DNA damage-induced Lok/CHK2 activation compromises germline stem cell self-renewal and lineage differentiation

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ABSTRACT
Stem cells in adult tissues are constantly exposed to genotoxic stress and also accumulate DNA damage with age. However, it remains largely unknown how DNA damage affects both stem cell self-renewal and differentiation. In this study, we show that DNA damage retards germline stem cell (GSC) self-renewal and progeny differentiation in a Lok kinase-dependent manner in the Drosophila ovary. Both heat-shock-inducible endonuclease I-Crel expression and X-ray irradiation can efficiently introduce double-strand breaks in GSCs and their progeny, resulting in a rapid GSC loss and a GSC progeny differentiation defect. Surprisingly, the elimination of Lok or its kinase activity can almost fully rescue the GSC loss and the progeny differentiation defect caused by DNA damage induced by I-Crel or X-ray. In addition, the reduction in bone morphogenetic protein signaling and Shotgun expression only makes a limited contribution to DNA damage-induced GSC loss. Finally, DNA damage also decreases the expression of the master differentiation factor Bam in a Lok-dependent manner, which helps explain the GSC progeny differentiation defect. Therefore, this study demonstrates, for the first time in vivo, that Lok kinase activation is required for the DNA damage-mediated disruption of adult stem cell self-renewal and lineage differentiation, and might also offer novel insight into how DNA damage causes tissue aging and cancer formation.

KEY WORDS: CHK2, Differentiation, Germline stem cell, Lok, Niche, Self-renewal

INTRODUCTION
Stem cells in adult tissues have the unique ability to self-renew and generate differentiated cells that replace lost cells caused by natural turnover, disease or injury, thus maintaining tissue homeostasis. Stem cells in some adult tissues, including skin and intestine, are constantly exposed to environmental toxins, UV light or other DNA-damaging agents (Signer and Morrison, 2013; Sperka et al., 2012). In addition, DNA damage accumulates in stem cells of aged tissues (Rossi et al., 2007; Sotiroupolou et al., 2010). DNA damage has been proposed to compromise self-renewal and proliferation, thus accelerating tissue aging and even degeneration (Signer and Morrison, 2013; Sperka et al., 2012). It has also long been linked to cancer formation possibly by enhancing formation of cancer stem cells (CSCs) (Clarke and Fuller, 2006; Reya et al., 2001; Rosen and Jordan, 2009). However, it remains unknown how DNA damage causes tissue aging and CSC formation.

In the Drosophila ovary, two or three GSCs are located at the niche, which is composed of adjacent cap cells and escort cells (Lin, 2002; Xie, 2013) (Fig. 1A). They can be easily identified by their location and the intracellular organelle known as the spectrosome: GSCs physically interact with cap cells and contain an anteriorly anchored spectrosome. They can be effectively distinguished from their immediate differentiating daughters, cystoblasts (CBs), because CBs are one cell away from cap cells and contain a randomly localized spectrosome. The niche provides bone morphogenetic protein (BMP) signals, encoded by dpp and gbb, which function within one cell diameter to repress bam expression directly and thereby maintain GSC self-renewal (Chen and McKearin, 2003a; Song et al., 2004; Xie and Spradling, 1998). In addition, niche-expressing E-cadherin (Shotgun in Drosophila) is also required for anchoring GSCs in the niche for long-term self-renewal (Song et al., 2002). Moreover, different levels of Shotgun can affect the ability of GSCs to compete for niche occupancy (Jin et al., 2008). Because CBs are positioned one cell away from the niche, BMP signaling is inactivated at multiple levels, allowing bam expression to be activated and further drive germ cell differentiation (Chen and McKearin, 2003a; Chen et al., 2011; Song et al., 2004). Bam is necessary and sufficient for GSC differentiation (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). Mechanistically, Bam is recruited to its target mRNAs through direct binding or its RNA-binding partners, such as Bgcn and Sxl, to repress target mRNA translation (Chau et al., 2009, 2012; Li et al., 2009, 2013). Bam works with Smurf to repress BMP signaling in GSC progeny by unknown mechanisms (Casanueva and Ferguson, 2004). Bam can also inactivate or convert the self-renewal functions of the translation initiation eIF4 complex, the deadenylase CCR4-NOT complex and the COP9 signalosome complex by directly binding to one component of these complexes, eIF-4A, CSN4 and Twin, respectively (Fu et al., 2015; Pan et al., 2014; Shen et al., 2009). Therefore, Bam controls GSC progeny differentiation via multiple independent mechanisms.

In organisms ranging from Drosophila to human, DNA damage causes the activation of the highly conserved checkpoint kinases CHK2 (CHEK2), ATM and ATR in various cell types, but their roles in the response of adult stem cells to DNA damage remain to be defined. Interestingly, ATM has been shown to be required for maintaining self-renewal of adult hematopoietic stem cells and germ line stem cells in mice in the absence of DNA damage (Ito et al., 2004; Takubo et al., 2008), and ATR has also been shown to be required in multiple tissue stem cells in mice (Ruzankina et al., 2007). In Drosophila, both Mei-41 (ATR) and Tefu (ATM) are required to maintain intestinal stem cells in the absence of DNA damage (Park et al., 2015). However, it remains unclear whether CHK2 (Lok in Drosophila) is also implicated in maintaining self-renewal of adult stem cells in the absence of DNA damage.
During meiosis, double-stranded DNA breaks are naturally generated to promote homologous recombination (Lake and Hawley, 2012). These breaks are efficiently repaired after recombination. However, persistent DNA damage caused by mutations in Piwi-interacting RNA (piRNA) pathway components, such as cuff, aub and armi, can evoke meiotic checkpoint activation, thus blocking oocyte development and normal egg pattern formation (Abdu et al., 2002; Chen et al., 2007; Klattenhoff et al., 2007). Meiotic checkpoint activation requires the function of the two highly conserved kinases, CHK2 and ATR. A recent study has shown that inactivation of CHK2 can reverse the female sterility caused by DNA damage in mice, indicating that CHK2 has a conserved role in DNA damage-induced checkpoint control in germ cells (Bolcun-Filas et al., 2014). Among piRNA mutants, only cuff mutant ovaries were reported to lose some GSCs, but it remains unclear whether the GSC loss caused by the cuff mutation is due to DNA damage or piRNA loss (Chen et al., 2007). In this study, we show that DNA damage causes GSC loss as well as the retardation of GSC progeny differentiation in a Lok-dependent manner. Stem cell loss could cause premature tissue aging, whereas the accumulation of ill-differentiated stem cell progeny could increase the chance of cancer stem cell formation (Clarke and Fuller, 2006; Rosen and Jordan, 2009; Signer and Morrison, 2013). Therefore, our findings might offer insight into how DNA damage leads to premature tissue aging and cancer formation in humans.

