DNA Damage-Induced CHK2 Activation Compromises Germline Stem Cell Self-Renewal and Lineage Differentiation

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Summary

Stem cells in adult tissues are constantly exposed to genotoxic stress and also accumulate DNA damage with age. DNA damage has been proposed to cause stem cell loss and cancer formation. However, it remains a mystery how DNA damage leads to both stem cell loss and cancer formation. In this study, we use germline stem cells (GSCs) in the *Drosophila* ovary to show that DNA damage retards stem cell self-renewal and lineage differentiation in a CHK2 kinase-dependent manner. 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 an accumulation of ill-differentiated GSC progeny. Elimination of CHK2 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. Surprisingly, checkpoint kinases ATM and ATR have distinct functions from CHK2 in GSCs in response to DNA damage. The reduction in BMP signaling and E-cadherin only makes limited contribution to DNA damage-induced GSC loss. Finally, DNA damage also decreases the expression of the master differentiation factor Bam in a CHK2-dependent manner, which helps explain the GSC progeny differentiation defect. Therefore, this study demonstrates, for the first time *in vivo*, that CHK2 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. Our study also demonstrates that inducible I-CreI is a convenient genetic system for studying DNA damage responses in stem cells.

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 composed of adjacent cap cells and escort cells (Lin, 2002; Xie, 2013) (Fig. 1A). They can be easily identified by their location and 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 BMP signals, encoded by *dpp* and *gbb*, which function within one cell diameter to directly repress *bam* expression and thereby maintain GSC self-renewal (Chen and McKearin, 2003a; Song et al., 2004; Xie and Spradling, 1998). In addition, niche-expressing E-cadherin is also required for anchoring GSCs in

the niche for long-term self-renewal (Song et al., 2002). Moreover, different levels of E-cadherin 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; Li et al., 2013). Bam works with dSmurf to repress BMP signaling in GSC progeny via 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, eIF4A, 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 the organisms ranging from *Drosophila* to man, DNA damage causes the activation of the highly conserved checkpoint kinases, CHK2, 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), whereas ATR has also been shown to be required in multiple tissue stem cells in mice (Ruzankina et al., 2007). Both ATR and

ATM are required to maintain intestinal stem cells in *Drosophila* in the absence of DNA damage (Park et al., 2015). However, it remains unclear if CHK2 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 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). The 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 if 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 CHK2-dependent manner. Stem cell loss could cause premature tissue aging, whereas the accumulation of ill-differentiated stem cell progeny could increase the chance for 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.

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-41*^{D3} (Banga et al., 1995); *UAS-shRNA* lines for *lok* (GL00020 and THU00402), *tefu* (HMS02790), *mei-41* (HMS02331; GL00284) and *grp* (HMS01573 and HMC05162) (Ni et al., 2009; Ni et al., 2011). Flies were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless specified.

Induction of DNA damage by I-CreI and X-ray

To induce DNA damage, flies were incubated at 37°C for 1 hour 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 20,700rad radiation (2070rad/min x 10min).

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, DSHB), rabbit polyclonal anti-β-galactosidase antibody (1:100, Cappel), 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), rabbit polyclonal anti-pS137 H2Av antibody (1:100, Rockland), rabbit monoclonal anti-Cleaved Caspase-3 (Asp175) (1:100, Cell signaling), rabbit monoclonal anti-pS423/425 Smad3 antibody (1:100, Epitomics), chicken polyclonal anti-GFP antibody (1:200, Invitrogen), rabbit anti-Anillin (generously provided by Dr. C. Field), and rabbit polyclonal anti-Fibrillarin (1:100, abcam). All images were taken with a Leica TCS SP5 confocal microscope.

Results

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^{o}C for one hour (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 the adult females one day (1d) and three days (3d) after one-hour 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 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 and 1C). 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 (1d 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 3d AHS (Fig. 1E and 1F). Additionally, GSCs undergo cell cycle arrest immediately after DNA damage, and then resume rapid cell proliferation after repairing DNA damage based on 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 HS) contain an average of 2.5 GSCs (Fig. 1G and 1H). Following DNA damage, the germaria maintain an average of 2.2, 1.0 and 1.2 GSCs 1d AHS, 3d AHS and 1w AHS, respectively (Fig. 1G-J). Consequently, 1w AHS, the germaria either contain one GSC or completely lose their GSCs (Fig. 1G and

1I). After DNA damage, some germaria also often contain more spectrosome-containing CB-like single germ cells than the control germaria (Fig. 1G and 1J). 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. Then we determined if DNA damage causes GSC loss due to apoptosis by examining the expression of the 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, the expression of the cleaved Caspase-3 was not detected in the examined 143 normal GSCs as well as 180 DNA damaged GSCs (118 GSCs 1d AHS and 62 GSCs 2d AHS) (Fig. 1K-M). Consistently, germline-specific *p35* overexpression cannot prevent the DNA damage-induced GSC loss (Fig. S3). Taken together, these results suggest that DNA damage causes GSC loss unlikely due to apoptosis although we could not 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 critical for protein synthesis; I-CreI-induced GSC loss could be due to decreased protein synthesis because protein synthesis is critical 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 unlikely affect rRNA production and thus protein synthesis.

