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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 heatshock-inducible endonuclease I-CreI 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-CreI 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; Sotiropoulou 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 germline 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.

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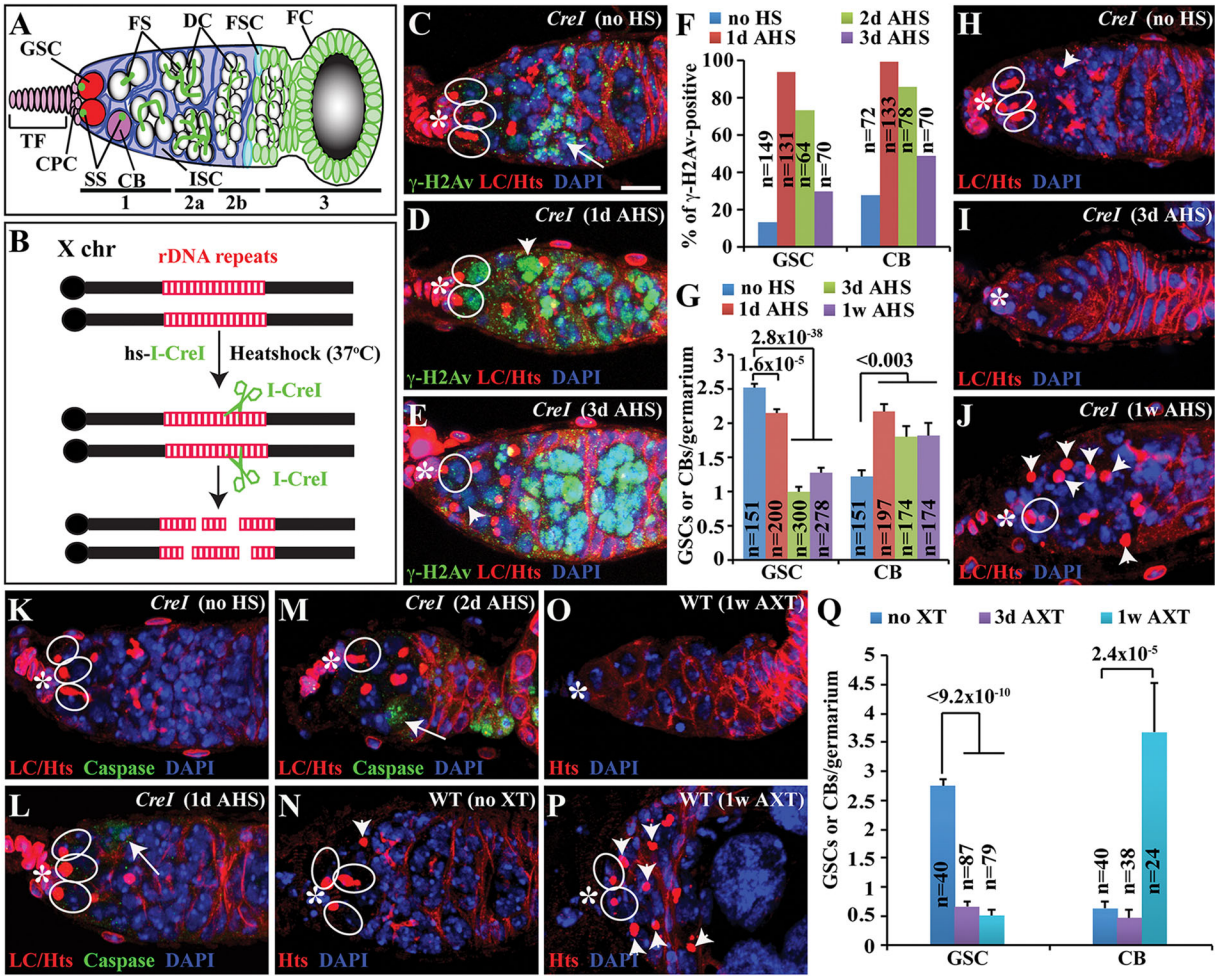

Fig. 1. DNA damage compromises GSC self-renewal and lineage differentiation. (A) Schematic of the germarium. CB, cystoblast; CPC, cap cell; DC, developing cyst; FC, follicle cell; FS, fusome; FSC, follicular stem cell; GSC, germline stem cell; ISC, inner sheath cell; SS, spectrosome; TF, terminal filament. (B) Applying endonuclease I-CreI to induce double-stranded breaks in rDNA on the X chromosome. (C-Q) Lamin C (LC) labels CPCs and TF in C-E and H-M, whereas Hts labels SS/FS in C-E and H-P. Ovals, arrowheads and asterisks indicate GSCs, CBs and CPCs, respectively. (C-F) GSCs are negative, whereas meiotic 16-cell cysts (arrow) are positive, for γ-H2Av without heatshock (no HS; C), whereas GSCs and CBs are γ-H2Av positive 1 day after 1 h heatshock (1 day AHS; D), but become γ-H2Av-negative 3 days AHS (E). (F) Percentage of γ-H2Av-positive GSCs and CBs in *CreI* flies with and without HS. (G-J) I-CreI-expressing germaria containing no GSCs 3 days AHS (I), or one GSC and six CBs 1 week AHS (J) in comparison with the control germarium containing three GSCs and one CB (H). (G) Number of GSCs and CBs per germarium in *CreI* flies with and without HS. (K-M) DNA-damaged GSCs remain negative for the expression of cleaved Caspase 3 at 1 day (L) and 2 days (M) AHS like the control GSCs (K). Arrows indicate cleaved Caspase 3-positive differentiated germ cells. (N-Q) Germaria containing no GSCs (O), or two GSCs and extra CBs (P) one week after 20,000 rad X-ray treatment (1 week AXT) in comparison with the control germarium carrying three GSCs and one CB (N). (Q) The number of GSCs and CBs per germarium in control flies with and without X-ray treatment (XT). In this figure and thereafter, I-CreI is abbreviated to CreI, error bars represent s.e.m. and *P*-values are calculated based on Student's *t*-test. *n*, number of germaria examined. Scale bar: 10 µm.

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

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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 heatshock 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 heatshock-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 heatshock 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* germaria [no heatshock (HS)] contain an average of 2.5 GSCs (Fig. 1G,H). Following DNA damage, the germaria 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 germaria either contain one GSC or completely lose their GSCs (Fig. 1G,I). After DNA damage, some germaria also often contain more spectrosome-containing CB-like single germ cells than the control germaria (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 germaria in comparison with the control germaria (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^{P6} heterozygous and homozygous females are viable and fertile, and their germaria carry two to three GSCs (Fig. 2A,B,E). lok^{P6} is a molecularly null allele (Abdu et al., 2002). Here, the 2-h heatshock was used to generate a more severe GSC loss phenotype for testing Lok requirement: most of the wild-type germaria 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 germaria 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^{P6} 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.