RESULTS

The inducible I-CreI system can efficiently introduce DNA damage in GSCs and their progeny, leading to GSC loss and retarded progeny differentiation

To investigate the effect of DNA damage on GSC self-renewal and differentiation, we used endonuclease I-CreI under the control of a heat-shock promoter (hs-I-CreI or CreI) to induce DNA damage in...
GSCs and their progeny by incubating female flies at 37°C for 1 h (Rong et al., 2002). The endonuclease I-CreI can introduce double-stranded DNA breaks in 18S ribosome gene repeats (Maggert and Golic, 2005; Royou et al., 2005) (Fig. 1B). In *Drosophila*, one rDNA locus on the X chromosome contains 18S, 5.8S and 28S repeats, and the other locus on the second chromosome contains 5S rDNA repeats (Richard et al., 2008). To evaluate the generation, repair and persistence of DNA damage in GSCs and their progeny, the ovaries from adult females 1 day and 3 days after a 1-h heat-shock treatment (AHS) were labeled for Hu li tai shao (Hts) and γ-H2Av. Hts is a protein marker for identifying germline-specific organelles, the spherical fusome or spectrosome in GSCs and CBs as well as the branched fusome in differentiated germ cell cysts (Lin et al., 1994), whereas γ-H2Av is a phosphorylated form of H2Av (His2Av) commonly used as a DNA damage marker (Jang et al., 2003). GSCs are identified by their direct contact with cap cells and the presence of an anteriorly anchored spectrosome, whereas CBs are identified by their one-cell distance from cap cells and the presence of a spectrosome (Xie, 2013) (Fig. 1A,C). Without I-CreI induction, γ-H2Av accumulates in the nucleus of meiotic germ cells with double-stranded DNA breaks, but is generally absent from GSCs and mitotic germ cell cysts (Fig. 1C). One day after heat-shock-mediated I-CreI induction (1 day AHS), extensive DNA damage can be detected in all cell types of ovaries, including GSCs and their early progeny (Fig. 1D). Although I-CreI can only cut 18S rRNA gene repeats, surprisingly, about 38 γH2Av foci exist in each GSC immediately after heat-shock treatment, suggesting that I-CreI might cut additional sites in GSCs (Fig. S1). Interestingly, DNA damage in GSCs appears to be more efficiently repaired than that in CBs and other differentiated GSC progeny because it is absent from GSCs but still persistent in CBs and cysts 3 days AHS (Fig. 1E,F). Additionally, GSCs undergo cell cycle arrest immediately after DNA damage, and then resume rapid cell proliferation after repairing DNA damage based on bromodeoxyuridine (BrdU) and phosphorylated histone H3 (pH3) expression (Fig. S2). These results indicate that I-CreI can efficiently induce DNA damage in GSCs, which can also be efficiently repaired.

Normally, the control *hs-I-CreI germlia* [no heat shock (HS)] contain an average of 2.5 GSCs (Fig. 1G,H). Following DNA damage, the germlia maintain an average of 2.2, 1.0 and 1.2 GSCs 1 day AHS, 3 days AHS and 1 week AHS, respectively (Fig. 1G-J). Consequently, 1 week AHS, the germlia either contain one GSC or completely lose their GSCs (Fig. 1G,J). After DNA damage, some germlia also often contain more spectrosome-containing CB-like single germ cells than the control germlia (Fig. 1G,J). These results indicate that DNA damage affects GSC maintenance and CB differentiation.

DNA damage-induced GSC loss could be due to apoptosis, differentiation or both. We determined whether DNA damage causes GSC loss due to apoptosis by examining the expression of cleaved Caspase-3 (an apoptosis indicator) and overexpressing the *Baculovirus* anti-apoptosis p35 gene. The cleaved Caspase-3 antibody is a reliable tool for identifying apoptotic cells in *Drosophila*, whereas p35 overexpression can effectively prevent Caspase-dependent apoptosis in *Drosophila* (Hay et al., 1994; Yu et al., 2002). Though it could be readily detected in differentiated germ cells or somatic cells, expression of cleaved Caspase-3 was not detected in the examined 143 normal GSCs as well as 180 DNA damaged GSCs (118 GSCs 1 day AHS and 62 GSCs 2 days AHS) (Fig. 1K-M). Consistent with this, germline-specific p35 overexpression could not prevent the DNA damage-induced GSC loss (Fig. S3). Taken together, these results suggest that the GSC loss caused by DNA damage is unlikely to be due to apoptosis, although we cannot completely rule out other forms of cell death.

One concern is that I-CreI-induced double-stranded breaks in the rDNA region could cause some deletion of rDNA repeats, resulting in the reduction of rRNA production. rRNAs are a component of ribosomes that are crucial for protein synthesis; I-CreI-induced GSC loss could be due to decreased protein synthesis because protein synthesis is essential for GSC maintenance (Sanchez et al., 2016; Shen et al., 2009; Zhang et al., 2014). First, our quantitative PCR results show that 18S rDNA repeat numbers and 18S rRNA levels remain unchanged in the I-CreI-expressing germlia in comparison with the control germlia (Fig. S4A-C). The nucleolus is the site of rRNA transcription and processing and of ribosome assembly; its size is correlated with rRNA production (Zhang et al., 2014). Our results show that DNA damage does not decrease the size of the nucleolus (Fig. S4D-H). Taken together, I-CreI-induced double-strand breaks in rDNA repeats is unlikely to affect rRNA production and thus protein synthesis.