Next, we determined if 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 20,000rad 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 causes GSC loss and increases the accumulation of CB-like single germ cells just like two-hour 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.

CHK2 is largely responsible for the DNA damage-induced GSC loss

DNA damage evokes cell cycle checkpoint activation through activation of 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, CHK2 and ATR, which are encoded by *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 if CHK2 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 2-3 GSCs (Fig. 2A, 2B and 2E). *lok*^{P6} is a molecularly null allele (Abdu et al., 2002). Here, the two-hour heatshock was used to generate a more severe GSC loss phenotype for testing CHK2 requirement: most of the germaria harbor 0 GSC 3d and 1w after the induction (Fig. 2C, 2E compared to 1G). By contrast, most of the *lok* heterozygous and homozygous germaria still maintain 2 GSCs 3d and 1w AHS (Fig. 2D-E). Similarly, both the heterozygous and homozygous *lok* mutations can significantly and drastically rescue the GSC loss caused by the 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 itself alone does not affect GSC maintenance (Fig. 2I-K). Quantitatively, *lok-i* and the deletion mutant *lok*^{P6} have similar rescue effect on the DNA damage-evoked GSC loss, suggesting that CHK2 is required intrinsically for the DNA damage-induced GSC loss (Fig. 2E and 2K). Therefore, DNA damage-induced GSC loss is largely CHK2-dependent.

We then investigated if ATR is also required for DNA damage-induced checkpoint activation in GSCs by examining GSC and CB numbers in the DNA-damaged *mei-41* mutant germaria. As the controls, both heterozygous and homozygous *mei-41* mutations appear to not have much effect on the GSC number in the absence of DNA damage (Fig. 3A, 3B and 3E). Contrary to the homozygous *lok* mutation, the homozygous *mei-41* mutation exacerbates the DNA damage-induced GSC loss phenotype (Fig. 3C-E). Consistently, 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 ATR is required for preventing DNA damage-induced GSC loss, and further suggest that ATR and CHK2 have distinct roles in mediating DNA damage-induced GSC loss.

ATM and CHK1 are encoded by *tefu* and *grp* in *Drosophila*, respectively. To determine if ATM and CHK1 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 GSC, and the *tefu-i* germaria contain one GSC on average (Fig. 3J and 3K). 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 CHK1 is dispensable for GSC maintenance (Fig. 3J and 3M). 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 0 GSC and 1 GSC, respectively (Fig. 3J, 3L and 3N). These results indicate that ATM is required for, but CHK1 is dispensable for, preventing DNA damage-induced GSC loss.

CHK2 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). Since ATR behaves differently from CHK2 in GSCs in response to DNA damage, we then determined if the kinase activity of CHK2 is required for DNA damage-induced GSC loss. To this end, we used the CAS9/CRSPR technique to introduce the point mutation into the endogenous *lok* locus,

which converts the residue D into A at the 286 amino acid of CHK2, to create the *lok*^{KD} allele, encoding a kinase-dead CHK2 (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 and 4C). Like *lok*^{P6}, both heterozygous and homozygous *lok*^{KD} mutations can significantly and drastically rescue the GSC loss induced by DNA damage produced by I-CreI (Fig. 4B and 4C). Similarly, both heterozygous and homozygous *lok*^{KD} mutations can significantly and drastically rescue the GSC loss induced by X-ray (Fig. 4D-F). All these results demonstrate that the kinase activity of CHK2 is critical for the DNA damage-induced GSC loss.