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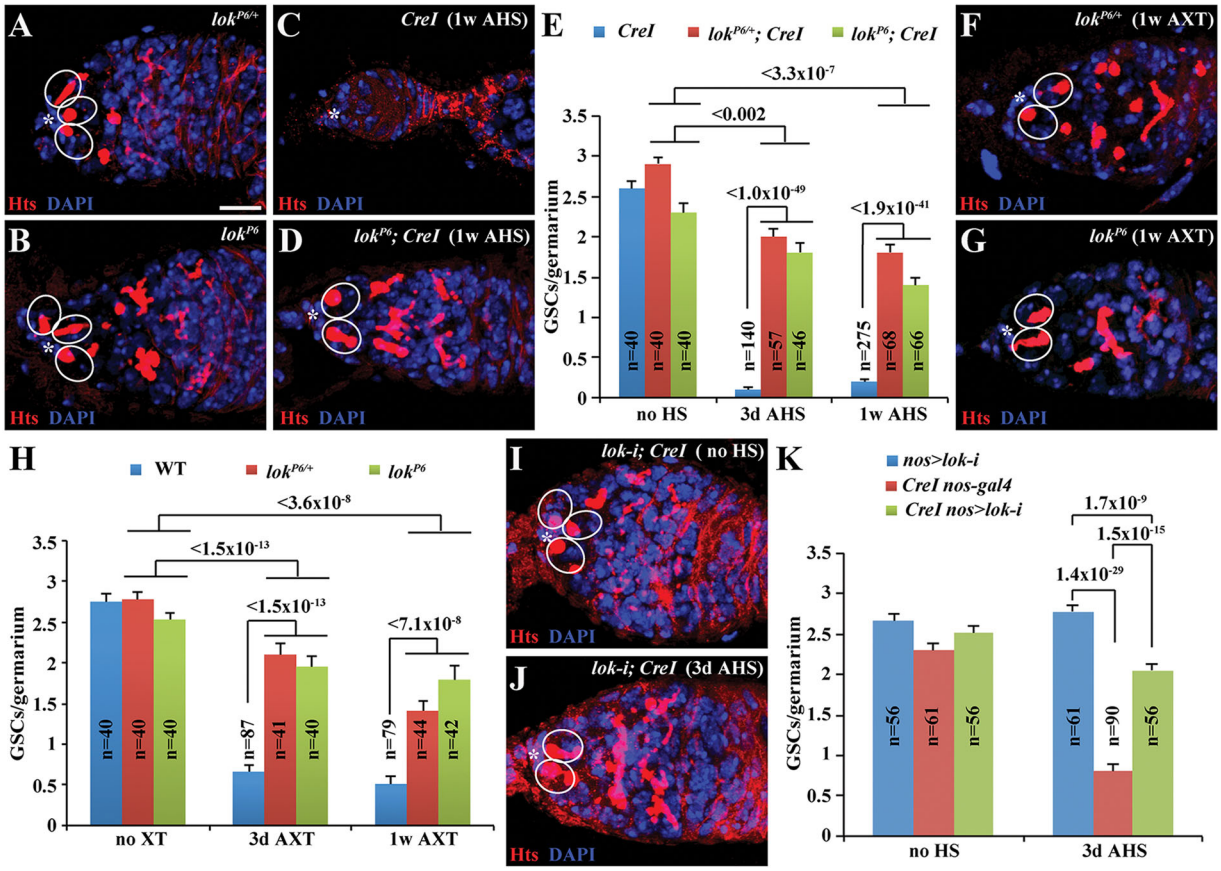

Fig. 2. CHK2 inactivation drastically rescues DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (A,B) *lok*^{P6} heterozygous (A) and homozygous (B) germaria each containing three GSCs. (C-E) DNA-damaged *lok*^{P6} homozygous germarium containing two GSCs (D) in comparison with DNA-damaged control one carrying no GSCs (C) 1 week after 2-h heatshock (1 week AHS). (E) Number of GSCs per germarium in *CreI*, *lok*^{P6} heterozygous and *lok*^{P6} homozygous flies with and without HS. (F-H) X-ray-treated *lok* heterozygous (F) and homozygous (G) germaria carrying two GSCs 1 week after X-ray treatment (AXT). (H) The number of GSCs per germarium in control, *lok*^{P6} heterozygous and *lok*^{P6} homozygous flies with and without X-ray treatment (XT). (I-K) Germline-specific *lok* knockdown (*lok-i*) significantly rescues the DNA damage-induced GSC loss. *lok-i* germaria containing three GSCs without DNA damage (no HS; I), and still maintaining two GSCs in the presence of DNA damage (3 days AHS; J). (K) The number of GSCs per germarium in *nos>lok-i*, *CreI nos-gal4* and *CreI nos>lok-i* flies with and without HS. Scale bar: 10 µm.

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* and *grp* 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 *lok*^{KD} allele, encoding a kinase-dead Lok (Ren et al., 2014). The heterozygous and homozygous *lok*^{KD} mutant germaria contain slightly more GSCs than the control and *lok*^{P6} mutant ones (Fig. 4A,C). Like *lok*^{P6}, both heterozygous and homozygous *lok*^{KD} mutations can significantly

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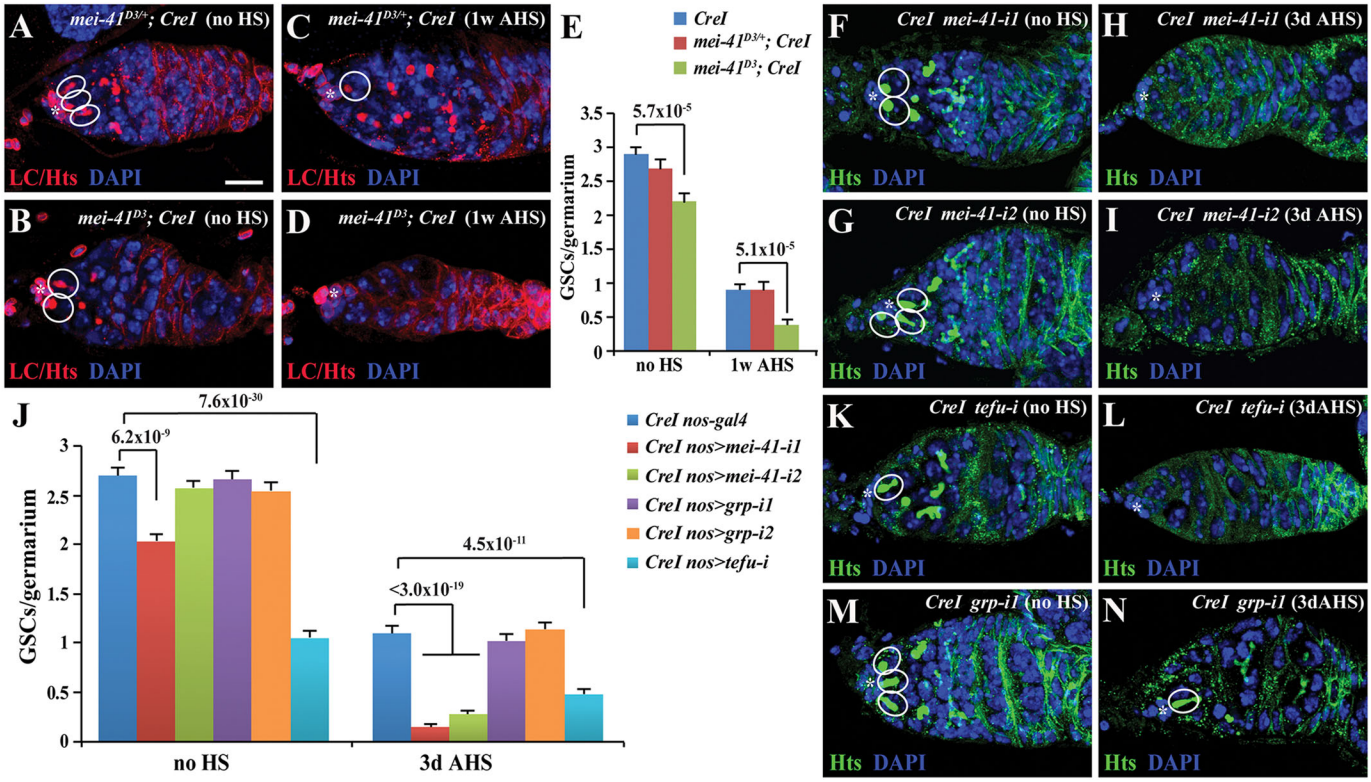