Next, we determined whether X-ray-induced DNA damage could also affect both GSC self-renewal and progeny differentiation. By testing different doses of X-ray irradiation for their effect on DNA damage and GSC development, we found that 20,000 rad X-ray irradiation could efficiently introduce DNA damage in GSCs, which was also efficiently repaired (Fig. S5). Consistently, the X-ray-induced DNA damage also caused GSC loss and increased the accumulation of CB-like single germ cells just like 2-h I-CreI expression-induced DNA damage (Fig. 1N-Q). These results further confirm that DNA damage compromises GSC self-renewal and progeny differentiation, and also indicate that the I-CreI system is a convenient genetic method for inducing DNA damage in GSCs.

**Lok is largely responsible for the DNA damage-induced GSC loss**

DNA damage evokes cell cycle checkpoint activation through activation of the highly conserved kinases ATM, ATR, CHK1 and CHK2 in mammalian cells, including stem cells; CHK1 and CHK2 function downstream of ATR and ATM, respectively (Sperka et al., 2012). In the *Drosophila* ovary, the homologues of CHK2 and ATR (Lok and Mei-41, respectively) have been implicated in DNA damage-induced meiotic checkpoint control in germ cells (Abdu et al., 2002; Chen et al., 2007; Klattenhoff et al., 2007). First, we tested whether Lok is required for the DNA damage-induced GSC loss and progeny differentiation defect. lok<sup>kn</sup> heterozygous and homozygous females are viable and fertile, and their germlia carry two to three GSCs (Fig. 2A,B,E). lok<sup>kn</sup> is a molecularly null allele (Abdu et al., 2002). Here, the 2-h heat-shock was used to generate a more severe GSC loss phenotype for testing Lok requirement: most of the wild-type germlia harbor no GSCs 3 days and 1 week after the induction (Fig. 2C,E compared with Fig. 1G). By contrast, most of the *lok* heterozygous and homozygous germlia still maintain two GSCs 3 days and 1 week AHS (Fig. 2D,E). Similarly, both the heterozygous and homozygous *lok* mutations can significantly and drastically rescue the GSC loss caused by X-ray-induced DNA damage (Fig. 2F-H). Interestingly, germline-specific *lok* knockdown (*lok*-I) also significantly and drastically rescues the GSC loss phenotype, although alone it does not affect GSC maintenance (Fig. 2I-K). Quantitatively, *lok*-I and the deletion mutant *lok*<sup>kn</sup> have similar rescue effect on DNA damage-evoked GSC loss, suggesting that Lok is required intrinsically for DNA damage-induced GSC loss (Fig. 2E,K). Therefore, DNA damage-induced GSC loss is largely Lok dependent.
We then investigated whether Mei-41 is also required for DNA damage-induced checkpoint activation in GSCs by examining GSC and CB numbers in DNA-damaged mei-41 mutant germaria. As the controls, both heterozygous and homozygous mei-41 mutations appear to have little effect on GSC number in the absence of DNA damage (Fig. 3A,B,E). In contrast to the homozygous lok mutation, the homozygous mei-41 mutation exacerbates the DNA damage-induced GSC-loss phenotype (Fig. 3C-E). Consistent with this, germline-specific mei-41 knockdown by the two RNAi lines drastically and significantly enhances DNA damage-induced GSC loss (Fig. 3F-J). These results demonstrate that Mei-41 is required for preventing DNA damage-induced GSC loss, and further suggest that Mei-41 and Lok have distinct roles in mediating DNA damage-induced GSC loss.

To determine whether the Drosophila homologues for ATM and CHK1 (Tefu and Grp, respectively) are also required for the DNA damage-induced GSC loss, we knocked down tefu andgrp expression specifically in germ cells. In the absence of DNA damage, germline-specific tefu knockdown (tefu-i) significantly decreases the number of GSCs, and the tefu-i germaria contain one GSC on average (Fig. 3J,K). This is consistent with the findings that ATM is required to maintain hematopoietic and germline stem cells in mice (Ito et al., 2004; Takubo et al., 2008). In the absence of DNA damage, germline-specific grp knockdown (grp-i) germaria carry 2.5 GSCs on average, indicating that Grp is dispensable for GSC maintenance (Fig. 3J,M). Interestingly, tefu-i, but not grp-i, significantly enhances the GSC loss caused by DNA damage, and thus most of the DNA-damaged tefu-i and grp-i germaria contain zero or one GSC, respectively (Fig. 3J,L,N). These results indicate that Tefu is required for, but Grp is dispensable for, preventing DNA damage-induced GSC loss.

Lok kinase activity is required for the DNA damage-induced GSC loss

ATR and ATM are known to function upstream to activate CHK2 kinase activity in response to DNA damage (Sperka et al., 2012). Because ATR behaves differently from CHK2 in GSCs in response to DNA damage, we sought to determine whether the kinase activity of Lok is required for DNA damage-induced GSC loss. To this end, we used the Cas9/CRISPR technique to introduce a point mutation into the endogenous lok locus, which converts the residue D into A at the 286 amino acid of Lok, to create the lokKD allele, encoding a kinase-dead Lok (Ren et al., 2014). The heterozygous and homozygous lokKD mutant germaria contain slightly more GSCs than the control and lokP6 mutant ones (Fig. 4A,C). Like lokP6, both heterozygous and homozygous lokKD mutations can significantly...
and drastically rescue the GSC loss induced by DNA damage produced by I-CreI (Fig. 4B,C). Similarly, both heterozygous and homozygous \( \text{lok}^{KD} \) mutations can significantly and drastically rescue the GSC loss induced by X-rays (Fig. 4D-F). All these results demonstrate that the kinase activity of Lok is crucial for DNA damage-induced GSC loss.