_p53_ is required to prevent the 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 if *p53* is also involved in DNA damage-induced GSC loss, we examined GSC numbers in the DNA damaged control and *p53* mutant germaria. Without DNA damage (no I-CreI expression), the *p53* homozygous mutant germaria have the average of 2.5 GSCs just like in the control and *p53* heterozygous germaria (Fig. 4G and 4I). Three days after DNA damage, the *p53* homozygous mutant germaria have the average of 1 GSC just like 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 and 4I). Since 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 E-cadherin 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 E-cadherin-mediated cell adhesion is required for retaining GSCs in the niche for continuous self-renewal (Song et al., 2002). To determine if DNA damage affects BMP signaling in GSCs, we examined the expression of *Dad-lacZ* and pMad in the control and DNA-damaged germaria. Niche-derived BMPs, Dpp and Gbb, function as short-range signals to activate Mad phosphorylation (pMad) and *Dad* activation in GSCs (Casanueva and Ferguson, 2004; Chen and McKearin, 2003a; Kai and Spradling, 2003; Song et al., 2004). In the control germaria, *Dad-lacZ* and pMad are specifically expressed in GSCs (Fig. 5A and 5C). In contrast, in the DNA-damaged germaria, *Dad-lacZ* and pMad expression is significantly downregulated in GSCs (Fig. 5A-D). To investigate if DNA damage also decreases E-cadherin accumulation in the GSC-niche junction, we quantified the E-cadherin expression levels based on immunofluorescence staining of E-cadherin in the control and DNA-damaged germaria as we previously reported (Jin et al., 2008). Indeed, E-cadherin 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 down-regulates BMP signaling and E-cadherin accumulation in GSCs.

To determine if decreased BMP signaling contributes to DNA damage-induced GSC loss, we investigated if 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 down-regulation might make some contributions to the DNA damage-induced GSC loss (Fig 5H-I').

To further determine if forced expression of E-cadherin can slow down the GSC loss caused by DNA damage, we used *nos-gal4* to drive *UASp-shg* expression in the DNA-damaged GSCs. Two independent *UASp-shg* transgenic lines in this study were used previously to overexpress E-cadherin in GSCs (Jin et al., 2008; Pan et al., 2007). As previously reported (Pan et al., 2007), forced expression of E-cadherin does not affect the GSC number in the absence of DNA damage (Fig. 5J and 5K). Similarly, forced

expression of E-cadherin does not rescue the DNA damage-induced GSC loss, suggesting that DNA damage might affect E-cadherin localization or function, but 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 E-cadherin-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 and 6B). By carefully examining the accumulation of CB-like cells 3d and 1w after DNA damage caused by either I-CreI or X-ray, we show that the DNA damaged germaria accumulate more CB-like cells during the 3d-1w period in comparison with the control ones (Fig. 6B and 6C; Fig. S6). Interestingly, the heterozygous lok^{P6} and lok^{KD} mutations enhance the emergence of the germ cell differentiation defect 3d after DNA damage, but do not enhance or suppress the germ cell differentiation defect 1w 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, 6F and 6G; Fig. S6). These results indicate that CHK2 activation also contributes to the DNA damage-induced germ cell differentiation defect.

To determine if 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 (Fig. 6H and 6J). 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 be detected in the CB. To determine how DNA damage might affect Bam protein expression in early GSC progeny, we examined its expression in 2-cell, 4-cell and 8-cell cysts of the control and DNA-damaged germaria. In the control germaria, 4-cell and 8-cell cysts strongly express Bam protein, which level is higher than that in 2-cell cysts (Fig. 7A-C' and 7J). However, in the DNA-damaged germaria, 2-cell, 4-cell and 8-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' and 7J). Consistent with the finding that CHK2 inactivation can rescue the DNA damage-induced CB differentiation defect, a homozygous *lok* mutation can restore Bam expression levels in 2-cell, 4-cell and 8-cell cysts in the DNA-damaged germaria to the levels comparable to those in the control germaria (Fig. 7G-J). These results suggest

that DNA damage decreases Bam protein accumulation in mitotic 2-cell, 4-cell and 8-cell cysts in a CHK2-dependent manner.

Then 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 the 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 in comparison with that in control ones, indicating that DNA damage affects *bam* transcription in CBs and mitotic cysts (Fig. 7K-M). To determine if 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* posttranscriptional regulation. As the 3'UTR control, DNA damage does not affect the expression of the *gfp* reporter fused with the *K10* 3'UTR in GSCs and mitotic cysts (Fig. 7N-P). In the control germaria (No HS), GFP protein expression is higher in mitotic cysts than that 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 in comparison 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 against aging, and could also be cellular targets 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, down-regulation of BMP signaling and E-cadherin-mediated GSC-niche adhesion as well as CHK2 activation-dependent GSC loss. In addition, CHK2 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, ATM, ATR, CHK1 and p53 do not work with CHK2 in DNA damage checkpoint control in *Drosophila* ovarian GSCs. Therefore, this study demonstrates that DNA damage-induced CHK2 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 CHK2 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 transient DNA damage causes GSC loss not through apoptosis based on two pieces of experimental evidence: first, DNA-damaged GSCs are not positive for the 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 due to self-renewal defects though we could not 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 E-cadherin-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 E-cadherin in GSCs. Since constitutively active BMP signaling alone or in combination with E-cadherin overexpression can only moderately rescue GSC loss caused by DNA damage, we conclude that decreased BMP signaling and apical E-