Fig. 3. Inactivation of Mei-41, Tefu or Grp fails to rescue the DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (A-E) The *mei-41*^{D3} homozygous mutation exacerbates the DNA damage-induced GSC loss. *mei-41*^{D3} heterozygous germaria containing three GSCs without DNA damage (no HS; A) and one GSC in the presence of DNA damage (1 week AHS; C), compared with *mei-41*^{D3} homozygous germaria containing two GSCs without DNA damage (no HS B) and no GSCs in the presence of DNA damage (1 week AHS; D). (E) Number of GSCs per germarium in control, *mei-41*^{D3} heterozygous and *mei-41*^{D3} homozygous flies with and without HS. (F-N) Germline-specific knockdown of *mei-41* or *tefu* enhances the DNA damage-induced GSC loss, but *grp* knockdown has no effect. Without DNA damage (no HS), germline-specific *mei-41* (F,G; two independent RNAi lines) and *grp* (M) knockdown germaria contain two or three GSCs, but *tefu* knockdown germarium carries one GSC (K). In the presence of DNA damage (3 days AHS), *mei-41* (H,I) and *tefu* (L) knockdown germaria contain no GSCs, but the *grp* knockdown germarium carries one GSC (N). (J) Number of GSCs per germarium in RNAi lines with and without HS. Scale bar: 10 µm.

and drastically rescue the GSC loss induced by DNA damage produced by I-CreI (Fig. 4B,C). Similarly, both heterozygous and homozygous *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 I-CreI expression), 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 *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, *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

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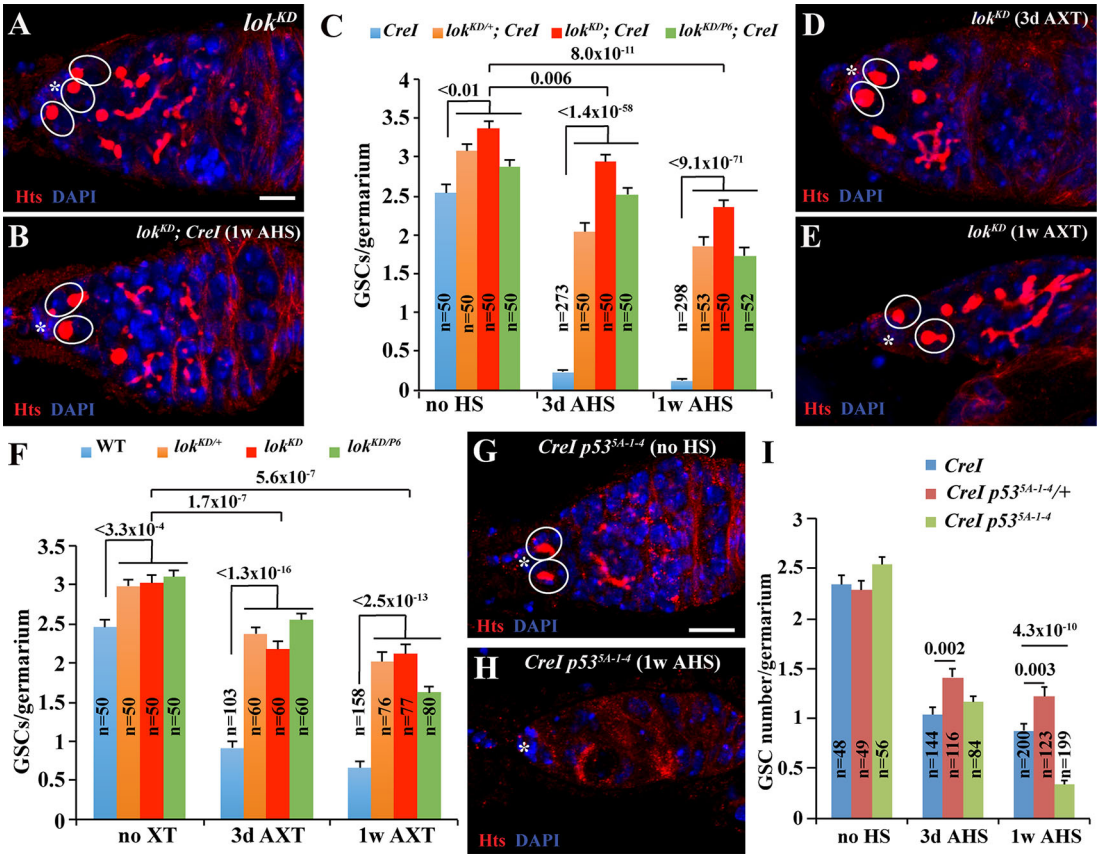

Fig. 4. Lok kinase function is required for the DNA damage-induced GSC loss, whereas p53 prevents DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (A-F) The kinase-dead *lok*^{KD} mutation rescues the DNA damage-induced GSC loss. *lok*^{KD} homozygous mutant germarium carrying three GSCs (A). *lok*^{KD} homozygous germaria still containing two GSCs 3 days (D) and 1 week (B,E) after DNA damage induced by I-CreI (B) or X-ray (D,E). (C,F) Number of GSCs per germarium in control (WT) and *lok* knockdown flies with and without DNA damage. (G-I) *p53* homozygous (G) germarium containing two GSCs in the absence of DNA damage (no HS), and DNA-damaged *p53* homozygous (H) germarium containing no GSCs 1 week AHS. (I) Number of GSCs per germarium in control, p53 heterozygous and p53 homozygous flies. Scale bars: 10 µm.