\( p53 \) is required to prevent DNA damage-induced GSC loss

In mammalian cells, DNA damage-induced activation of ATM-CHK2 and ATR-CHK1 results in p53 protein phosphorylation, which uses transcription-dependent and independent mechanisms to slow down cell cycle for DNA repair (Lord and Ashworth, 2012; Sperka et al., 2012). Recent studies have also shown that p53 activity in \( Drosophila \) ovarian GSCs is activated in response to DNA damage (Lu et al., 2010; Wylie et al., 2014). To determine whether p53 is also involved in DNA damage-induced GSC loss, we examined GSC numbers in DNA-damaged control and p53 mutant germaria. Without DNA damage (no HS), the p53 homozygous mutant germaria have an average of 2.5 GSCs just like in the control and p53 heterozygous germaria (Fig. 4G,I). Three days after DNA damage, the p53 homozygous mutant germaria have an average of one GSC, as in the control germaria, though the p53 heterozygous germaria have slightly more GSCs (Fig. 4I). Interestingly, one week after DNA damage, the p53 homozygous mutant germaria have significantly fewer GSCs than the control germaria (Fig. 4H,I). As p53 is activated in GSCs in response to DNA damage (Lu et al., 2010; Wylie et al., 2014), these results suggest that p53 upregulation plays a role in preserving GSCs in the presence of DNA damage.

Reduced BMP signaling and Shotgun might contribute to DNA damage-induced GSC loss

BMP signaling is important for maintaining GSC self-renewal at least in part by repressing the expression of \( \text{bam} \), which controls CB differentiation (Chen and McKearin, 2003a; Song et al., 2004; Xie and Spradling, 1998), whereas Shotgun-mediated cell adhesion is required for retaining GSCs in the niche for continuous self-renewal (Song et al., 2002). To determine whether DNA damage affects BMP signaling in GSCs, we examined the expression of Dad-lacZ and phosphorylated Mad (pMad) in the control and DNA-damaged germaria. The niche-derived BMPs Dpp and Gbb function as short-range signals to activate Mad phosphorylation and Dad activation in GSCs (Casanueva and Ferguson, 2004; Chen and McKearin, 2003a; Kai and Spradling, 2003; Song et al., 2004). In control germaria, Dad-lacZ and pMad are specifically expressed in GSCs (Fig. 5A,C). By contrast, in the DNA-damaged germaria (3 days AHS), Dad-lacZ and pMad expression is significantly downregulated in GSCs (Fig. 5A-D). To investigate whether DNA damage also decreases Shotgun accumulation in the GSC-niche junction, we quantified the Shotgun expression levels based on immunofluorescence staining of Shotgun in control and DNA-damaged germaria, as we previously reported (Jin et al., 2008). Indeed, Shotgun...
accumulation at the GSC-niche junction decreases in the damaged germaria in comparison with the germaria without DNA damage (Fig. 5E,F). These results demonstrate that DNA damage downregulates BMP signaling and Shotgun accumulation in GSCs.

To determine whether decreased BMP signaling contributes to DNA damage-induced GSC loss, we investigated whether germ cell-specific expression of two constitutively active BMP type I receptors, Tkv* and Sax*, could rescue the DNA damage-induced GSC-loss phenotype. Expression of Tkv* and Sax* can completely block CB differentiation, causing formation of GSC-like tumors (Casanueva and Ferguson, 2004; Jin et al., 2008). The DNA-damaged germaria contain one GSC on average in comparison with two or three GSCs in the control germaria containing no DNA damage (Fig. 5G-H). Although germline-specific Tkv* and Sax* expression blocks CB differentiation, the number of endogenous GSCs, which are in direct contact with cap cells, is still two or three, as in the wild-type control (Jin et al., 2008). The DNA-damaged germaria contain one GSC on average in comparison with two or three GSCs in the control germaria containing no DNA damage (Fig. 5G-H). Although germline-specific Tkv* and Sax* expression blocks CB differentiation, the number of endogenous GSCs, which are in direct contact with cap cells, is still two or three, as in the wild-type control (Jin et al., 2008). Following germline-specific Tkv* and Sax* expression, the DNA-damaged germaria contain one or two GSCs with an average of 1.5 GSCs, which is significantly more than those GSCs in DNA-damaged germaria, indicating that BMP signaling downregulation might make some contributions to the DNA damage-induced GSC loss (Fig. 5H-I).

To investigate further whether forced expression of Shotgun can slow down the GSC loss caused by DNA damage, we used nos-gal4 to drive UASp-shg expression in the DNA-damaged GSCs. The two independent UASp-shg transgenic lines used in this study were used previously to overexpress Shotgun in GSCs (Jin et al., 2008; Pan et al., 2007). As previously reported (Pan et al., 2007), forced expression of Shotgun does not affect the GSC number in the absence of DNA damage (Fig. 5J,K). Similarly, forced expression of Shotgun does not rescue the DNA damage-induced GSC loss, suggesting that DNA damage might affect Shotgun localization or function, and not simply expression (Fig. 5J-K). Interestingly, shg overexpression can slightly but significantly increase the rescue effect of tkv* overexpression on the DNA damage-induced GSC loss (Fig. 5L). Taken together, these results suggest that DNA damage decreases BMP signaling and Shotgun-mediated cell adhesion, which might partly contribute to the GSC loss.