cadherin accumulation might partly contribute to the DNA damage-induced GSC loss. Therefore, our findings suggest that DNA damage-mediated down-regulation of BMP signaling and E-cadherin-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, ATR, but not ATM, is required for checkpoint activity, indicating that ATM and ATR could have different functions in germ cells (Joyce et al., 2011). Both ATR and CHK2 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), while CHK1 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, CHK2 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 if CHK2 behaves similarly in mammalian stem cells

in response to DNA damage. Second, ATM 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 if ATM also prevents the oxidative stress in *Drosophila* GSCs as in mouse hematopoietic stem cells. Third, ATR is dispensable for normal GSC maintenance, but it protects GSCs in the presence of DNA damage. Although CHK2 and ATR 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, CHK1 is dispensable for GSC self-renewal in the absence and presence of DNA damage. Consistent with our findings, the females homozygous for *grp*, encoding CHK1 in *Drosophila*, can still normally 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 CHK2 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 CHK2-dependent manner because CHK2 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. Consistently, Bam protein expression is significantly decreased in DNA damaged mitotic cysts in comparison with control ones. Interestingly, CHK2 inactivation can also fully restore Bam protein expression levels in the DNA-damaged mitotic cysts. Taken together, CHK2 activation is largely responsible for Bam down-regulation 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.

Acknowledgements

We would like to thank D. Chen, B. Sullivan, Developmental Studies Hybridoma Bank and Bloomington *Drosophila* Stock Center for reagents; the Xie laboratory members for stimulating discussions and critical comments, and L. Gutchewsky for administrative assistance. This work is supported by Stowers Institute for Medical Research (T. X.).

Authors' 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.).

Competing Interests: All the authors also declare no competing interests.