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). 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*^{P6} and *lok*^{KD} mutations enhance the emergence of the germ cell differentiation defect 3 days after DNA damage, but do not enhance or suppress the germ cell

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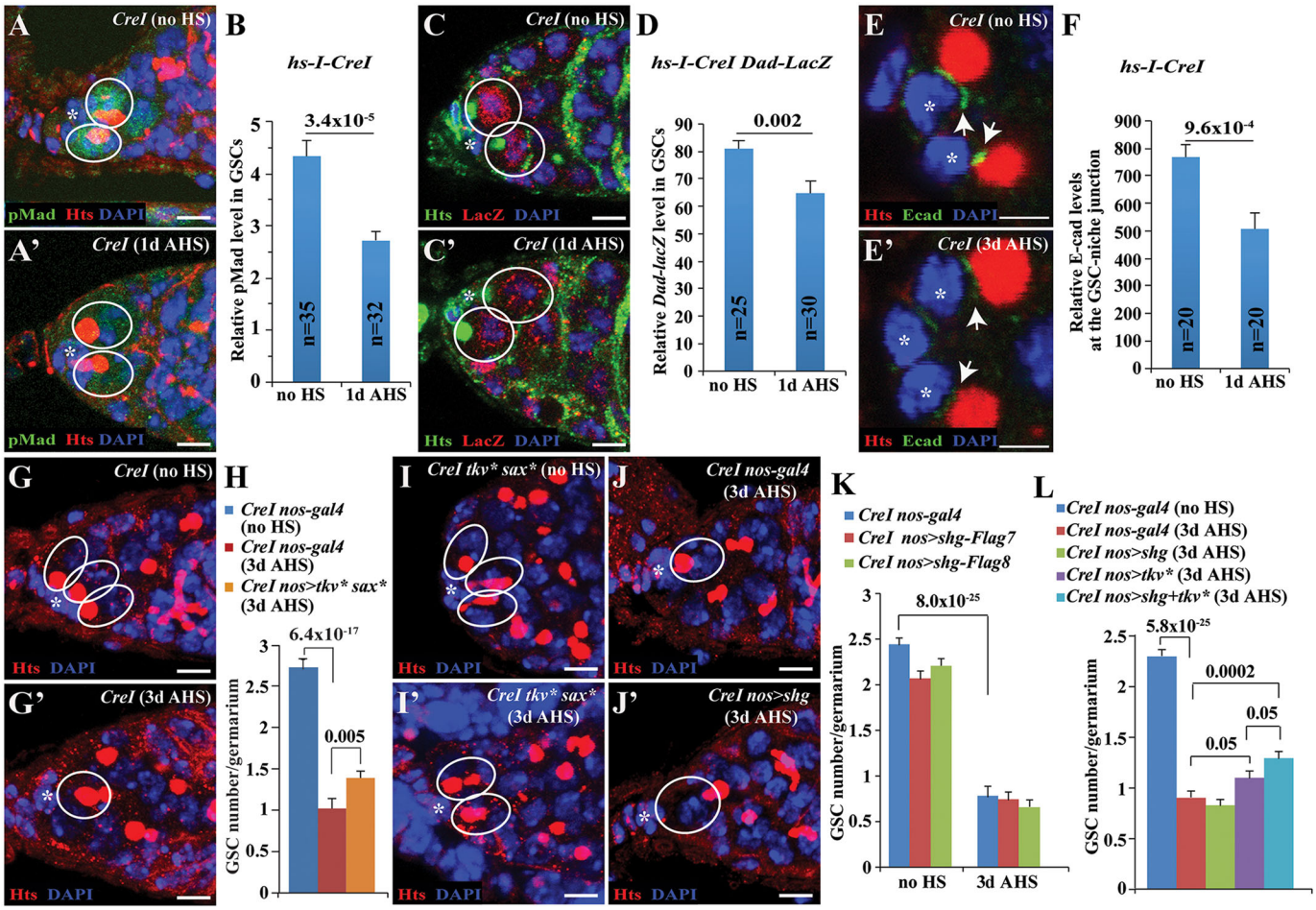

Fig. 5. Downregulation of BMP signaling and Shotgun accumulation partly contributes to the DNA damage-induced GSC loss. (A-D) GSCs show significantly lower pMad (A,A′) and *Dad-lacZ* (C,C′) expression in the presence of DNA damage (1 day AHS; A′,C′) than in the control GSCs (no HS; A,C). (B,D) Quantification of pMad (B) and *Dad-lacZ* (D) levels in GSCs. (E-F) GSCs show significantly lower Shotgun (Ecad) accumulation at the GSC-niche junction (arrowheads) in the presence of DNA damage (3 days AHS; E′) than in the control GSCs (no HS; E). (F) Quantification of Shotgun (E-cad) levels in the GSC-niche junction. (G-I′) Control germaria containing three GSCs without DNA damage (no HS; G), but one GSC in the presence of DNA damage (3 days AHS; G′). Tkv*/Sax*-expressing germaria containing three GSCs without DNA damage (no HS; I) and two GSCs in the presence of DNA damage (3 days AHS; I′). (H) Number of GSCs per germarium in *CreI nos-gal4* (no HS), *CreI nos-gal4* (3 days AHS), *CreI nos>tkv*sax**. (J-K) Germline-specific Shotgun overexpression fails to rescue the DNA damage-induced GSC loss. In the presence of DNA damage (3 days AHS), both the control germarium (J) and Shotgun-overexpressing germarium (J′) contain one GSC. (K) Number of GSCs per germarium in control and Shotgun-overexpressing flies with and without HS. (L) Quantification of GSC number in *CreI nos-gal4* (no HS), *CreI nos-gal4* (3 days AHS), *CreI nos>shg*, *CreI nos>tkv**, *CreI nos>shg+tkv** shows that germline-specific Shotgun overexpression can enhance the rescue effect of constitutive BMP signaling on the DNA damage-induced GSC loss. Scale bars: 25 µm (A,A′,C,C′,G,G′,I-J′); 10 µm (E,E′).

differentiation defect 1 week after DNA damage (Fig. 6C-E; Fig. S6). By contrast, the homozygous lok^{P6} and lok^{KD} 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′,J). 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′,J). Consistent with