DNA damage disrupts the Bam-dependent differentiation of GSC progeny
As mentioned earlier, DNA damage can increase CB-like single germ cells (Fig. 6A,B). By carefully examining the accumulation of CB-like cells 3 days and 1 week after DNA damage caused by either I-CreI or X-rays, we show that the DNA-damaged germaria accumulate more CB-like cells during the 3 days-1 week period compared with the control germaria (Fig. 6B,C; Fig. S6). Interestingly, the heterozygous lok* and lokKD mutations enhance the emergence of the germ cell differentiation defect 3 days after DNA damage, but do not enhance or suppress the germ cell
differentiation defect 1 week after DNA damage (Fig. 6C-E; Fig. S6). By contrast, the homozygous lokP6 and lokKD mutations significantly suppress the germ cell differentiation defect caused by I-CreI-induced or X-ray-induced DNA damage (Fig. 6C,F,G; Fig. S6). These results indicate that Lok activation also contributes to the DNA damage-induced germ cell differentiation defect.

To determine whether the DNA damage-induced germ cell differentiation defect is also Bam dependent, we quantified the CB number in the DNA-damaged control and bam heterozygous germaria. Bam is necessary and sufficient for CB differentiation into germline cysts in the Drosophila ovary (McKearin and Spradling, 1990; Ohlstein and McKearin, 1997). The bam heterozygous germaria accumulate slightly more CB-like cells than wild type. (Fig. 6H,J). By contrast, DNA-damaged bam heterozygous germaria contain significantly more CBs than the bam heterozygous germaria and the DNA-damaged wild-type germaria (Fig. 6H-J). In addition, forced bam expression can also sufficiently induce the differentiation of the accumulated CB-like cells caused by X-ray-induced DNA damage (Fig. 6K-N). These results indicate that DNA damage compromises Bam-dependent GSC progeny differentiation.

**DNA damage decreases Bam protein accumulation at least at two different levels**

Although Bam function is essential for the CB to develop into a cyst, its protein levels are difficult to detect in the CB. To determine how DNA damage might affect Bam protein expression in early GSC progeny, we examined its expression in two-cell, four-cell and eight-cell cysts of control and DNA-damaged germaria. In the control germaria, four-cell and eight-cell cysts strongly express Bam protein, at a higher level than in two-cell cysts (Fig. 7A-C). However, in the DNA-damaged germaria, two-cell, four-cell and eight-cell cysts express significantly less Bam protein than those in the control germaria, indicating that DNA damage decreases Bam protein expression in mitotic cysts (Fig. 7D-F). Consistent with
the finding that Lok inactivation can rescue the DNA damage-induced CB differentiation defect, a homozygous lok mutation can restore Bam expression levels in two-cell, four-cell and eight-cell cysts in the DNA-damaged germaria to levels comparable to those in the control germaria (Fig. 7G-J). These results suggest that DNA damage decreases Bam protein accumulation in mitotic two-cell, four-cell and eight-cell cysts in a Lok-dependent manner.

Next, we determined how DNA damage affects Bam protein expression in mitotic cysts. Bam-gfp is the gfp gene under the control of the bam promoter for studying bam transcription regulation (Chen and McKearin, 2003b). In control germaria, bam-gfp is repressed in GSCs and upregulates its expression in CBs and mitotic cysts (Fig. 7K). Surprisingly, its expression is significantly reduced in DNA-damaged CBs and mitotic cysts compared with that in controls, indicating that DNA damage affects bam transcription in CBs and mitotic cysts (Fig. 7K-M). To determine whether DNA damage affects bam expression at the post-transcriptional level in mitotic cysts, we generated Pnos-gfp-bam3′UTR, the gfp gene fused with the bam 3′UTR under the control of the nos gene promoter, for studying bam post-transcriptional regulation. In the 3′UTR control (gfp reporter fused with the K10 3′UTR), DNA damage does not affect the expression of GFP in GSCs and mitotic cysts (Fig. 7N-P).

DISCUSSION

Stem cells in adult tissues are responsible for generating new cells to combat aging, but could also be responsible for tumor formation. Although aged stem cells have been shown to accumulate DNA damage, it remains largely unclear how DNA damage affects stem cell self-renewal and differentiation. A previous study has reported that upon weak irradiation apoptotic differentiated GSC progeny can prevent GSC loss by activating Tie-2 receptor tyrosine kinase signaling (Xing et al., 2015). In this study, we show that temporally
Introduced DNA double-stranded breaks cause premature GSC loss and slow down GSC progeny differentiation (Fig. 7T). Mechanistically, DNA damage causes GSC loss at least via two independent mechanisms: downregulation of BMP signaling and Shotgun-mediated GSC-niche adhesion as well as Lok activation-dependent GSC loss. In addition, Lok activation also decreases Bam protein expression by affecting its gene transcription and translation, slowing down CB differentiation into mitotic cysts and thus causing the accumulation of CB-like cells. Surprisingly, unlike in many somatic cell types, Tefu, Mei-41, Grp and p53 do not work with Lok in DNA-damage checkpoint control in Drosophila ovarian GSCs. Therefore, this study demonstrates that DNA damage-induced Lok activation causes premature GSC loss and also retards GSC progeny differentiation (Fig. 7T). Our findings could also offer insight into how DNA damage affects stem cell-based tissue regeneration. In addition, this study also shows that the inducible I-CreI system is a convenient method for studying stem cell responses to transient DNA damage because it does not require any expensive irradiation equipment as the X-ray radiation does.

**DNA damage-induced Lok activation is primarily responsible for GSC loss**

DNA damage normally leads to cell apoptosis to eliminate potential cancer-forming cells (Lord and Ashworth, 2012; Sperka et al., 2012). In this study, we show that the GSC loss caused by transient DNA damage is not a result of apoptosis based on two pieces of experimental evidence. First, DNA-damaged GSCs are not positive for cleaved Caspase-3, a widely used apoptosis marker. Second, forced expression of a known apoptosis inhibitor, p35, does not show any rescue effect on DNA damage-induced GSC loss. Thus, DNA damage-induced GSC loss is likely to be due to self-renewal defects though we cannot rule out the possibility that other forms of...
cell death are responsible. p53 is known to be required for DNA damage-induced apoptosis from flies to humans (Slee et al., 2004); this study, however, demonstrates that p53 prevents the DNA damage-induced GSC loss. Vacating DNA-damaged GSCs from the niche via differentiation might allow their timely replacement and restoration of normal stem cell function. Therefore, our findings argue strongly that DNA damage primarily compromises self-renewal, thus causing GSC loss.