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Figures

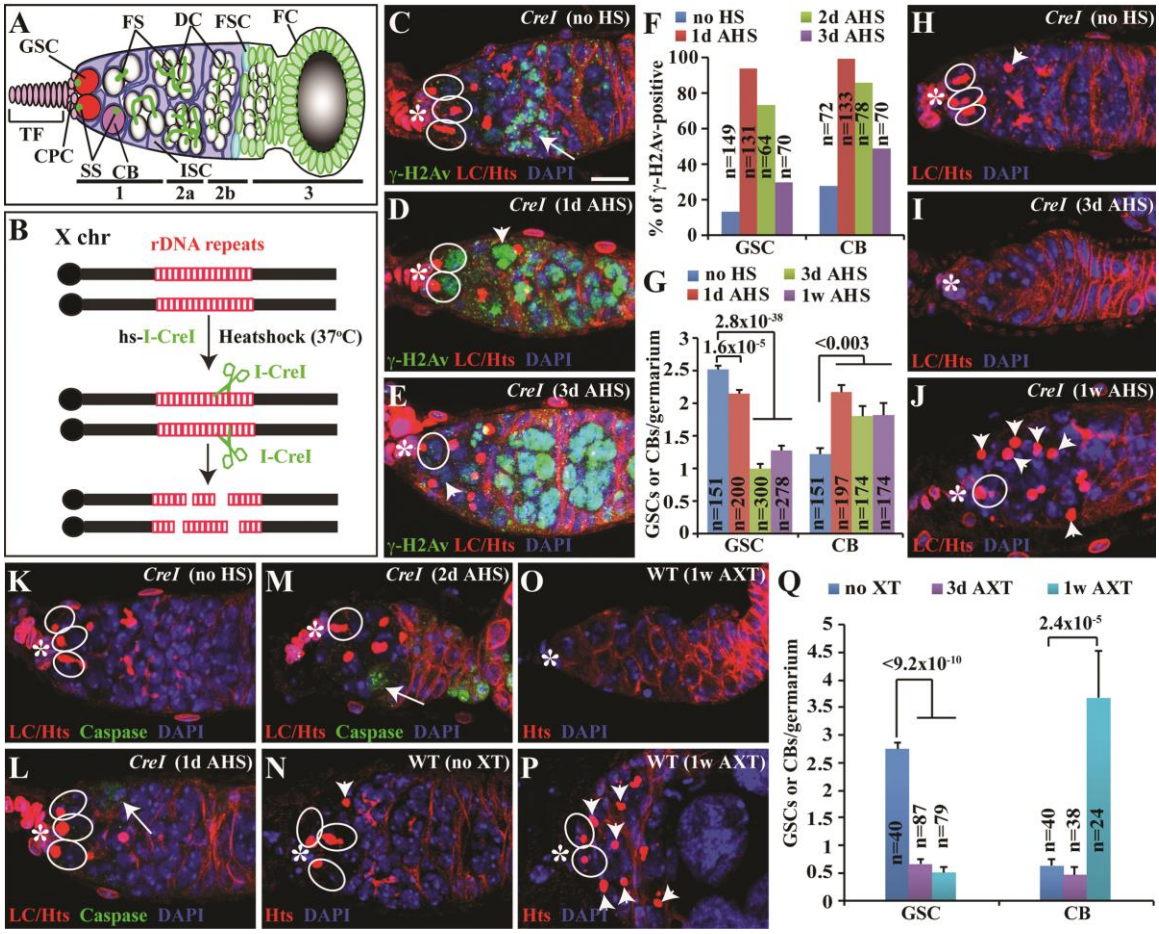


Figure 1. DNA damage compromises GSC self-renewal and lineage differentiation. (**A**) Schematic diagram of the germarium. Abbreviations: TF (terminal filament); GSC (germline stem cell); CPC (cap cell); CB (cystoblast); SS (spectrosome); FS (fusome); DC (developing cyst); ISC (inner sheath cell); FSC (follicular stem cell); FC (follicle cell). (**B**) Applying endonuclease I-CreI to induce double-stranded breaks in rDNA on the X chromosome. 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 one day after one-hour heatshock (1d AHS, **D**), but become negative 3d AHS (**E**). **F**: % of γ-H2Av-positive GSCs. (**G**-**J**) I-CreI-expressing germaria contain 0 GSC 3d AHS (**I**), or 1 GSC and 6 CBs 1w AHS (**J**) in comparison with the control one containing 3 GSCs and 1 CB (**H**). **G**: GSC and CB quantification results. (**K**-**M**) DNA-damaged GSCs remain negative for the expression of cleaved Caspase 3 1d (**L**) and 2d (**M**) AHS like the control GSCs (**K**). Arrows indicate cleaved Caspase 3-positive differentiated germ cells. (**N**-**Q**) Germaria contain 0 GSC (**O**), or 2 GSCs and extra CBs (**P**) one week after 20000rad X-ray treatment (1w AXT) in comparison with the control one carrying 3 GSCs and 1 CB (**N**). **Q**: GSC quantification results. In this figure and thereafter, I-CreI is abbreviated to CreI, error bars represent standard errors and *P* values are calculated based on Student's *t-test*. Scale bar: 10μm.

