevic, B.R., and T.T. Su. 2004. Relative contribution of DNA repair, cell cycle checkpoints, and cell death to survival after DNA damage in *Drosophila* larvae. *Curr. Biol.* 14:23–32. http://dx.doi.org/10.1016/j.cub.2003.12.032

Jang, J.K., D.E. Sherizen, R. Bhagat, E.A. Manheim, and K.S. McKim. 2003. Relationship of DNA double-strand breaks to synapsis in *Drosophila*. *J. Cell Sci.* 116:3069–3077. http://dx.doi.org/10.1242/jcs.00614

Joyce, E.F., and K.S. McKim. 2009. *Drosophila* PCH2 is required for a pachytene checkpoint that monitors double-strand-break-independent events leading to meiotic crossover formation. *Genetics*. 181:39–51. http://dx.doi.org/10.1534/genetics.108.093112

Keeney, S. 2001. Mechanism and control of meiotic recombination initiation. *Curr. Top. Dev. Biol.* 52:1–53. http://dx.doi.org/10.1016/S0070-2153(01)52008-6

King, R.C. 1970. Ovarian Development in *Drosophila melanogaster*. Academic Press, New York. 227 pp.

Kusch, T., L. Florens, W.H. Macdonald, S.K. Swanson, R.L. Glaser, J.R. Yates III, S.M. Abmayr, M.P. Washburn, and J.L. Workman. 2004. Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. *Science*. 306:2084–2087. http://dx.doi.org/10.1126/science.1103455

Lantz, V., J.S. Chang, J.I. Horabin, D. Bopp, and P. Schedl. 1994. The *Drosophila* orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. *Genes Dev.* 8:598–613. http://dx.doi.org/10.1101/gad.8.5.598

Laurençon, A., A. Purdy, J. Sekelsky, R.S. Hawley, and T.T. Su. 2003. Phenotypic analysis of separation-of-function alleles of MEI-41, *Drosophila* ATM/ATR. *Genetics*. 164:589–601.

Lee, N., H. Erdjument-Bromage, P. Tempst, R.S. Jones, and Y. Zhang. 2009. The H3K4 demethylase lid associates with and inhibits histone deacetylase Rpd3. *Mol. Cell. Biol.* 29:1401–1410. http://dx.doi.org/10.1128/MCB.01643-08

Madigan, J.P., H.L. Chotkowski, and R.L. Glaser. 2002. DNA double-strand break-induced phosphorylation of *Drosophila* histone variant H2Av helps prevent radiation-induced apoptosis. *Nucleic Acids Res.* 30:3698–3705. http://dx.doi.org/10.1093/nar/gkf496

McCaffrey, R., D. St Johnston, and A. González-Reyes. 2006. *Drosophila* mus301/spindle-C encodes a helicase with an essential role in double-strand DNA break repair and meiotic progression. *Genetics*. 174:1273–1285. http://dx.doi.org/10.1534/genetics.106.058289

McKim, K.S., and A. Hayashi-Hagihara. 1998. *mei-W68* in *Drosophila melanogaster* encodes a Spo11 homolog: Evidence that the mechanism for initiating meiotic recombination is conserved. *Genes Dev.* 12:2932–2942. http://dx.doi.org/10.1101/gad.12.18.2932

McKim, K.S., E.F. Joyce, and J.K. Jang. 2009. Cytological analysis of meiosis in fixed *Drosophila* ovaries. *Methods Mol. Biol.* 558:197–216. http://dx.doi.org/10.1007/978-1-60761-103-5_12

Mehrotra, S., and K.S. McKim. 2006. Temporal analysis of meiotic DNA double-strand break formation and repair in *Drosophila* females. *PLoS Genet.* 2:e200. http://dx.doi.org/10.1371/journal.pgen.0020200

Page, S.L., and R.S. Hawley. 2001. *c(3)G* encodes a *Drosophila* synaptonemal complex protein. *Genes Dev.* 15:3130–3143. http://dx.doi.org/10.1101/gad.935001