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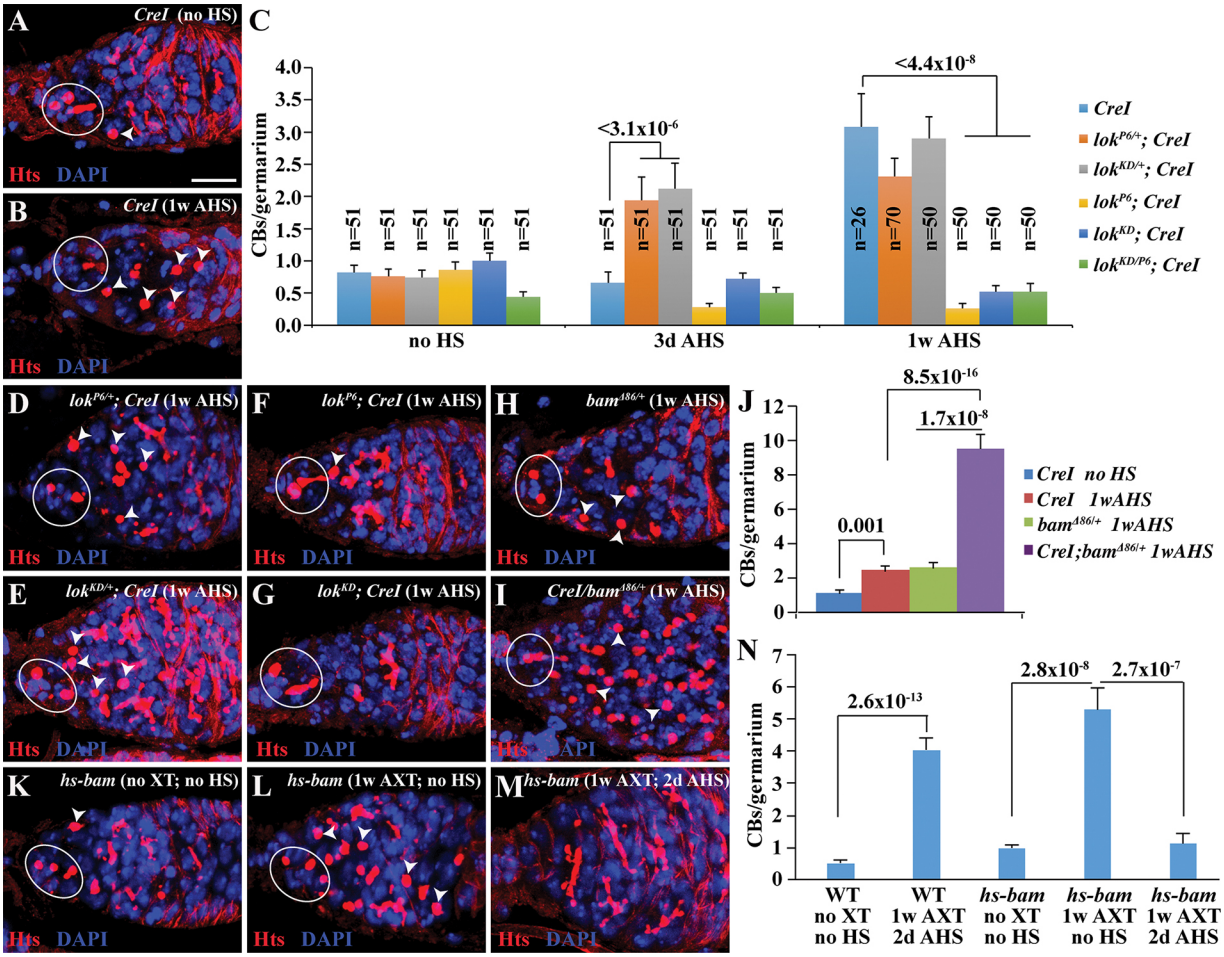

Fig. 6. DNA damage slows down GSC progeny differentiation by decreasing Bam function in a Lok-dependent manner. Ovals indicate GSCs and CPCs, whereas arrowheads indicate CBs. (A-G) DNA-damaged germarium (B) containing excess CBs 1 week AHS in comparison with a control germarium carrying one CB (A). DNA-damaged lok^{P6} (D) and lok^{KD} (E) heterozygous germaria containing excess CBs 1 week AHS, compared with DNA-damaged lok^{P6} (F) and lok^{KD} (G) homozygous germaria containing one or no CB 1 week AHS. (C) Number of CBs per germarium in control and in heterozygous and homozygous lok^{P6} and lok^{KD} flies. (H-J) DNA-damaged *bam* heterozygous germarium (I) accumulates more CBs than the control *bam* heterozygous germarium (H). (J) Number of CBs per germarium in *CreI* no HS, *CreI 1wAHS*, *bam*/+ and *CreI;bam*/+. (K-N) Forced *bam* expression (M) can drastically decrease the accumulation of CBs in the X-ray-damaged germarium (L) compared with the control germarium (K). (N) Number of CBs per germarium in control (WT) and *bam*-overexpressing flies with and without HS- and X-ray-induced DNA damage. Scale bars: 10 µm.

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). In the *Pnos-gfp-bam3′UTR* control germaria (no HS), GFP protein expression is higher in mitotic cysts than in GSCs and 16-cell cysts, indicating that *bam* expression is also regulated partly via its 3′UTR (Fig. 7Q). Interestingly, GFP protein expression, but not *gfp* mRNA expression, is reduced significantly in the DNA-damaged mitotic cysts compared with the control ones (Fig. 7Q-S′). These results indicate that DNA damage decreases Bam protein expression at least at transcriptional and translational levels.

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

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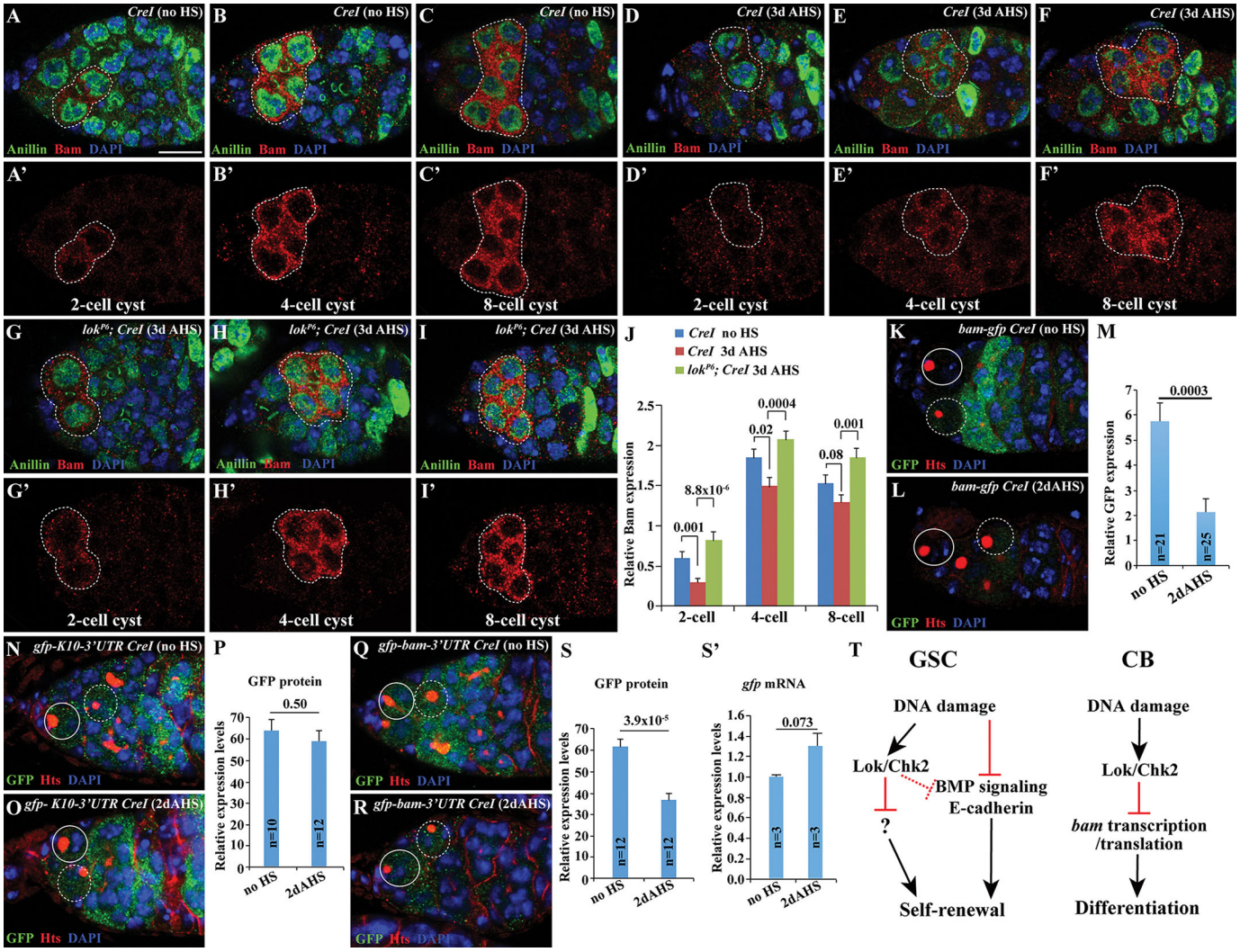