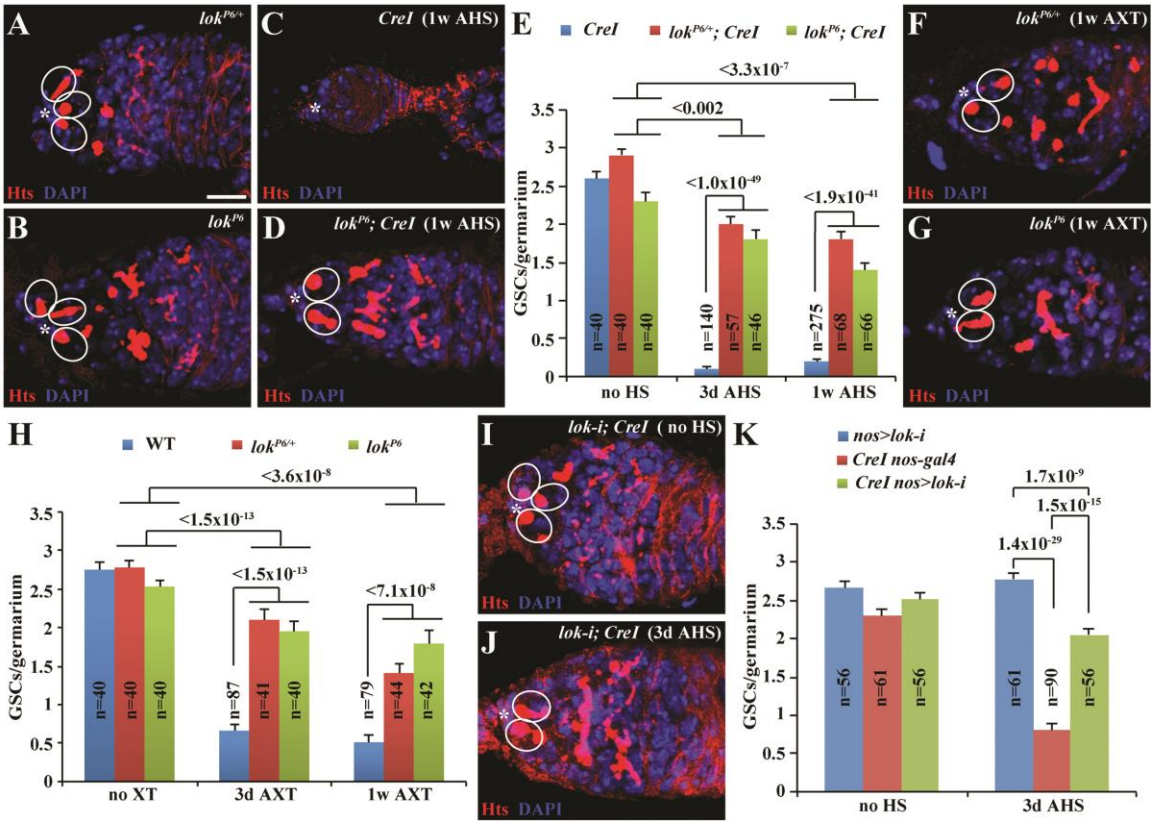


Figure 2. CHK2 inactivation drastically rescues DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (**A**, **B**) *lok*^{P6} heterozygous (**A**) and homozygous (**B**) germaria contains 3 GSCs. (**C**-**E**) DNA damaged *lok*^{P6} homozygous germarium contains 2 GSCs (**D**) in comparison with DNA damaged control ones carrying 0 GSC (**C**) one week after two-hour heatshock (1w AHS). **E**: GSC quantification results. (**F**-**H**) X-ray-treated *lok* heterozygous (**F**) and homozygous (**G**) germaria carry 2 GSCs 1w AXT. **H**: GSC quantification results. (**I**-**K**) Germline-specific *lok* knockdown (*lok-i*) significantly rescues the DNA damage-induced GSC loss. *lok-i* germaria contain 3 GSCs without DNA damage (no HS, **I**), and still maintain 2 GSCs in the presence of DNA damage (3d AHS, **J**). **K**: GSC quantification results. Scale bar: 10μm.