Both niche-activated BMP signaling and Shotgun-mediated cell adhesion are essential for GSC self-renewal (Chen and McKearin, 2003a; Song et al., 2004; Song and Xie, 2002; Xie and Spradling, 1998). Consistent with the idea that DNA damage compromises GSC self-renewal, it significantly decreases BMP signaling activity and apical accumulation of Shotgun in GSCs. As constitutively active BMP signaling alone or in combination with Shotgun overexpression can only moderately rescue GSC loss caused by DNA damage, we conclude that decreased BMP signaling and apical Shotgun accumulation might partly contribute to the DNA damage-induced GSC loss. Therefore, our findings suggest that DNA damage-mediated downregulation of BMP signaling and Shotgun-mediated adhesion only moderately contributes to the GSC loss.

DNA damage leads to checkpoint activation and cell cycle slowdown, thus giving more time for repairing DNA damage. In various cell types, ATM-CHK2 and ATR-CHK1 kinase pathways are responsible for DNA damage-induced checkpoint activation (Callegari and Kelly, 2007; Kastan and Bartek, 2004; Sperka et al., 2012). During Drosophila meiosis, Mei-41, but not Tefu, is required for checkpoint activity, indicating that Tefu and Mei-41 could have different functions in germ cells (Joyce et al., 2011). Both Mei-41 and Lok have been shown to be required for DNA damage-evoked checkpoint control in Drosophila germ cells and embryonic cells (Abdu et al., 2002; Chen et al., 2007; Klattenhoff et al., 2007; Masrouha et al., 2003), and Grp can control the entry into the anaphase of cell cycle in response to DNA damage, the G2-M checkpoint activation as well as the Drosophila midblastula transition (de Vries et al., 2005; Royou et al., 2005; Takada et al., 2007). In this study, we have shown that these four checkpoint kinases function differently in GSCs. First, Lok is required for DNA damage-induced GSC loss, but is dispensable for normal GSC maintenance. Particularly, inactivation of its kinase activity can almost fully rescue DNA damage-induced GSC loss. Interestingly, inactivation of CHK2 function can also rescue the female germ cell defect caused by DNA damage in the mouse ovary, indicating that CHK2 function in DNA-damage checkpoint activation is conserved at least in female germ cells (Bolcun-Filas et al., 2014). However, it remains unclear whether CHK2 behaves similarly in mammalian stem cells in response to DNA damage. Second, Tefu promotes GSC maintenance in the absence and presence of DNA damage. This is consistent with the finding that ATM is required for the maintenance of mouse male germ line stem cells and hematopoietic stem cells (Ito et al., 2004; Takubo et al., 2008). It will be interesting to investigate whether Tefu also prevents the oxidative stress in Drosophila GSCs as in mouse hematopoietic stem cells. Third, Mei-41 is dispensable for normal GSC maintenance, but it protects GSCs in the presence of DNA damage. Although Lok and Mei-41 behave similarly in DNA-damage checkpoint control during meiosis and late germ cell development (Joyce et al., 2011; Klattenhoff et al., 2007), they behave in an opposite way in GSCs in response to DNA damage. Finally, Grp is dispensable for GSC self-renewal in the absence and presence of DNA damage. Consistent with our findings, the females homozygous for grp can still lay eggs, but those eggs could not develop normally (Fogarty et al., 1997; Sibon et al., 1997). It will be of great interest in the future to figure out how CHK2 inactivation prevents DNA damage-induced GSC loss and how ATM and ATR inactivation promotes DNA damage-induced GSC loss at the molecular level. A further understanding of the functions of CHK1, ATM and ATR in stem cell response to DNA damage will help preserve aged stem cells and prevent their transformation into CSCs.

**DNA damage-evoked Lok activation retards GSC progeny differentiation by decreasing Bam expression at least at two levels**

This study has also revealed a novel mechanism of how DNA damage affects stem cell differentiation. Bam is a master differentiation regulator controlling GSC-CB and CB-cyst switches in the Drosophila ovary: CB-like single germ cells accumulate in bam mutant ovaries, whereas forced Bam expression sufficiently drives GSC differentiation (McKearin and Ohlstein, 1995; Ohlstein and McKearin, 1997). In this study, we show that DNA damage causes the accumulation of CB-like cells in a Lok-dependent manner because Lok inactivation can fully rescue the germ cell differentiation defect caused by DNA damage. In addition, a heterozygous bam mutation can drastically enhance, and forced bam expression can completely repress, the DNA damage-induced germ cell differentiation defect, indicating that DNA damage disrupts Bam-dependent differentiation pathways. Consistent with this, Bam protein expression is significantly decreased in DNA-damaged mitotic cysts in comparison with control ones. Interestingly, Lok inactivation can also fully restore Bam protein expression levels in the DNA-damaged mitotic cysts. Taken together, Lok activation is largely responsible for Bam downregulation in DNA-damaged mitotic cysts, which can mechanistically explain the DNA damage-induced germ cell differentiation defect.

We have further revealed that DNA damage decreases Bam protein expression at least at two different levels. First, we used the bam transcription reporter bam-gfp to show that DNA damage decreases bam transcription in CBs and mitotic cysts. Second, we generated the posttranscriptional reporter Pnos-GFP-bam′UTR to show that DNA damage decreases Bam protein expression via its 3′ UTR in CBs and mitotic cysts at the level of translation. Although the detailed molecular mechanisms underlying regulation of Bam protein expression by DNA damage await future investigation, our findings demonstrate that DNA damage causes the GSC progeny differentiation defect by decreasing Bam protein expression at transcriptional and translational levels. Taken together, our findings from Drosophila ovarian GSCs could offer important insight into how DNA damage affects stem cell-based tissue regeneration, and have also established Drosophila ovarian GSCs as a new paradigm for studying how DNA damage affects stem cell behavior at the molecular level. Because many stem cell regulatory strategies are conserved from Drosophila to mammals (Li and Xie, 2005; Morrison and Spradling, 2008), what we have learned from this study should help understand how mammalian adult stem cells respond to DNA damage.