Fig. 7. DNA damage decreases Bam protein expression in mitotic cysts. (A-J) In comparison with control two-cell (A,A′), four-cell (B,B′) and eight-cell (C,C′) cysts (dashed lines), DNA-damaged (3 days AHS) two-cell (D,D′), four-cell (E,E′) and eight-cell (F,F′) cysts (dashed lines) have significantly decreased Bam protein expression, but DNA-damaged (3 day AHS) homozygous *lok* mutant two-cell (G,G′), four-cell (H,H′) and eight-cell (I,I′) cysts (dotted lines) have normal Bam protein expression. (J) Bam protein expression in control and homozygous *lok* mutant flies with and without HS. (K-M) *bam-gfp* expression is significantly decreased in a DNA-damaged CB (dashed circle, L) compared with a control CB (dashed circle, K). (M) GFP intensity in *bam-gfp CreI* flies with and without HS. (N-P) *Pnos-gfp-K10-3′UTR* expression remains unchanged in control (dashed circle, N) and DNA-damaged (dashed circle, O) CBs. (P) GFP intensity in *gfp-K10-3′UTR CreI* flies with and without HS. (Q-S′) *Pnos-gfp-bam3′UTR* expression is significantly decreased in a DNA-damaged CB (dashed circle, R) compared with a control CB (dashed circle, Q). (S,S′) GFP intensity and *gfp* mRNA quantification in *gfp-bam-3′UTR CreI* flies with and without HS. (T) A working model explaining how DNA damage affects GSC self-renewal and progeny differentiation mechanistically. Scale bars: 10 µm.

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

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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 germline 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 CHK2, 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-bam3′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^{p6} (Abdu et al., 2002; Takada et al., 2003); $mei\text{-}41^{D3}$ (Banga 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 lok^{KD} 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-CreI 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 rRNA 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), 1B1], rabbit polyclonal anti-β-galactosidase antibody (1:100; 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 H2Av antibody (1:100; Rockland, 600401914), rabbit monoclonal anti-Cleaved Caspase-3 (Asp175) (1:100; Cell Signaling, 39579S), rabbit monoclonal anti-pS423/425 Smad3 antibody (1:100; Epitomics, 1880-1), chicken polyclonal anti-GFP antibody (1:200; Invitrogen, A10262), rabbit anti-Anillin (generously provided by Dr C. Field, Harvard University), and rabbit polyclonal anti-Fibrillarin (1:100; Abcam, ab5821). 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

Concept, data analysis and manuscript preparation: X.M. and T.X.; performed experiments and generation of reagents: X.M., Y.H., X.S., T.D., Z.Y. and J.N.

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Supplementary information

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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 Biosysterms 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	
Name	Sequence
18S-F	AGCCTGAGAAACGGCTACCA
18S-R	AGCTGGGAGTGGGTAATTTACG
5S set1-F	GACCATACCACGCTGAATA
5S set1-R	CCCGACGCTGCTTAAT
5S set2-F	CGCTGAATACATCGGTTCT
5S set2-R	CGCGGTGTTCCCAAG

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 CACCATGTGTGTAATTTTTCCGGTAGAAATCG and TTTAATTGTGTTTAATATTACATTTTTGTTGAG, 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

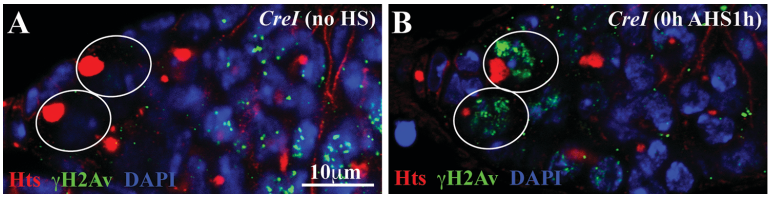
The 286th residue Asp in the transcript CHK2-PB, which is responsible for CHK2 kinase activity, was mutated into Ala to generate *lok^{KD}* by modifying the codon GAC to GCC. The sgRNA target site was "GTCAGGCTTAAGGTCACGAT**GGG**" (PAM in bold). To target this genomic site, we constructed the *lok^{KD}* 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, CTAGCTAGCTCAGAACCCACAAGAGCAG, GGAAGATCTCGGAATGG-TTTGCTGAAGA, CAACTACCTAGGTTCTACCTTTCAGGCATCACACATCGTGC-CCTTAAGCCTG and CAGGCTTAAGGGCACGATGTGTGATGCCTGAAAGGTAG-AACCTAGGTAGTTG, 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 lok^{KD} donor as a selection marker. The lok^{KD} donor and sgRNA plasmid were injected into *{nos-Cas9}attP2* embryos at the concentration of 300ng/µl and 100ng/µl, respectively. The lok^{KD} mutant lines were identified by sequencing PCR products using primers CTAGCTAGCTCAGAACCCACAAGAGCAG and GGAAGATCTCGGAATGGTTTGCTGAAGA.

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^{1118}) 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



C

treatment	No. of γH2Av foci	No. of GSCs examined
no HS	0	26
0h AHS1h	37.7±6.6	26

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.