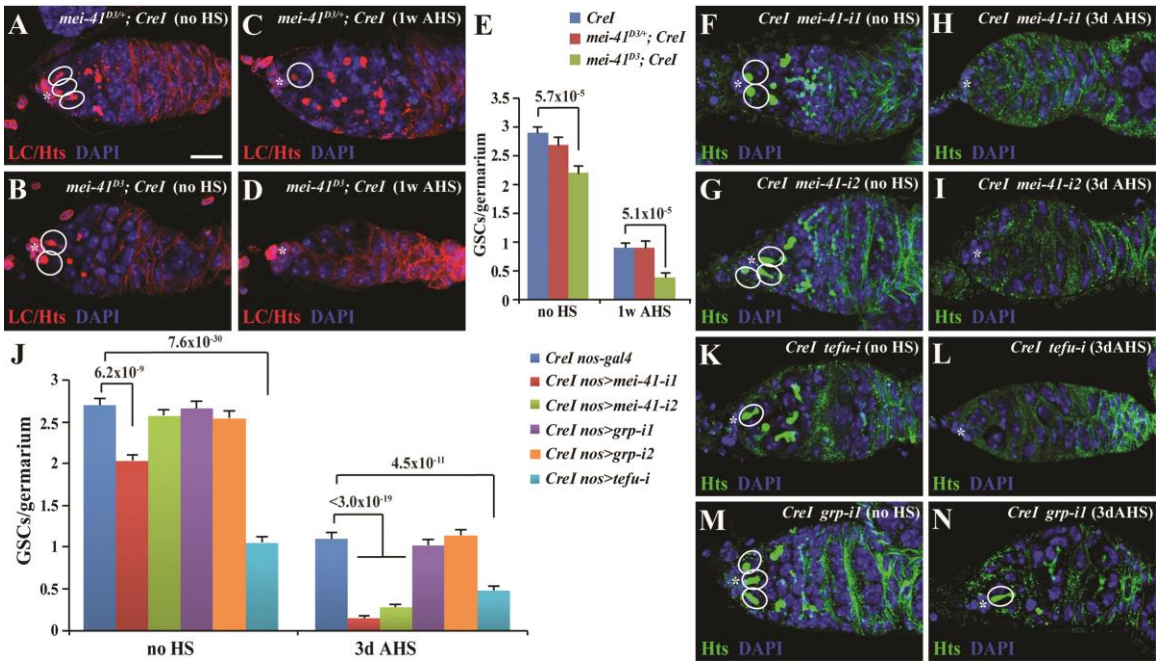


Figure 3. Inactivation of ATR, ATM or CHK1 fails to rescue the DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (**A**-**E**) The *mei-41*^{D3} homozygous mutation exacerbates the DNA damage-induced GSC loss. *mei-41*^{D3} heterozygous germaria contain 3 GSCs without DNA damage (no HS, **A**) and 1 GSC in the presence of DNA damage (1w AHS, **C**), whereas *mei-41*^{D3} homozygous germaria contain 2 GSCs without DNA damage (no HS, **B**) and 0 GSC in the presence of DNA damage (1w AHS, **D**). **E**: GSC quantification results. (**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** and **G**; two independent RNAi lines) and *grp* (**M**) knockdown germaria contain 2 or 3 GSCs, but *tefu* knockdown germarium carries 1 GSC (**K**). In the presence of DNA damage (3d AHS), *mei-41* (**H** and **I**) and *tefu* (**L**) knockdown germaria contain 0 GSC, but the *grp* knockdown germarium carries 1 GSC (**N**). **J**: GSC quantification results. Scale bar: 10μm.