Pedersen, M., S. Tiong, and S.D. Campbell. 2010. Molecular genetic characterization of *Drosophila* ATM conserved functional domains. *Genome*. 53:778–786. http://dx.doi.org/10.1139/G10-067

Queenan, A.M., G. Barcelo, C. Van Buskirk, and T. Schüpbach. 1999. The transmembrane region of Gurken is not required for biological activity, but is necessary for transport to the oocyte membrane in *Drosophila*. *Mech. Dev.* 89:35–42. http://dx.doi.org/10.1016/S0925-4773(99)00196-3

Queiroz-Machado, J., J. Perdigão, P. Simões-Carvalho, S. Herrmann, and C.E. Sunkel. 2001. tef: A mutation that causes telomere fusion and severe genome rearrangements in *Drosophila* melanogaster. *Chromosoma*. 110:10–23. http://dx.doi.org/10.1007/s004120000116

Shiloh, Y. 2006. The ATM-mediated DNA-damage response: Taking shape. *Trends Biochem. Sci.* 31:402–410. http://dx.doi.org/10.1016/j.tibs.2006.05.004

Sibon, O.C., A. Laurençon, R. Hawley, and W.E. Theurkauf. 1999. The *Drosophila* ATM homologue Mei-41 has an essential checkpoint function at the midblastula transition. *Curr. Biol.* 9:302–312. http://dx.doi.org/10.1016/S0960-9822(99)80138-9

Silva, E., S. Tiong, M. Pedersen, E. Homola, A. Royou, B. Fasulo, G. Siriaco, and S.D. Campbell. 2004. ATM is required for telomere maintenance and chromosome stability during *Drosophila* development. *Curr. Biol.* 14: 1341–1347. http://dx.doi.org/10.1016/j.cub.2004.06.056

Song, Y.H., G. Mirey, M. Betson, D.A. Haber, and J. Settleman. 2004. The *Drosophila* ATM ortholog, dATM, mediates the response to ionizing radiation and to spontaneous DNA damage during development. *Curr. Biol.* 14:1354–1359. http://dx.doi.org/10.1016/j.cub.2004.06.064

Spradling, A.C. 1993a. Developmental Genetics of Oogenesis. In the Development of Drosophila melanogaster. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1564 pp.

Spradling, A.C. 1993b. Germline cysts: Communes that work. *Cell*. 72:649–651. http://dx.doi.org/10.1016/0092-8674(93)90393-5

Staeva-Vieira, E., S. Yoo, and R. Lehmann. 2003. An essential role of DmRad51/SpnA in DNA repair and meiotic checkpoint control. *EMBO J.* 22:5863–5874. http://dx.doi.org/10.1093/emboj/cdg564

Stucki, M., and S.P. Jackson. 2006. gammaH2AX and MDC1: Anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst.)*. 5:534–543. http://dx.doi.org/10.1016/j.dnarep.2006.01.012

Walker, M.Y., and R.S. Hawley. 2000. Hanging on to your homolog: The roles of pairing, synapsis and recombination in the maintenance of homolog adhesion. *Chromosoma*. 109:3–9. http://dx.doi.org/10.1007/s004120050407

Ward, I.M., and J. Chen. 2001. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J. Biol. Chem.* 276:47759–47762. http://dx.doi.org/10.1074/jbc.M009785200

Wyman, C., and R. Kanaar. 2006. DNA double-strand break repair: All's well that ends well. *Annu. Rev. Genet.* 40:363–383. http://dx.doi.org/10.1146/annurev.genet.40.110405.090451

Xu, Y., and D. Baltimore. 1996. Dual roles of ATM in the cellular response to radiation and in cell growth control. *Genes Dev.* 10:2401–2410. http://dx.doi.org/10.1101/gad.10.19.2401

Youds, J.L., and S.J. Boulton. 2011. The choice in meiosis - defining the factors that influence crossover or non-crossover formation. *J. Cell Sci.* 124:501–513. http://dx.doi.org/10.1242/jcs.074427

Zou, L., and S.J. Elledge. 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*. 300:1542–1548. http://dx.doi.org/10.1126/science.1083430