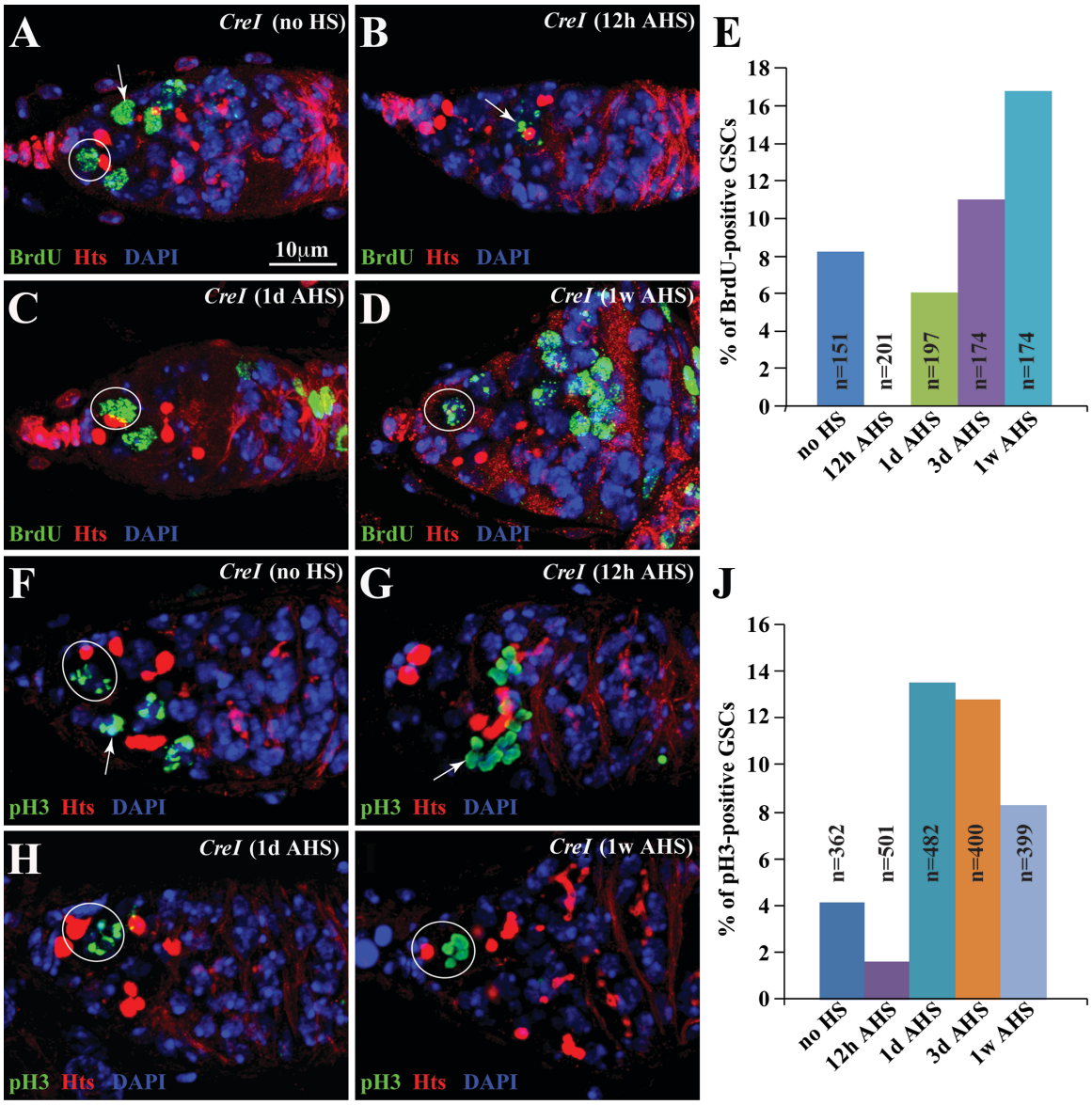


Figure S2. GSCs resume their proliferation one day after DNA damage. Ovals indicate GSCs, whereas arrows highlight mitotic cysts. (**A-E**) GSCs in the *hs-I-CreI* germarium are negative for BrdU labeling 12h AHS (**B**) and then become positive again 1d AHS (**C**) and 1w AHS (**D**) as in the control (no HS, **A**). **E**: BrdU-positive GSC quantification results. BrdU-positive GSCs undergo DNA replication. (**F-J**) GSCs in the *hs-I-CreI* germarium 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.

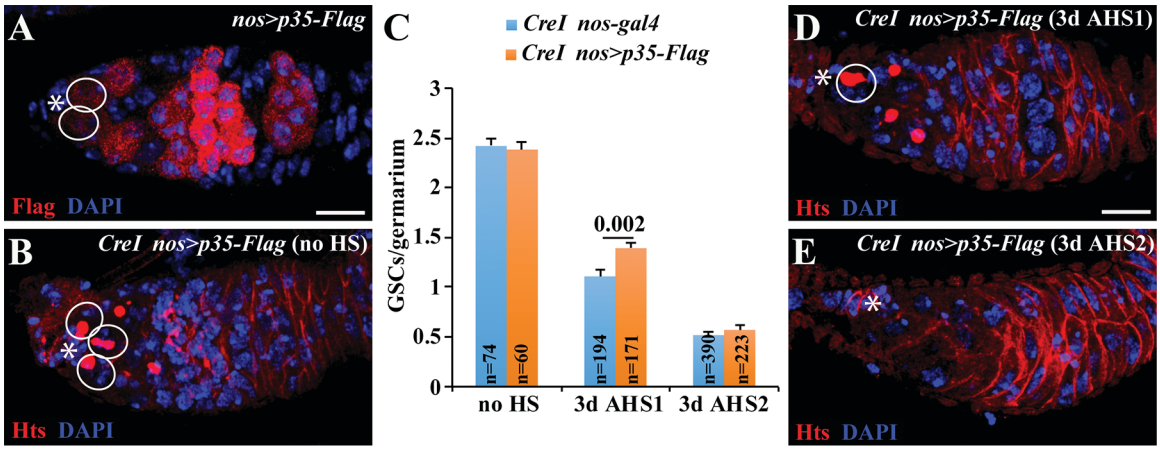


Figure S3. Germline-specific overexpression of the Baculovirus anti-apoptosis gene *p35* fails to rescue the DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (**A**) *nos-gal4*-driven expression of the C-terminal Flag-tagged *p35* (*nos>p35-Flag*) specifically in 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.