**MATERIALS AND METHODS**

**Drosophila strains and culture**

The Drosophila stocks used in this study include: hs-I-CreI (Rong et al., 2002); lok′ (Abdu et al., 2002; Takada et al., 2003; mei-41D15C (Bang et al., 1995); UAS-shRNA lines for lok (GL00020 and THU00402), tefu (HMS02790), mei-41 (HMS02331 and GL00284) and grp (HMS01573).
and HMC05162) (Ni et al., 2009, 2011). Flies were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless otherwise specified. For generation of the kinase-dead lokKD mutant strain, and the Pnos-GFP-bam 3’ UTR and UASp-p35-Flag transgenic strains, see supplementary Materials and Methods.

Induction of DNA damage by I-Cre and X-rays

To induce DNA damage, flies were incubated at 37°C for 1 h or two consecutive hours and then maintained at 25°C for 3 days or 1 week before dissection and immunostaining. For the X-ray radiation, adult females were treated in a Faxitron X-ray machine model CP160 for 10 min to produce a total of 20,700 rad radiation (2070 rad/min×10 min). See supplementary Materials and Methods for further details of determination of RNA gene copy number and rRNA expression.

Immunohistochemistry

Immunohistochemistry was performed according to our previously published procedures (Song et al., 2002; Xie and Spradling, 1998). The following antibodies were used in this study: mouse monoclonal anti-Hts antibody [1:50; Developmental Studies Hybridoma Bank (DSHB), B81]; rabbit polyclonal anti-β-galactosidase antibody [1:100; DSHB, Cappel, 55976], mouse monoclonal anti-Bam antibody [1:3; DSHB], mouse monoclonal anti-lamin C antibody [LC28.26; 1:3; DSHB], rat monoclonal anti-E-cadherin antibody [1:5; DSHB, DCAD2], rabbit polyclonal anti-pS137 H2A antibody [1:100; Rockland, 60041914], rabbit monoclonal anti-Cleaved Caspase-3 (Asp175) antibody [1:200; Invitrogen, A10262], rabbit anti-Anillin (generously provided by Dr. C. Field, Harvard University), and rabbit polyclonal anti-Fibrillarin [1:100; Abcam, ab8281]. All images were taken with a Leica TCS SP5 confocal microscope.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.141069.supplemental

References


Supplemental Information

Materials and Methods

*rRNA gene copy number and rRNA expression*

For rDNA qPCR, genomic DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. A total of 4ng of genomic DNA was used for each qPCR reaction of 10µl. For rRNA RT-qPCR, total RNAs were extracted with Trizol, purified by organic extraction followed by isopropanol precipitation and treated with DNase to remove DNA contamination. 500ng of RNAs were then used to produce cDNAs with oligo(dT) primers and random hexamers using SuperScript III Reverse Transcriptase (Life Technologies). Fluorescence-based quantitative real-time PCR (qPCR) was performed to assay levels of 18S and 5S with tbp, gapdh and rpl32 as internal controls. cDNAs from SuperScript III Reverse Transcription were diluted 1:500 and 2ul aliquots of each cDNA sample were added to 5ul of 2x power SYBR Green PCR Master Mix (Applied Biosystems part No.: 4367659, Lot No.: 1305403), 0.5µl each of 10nm Forward & Reverse primer and 2ul of water in a 384-well plate. The resulting reactions were sealed, centrifuged, and cycled on an ABI 7900HT according to the instrument’s standard protocol. Analysis of the fluorescence curves was done using ABI’s SDS2.4 software. The Ct values were analyzed using the Biogazelle qBase Plus version 2.4 software to generate normalized relative quantities using assays for endogenous controls.
### Primers for qPCRs

<table>
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<tr>
<th>Name</th>
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<tr>
<td>18S-F</td>
<td>AGCCTGAGAAACGGCTACCA</td>
</tr>
<tr>
<td>18S-R</td>
<td>AGCTGGGAGTGGGTAATTACGG</td>
</tr>
<tr>
<td>5S set1-F</td>
<td>GACCATAACCACGCTGAATA</td>
</tr>
<tr>
<td>5S set1-R</td>
<td>CCCGACGCTGCTTAAT</td>
</tr>
<tr>
<td>5S set2-F</td>
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</tr>
<tr>
<td>5S set2-R</td>
<td>CGCGGTGTTCACCAAG</td>
</tr>
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</table>

**Generation of the UASp-p35-Flag transgenic fly strain**

The coding sequence of p35 was PCR-amplified from the genomic DNA of the UAS-p35 flies using the primers CACCATGTGTGTAATTTTTTCCGGTAGAAATCG and TTTAATTGTGTTTAATATTACATTTTTTGTTAG, and was then cloned into the pENTR/D-TOPO vector (Invitrogen, K2400-20). The p35-pENTR was then recombined with the pPWF destination vector (Invitrogen, LR clonase II, 11791-020) to generate the UASp-p35-Flag plasmid.