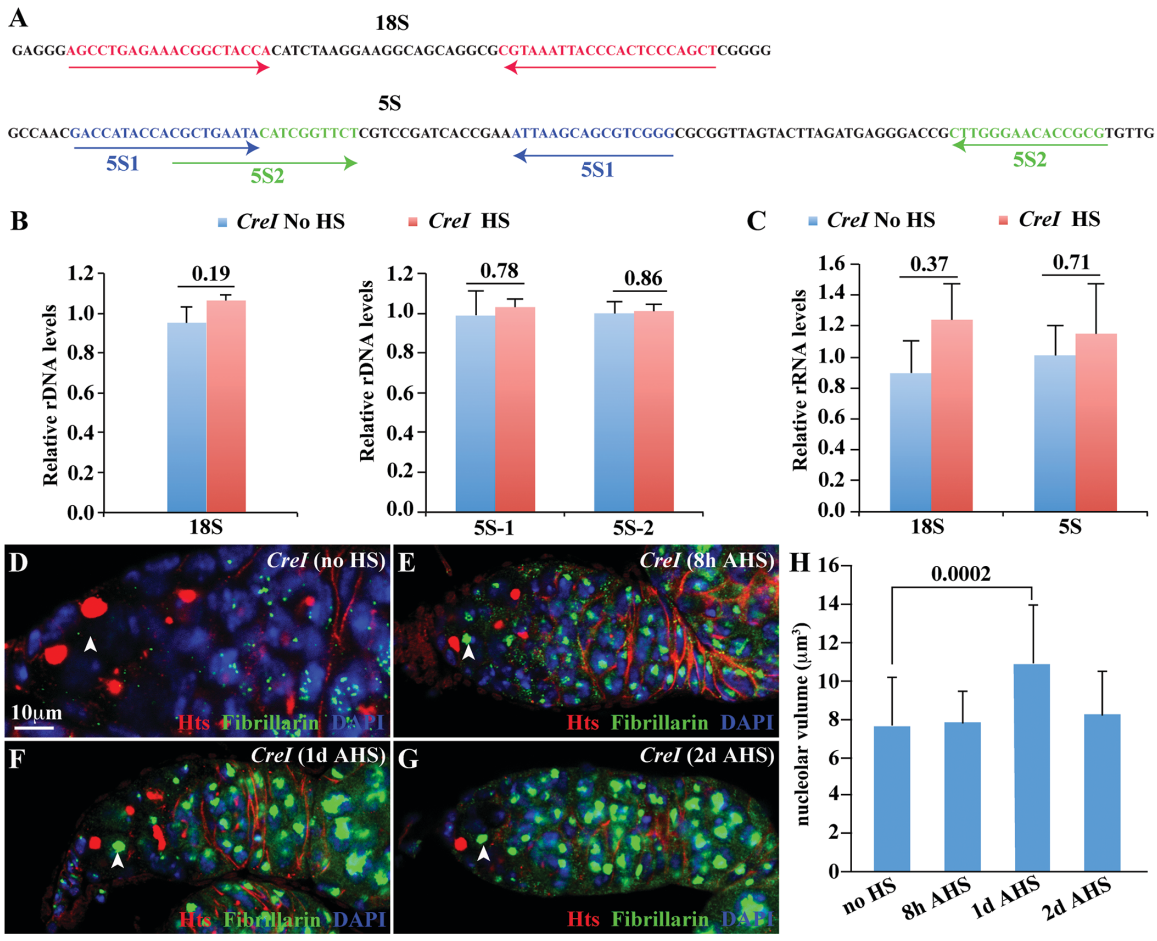


Figure S4. I-CreI-induced double-stranded DNA breaks do not affect *18S* rDNA repeats, 18S rRNA production and thus nucleolus volume. (**A**) Primer pairs for quantitative PCRs to detect the copy numbers of *18S* and *5S* genes. (**B**) Quantitative PCR results show that I-CreI-mediated double-stranded 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 gernarium (no HS, **D**). **H**: nucleolus volume quantification results. Scale bar: 10μm.

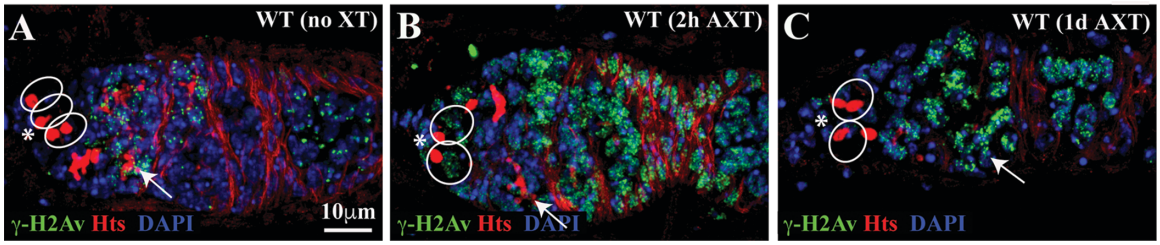


Figure S5. X-ray irradiation can efficiently introduce DNA damage into GSCs and their progeny. Ovals and asterisks indicate GSCs and CPCs, respectively. (**A**) Wild-type (WT) germarium contains γ-H2Av-negaitve GSCs and γ-H2Av-positive meiotic germ cells (arrow) without X-ray treatment (no XT). (**B**) 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-negaitve GSCs and γ-H2Av-positive differentiated germ cells (arrow) 1d AXT, indicating that DNA damage has been successfully repaired in GSCs. Scale bar: 10μm.

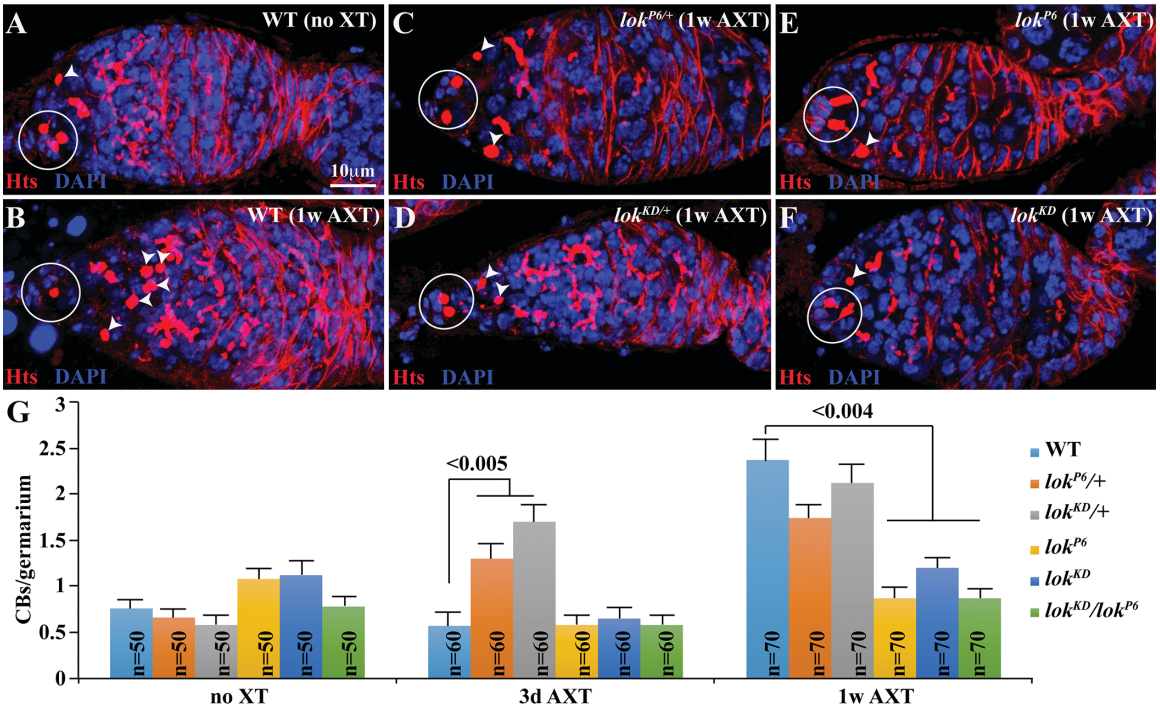


Figure S6. CHK2 inactivation can rescue the germ cell differentiation defect caused by X-ray-induced DNA damage. Circles and arrowheads indicate CPCs/GSCs and CBs, respectively. (**A**, **B**) X-ray-treated germarium (1w AXT, **B**) contains excess CBs in comparison with the control untreated germarium 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.