**Generation of the kinase-dead lok\(^{KD}\) mutant by CAS9/CRSPR**

The 286\(^{th}\) residue Asp in the transcript CHK2-PB, which is responsible for CHK2 kinase activity, was mutated into Ala to generate lok\(^{KD}\) by modifying the codon GAC to GCC. The sgRNA target site was “GTCAGGCTTAAGGTCACGATGGG” (PAM in bold). To target this genomic site, we constructed the lok\(^{KD}\) donor and the U6B promoter-driven sgRNA plasmid. The lok\(^{KD}\) donor was generated by PCR on the Drosophila genomic DNA with AccuPrime™ Pfx DNA Polymerase (Invitrogen, 12344-024) using
primers, CTAGCTAGCTCAGAACCACAAGAGCAG, GGAAGATCTCGGAATGTTTGCTGAAGA, CAACTACCTAGGTTCTACCTTTTCAGGCATCACATCGTGCCTTAAGCCTG and CAGGCTTAAGGGCAGATGTGTGATGCTGAAAGGTAGAACCTAGGTAGTTG, and was further cloned into the pBluescript plasmid cut by NheI and BglII. There is one AvrII site near the sgRNA target site in the lokKD donor as a selection marker. The lokKD donor and sgRNA plasmid were injected into {nos-Cas9}attP2 embryos at the concentration of 300ng/µl and 100ng/µl, respectively. The lokKD mutant lines were identified by sequencing PCR products using primers CTAGCTAGCTCAGAACCACAAGAGCAG and GGAAGATCTCGGAATGTTTGCTGAAGA.

Generation of Pnos-GFP-bam 3’UTR transgenic reporter flies

To construct Pnos-eGFP-bam 3’UTR, we amplified the bam 3’UTR from the Drosophila cDNA libraries (w^{118}) using a pair of primers (one with a BamHI cutting site and the other with a SpeI cutting site). The amplified bam 3’UTR DNA was cut with BamHI and SpeI, and was then cloned into the BamHI-SpeI site of the pVALIUM-Pnos-eGFP-nos 3’UTR vector to replace the nos 3’UTR. The pVALIUM-Pnos-eGFP-bam 3’UTR construct was then introduced into the attP site in the Drosophila strain (BL#24482) using PhiC31 integrase-mediated transgenesis by Rainbow Company Inc.
Supplemental figures

Figure S1. I-CreI expression can induce double-stranded DNA breaks. Ovals indicate GSCs. (A-C) GSCs in the hs-I-CreI germarium contain γ-H2Av-positive foci immediately after one-hour heatshock (0h AHS1h, B) in comparison with those GSCs in the control germarium (no HS, A). C: γ-H2Av foci quantification results. Scale bar: 10µm.
Figure S2. GSCs resume their proliferation one day after DNA damage. Ovals indicate GSCs, whereas arrows highlight mitotic cysts. (A-E) GSCs in the hs-I-CreI gerarium are negative for BrdU labeling 12h AHS (B) and then become positive again 1d AHS (C) and 1w AHS (D) as in the control (no HS, A). E: BrdU-positive GSC quantification results. BrdU-positive GSCs undergo DNA replication. (F-J) GSCs in the hs-I-CreI gerarium are negative for pH3 expression 12h AHS (G) and then become positive again 1d AHS (H) and 1w AHS (I) as in the control (no HS, F). J: pH3-positive GSC quantification results. pH3-positive GSCs are in late G2 phase of the cell cycle or in mitosis. Scale bar: 10µm.
Figure S3. Germline-specific overexpression of the Baculovirus anti-apoptosis gene *p35* fails to rescue the DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (A) *nos-gal4*-driven expression of the C-terminal Flag-tagged *p35* (*nos>*p35-Flag) specifically in germ cells, including GSCs. (B-E) *nos>*p35-Flag germaria carry 3 GSCs in the absence of DNA damage (no HS, B), but contain 1 GSC 3 days after one-hour heatshock (3d AHS1, D) or 0 GSC 3 days after two-hour heatshock (3d AHS2, E). C: GSC quantification results. Scale bar: 10µm.
Figure S4. I-CreI-induced double-stranded DNA breaks do not affect 18S rDNA repeats, 18S rRNA production and thus nucleolus volume. (A) Primer pairs for quantitative PCRs to detect the copy numbers of 18S and 5S genes. (B) Quantitative PCR results show that I-CreI-mediated double-stranded DNA breakage does not affect the copy numbers of 18S and 5S genes. (C) Quantitative RT-PCR results show that I-CreI-mediated double-stranded DNA breakage does not affect the production of 18S and 5S rRNAs. (D-H) The nucleolus size does not change in GSCs in the hs-I-CreI germaria 8h AHS (E), 1d AHS (F) and 2d AHS (G) in comparison with those in the control germarium (no HS, D). H: nucleolus volume quantification results. Scale bar: 10µm.
Figure S5. X-ray irradiation can efficiently introduce DNA damage into GSCs and their progeny. Ovals and asterisks indicate GSCs and CPCs, respectively. (A) Wild-type (WT) germarium contains γ-H2Av-negative GSCs and γ-H2Av-positive meiotic germ cells (arrow) without X-ray treatment (no XT). (B) Germarium contains γ-H2Av-positive germ cells, including 2 GSCs and differentiated germ cells (arrow) 2 hours after 20000rad X-ray treatment (2h AXT). (C) Germarium contains γ-H2Av-negative GSCs and γ-H2Av-positive differentiated germ cells (arrow) 1d AXT, indicating that DNA damage has been successfully repaired in GSCs. Scale bar: 10µm.
Figure S6. CHK2 inactivation can rescue the germ cell differentiation defect caused by X-ray-induced DNA damage. Circles and arrowheads indicate CPCs/GSCs and CBs, respectively. (A, B) X-ray-treated germarium (1w AXT, B) contains excess CBs in comparison with the control untreated germarium containing 1 CB (no XT, A). (C, D) X-ray-treated $lok^{P6}$ (1w AXT, C) and $lok^{KD}$ (1w AXT, D) heterozygous germaria contain 2 CBs. (E, F) X-ray-treated $lok^{P6}$ (1w AXT, E) and $lok^{KD}$ (1w AXT, F) homozygous germaria contain 1 CB. G: CB quantification results. Please note that both $lok^{P6}$ and $lok^{KD}$ heterozygous mutations actually promote the germ cell differentiation defect 3d AXT. Scale bar: 10µm.