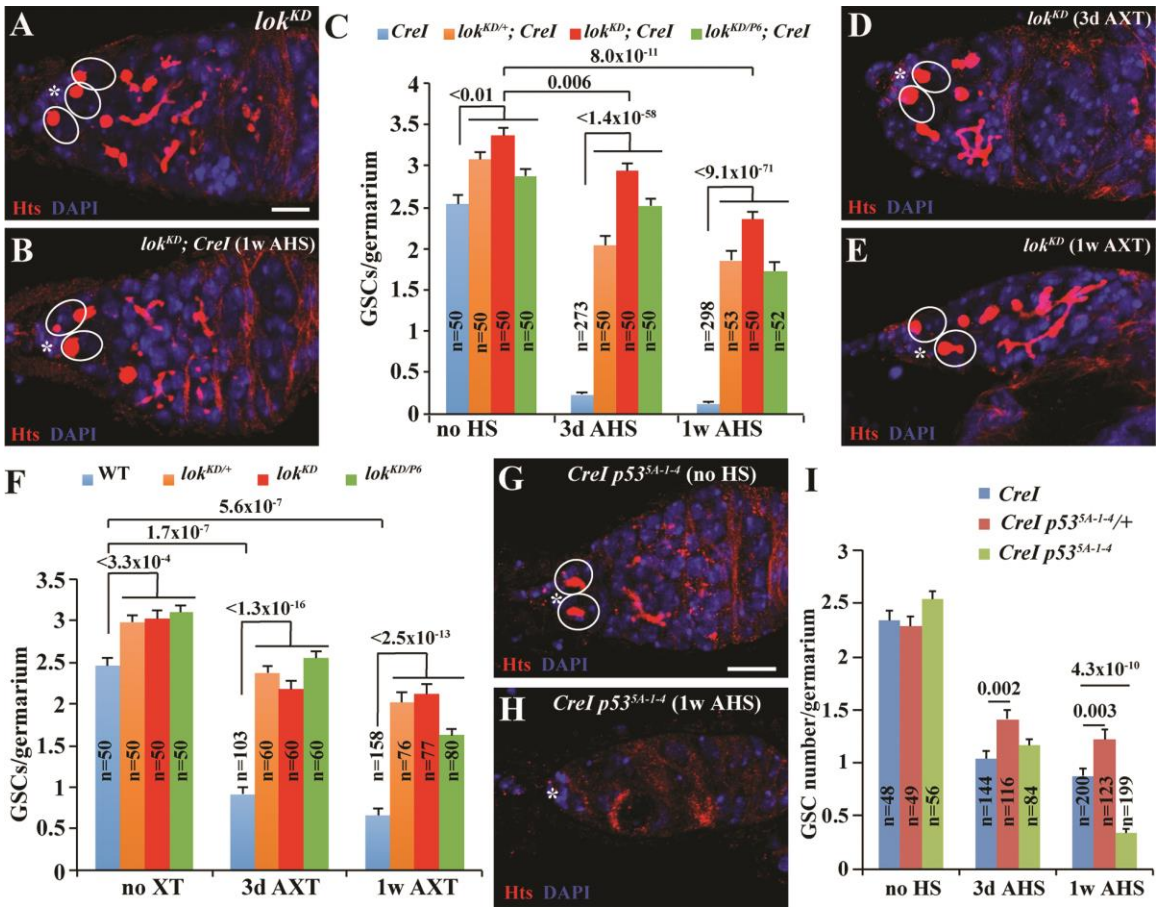


Figure 4. CHK2 kinase function is required for the DNA damage-induced GSC loss, whereas p53 prevents DNA damage-induced GSC loss. Ovals and asterisks indicate GSCs and CPCs, respectively. (**A-F**) The kinase-dead *lok*^{KD} mutation rescues the DNA damage-induced GSC loss. The *lok*^{KD} homozygous mutant germarium carries 3 GSCs (**A**). *lok*^{KD} homozygous germaria still contain 2 GSCs 3d (**D**) and 1w (**B, E**) after DNA damage induced by I-CreI (**B**) or X-ray (**D**, **E**). **C** and **F**: GSC quantitative results. (**G-I**) *p53* homozygous (**G**) germarium contains two GSCs in the absence of DNA damage (no HS), whereas DNA-damaged *p53* homozygous (**H**) germarium contains 0 GSC 1w AHS. **I**: GSC quantification results. Scale bars: 10μm.

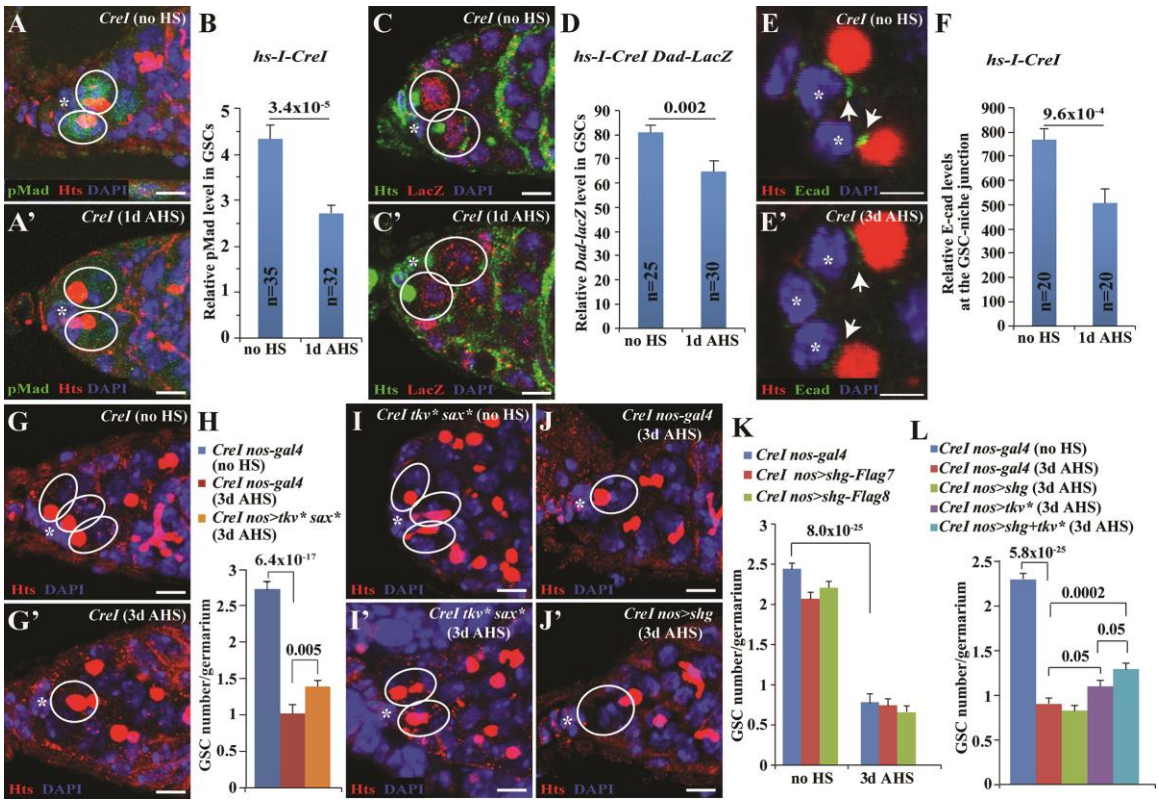


Figure 5. Downregulation of BMP signaling and E-cadherin accumulation partly contributes to the DNA damage-induced GSC loss. (**A**-**D**) GSCs show significantly lower pMad (**A**, **A'**) and *Dad-lacZ* (**C**, **C'**) expression in the presence of DNA damage (1d AHS, **A'**, **C'**) than the control GSCs (no HS, **A**, **C**). **B** and **D** show pMad and *Dad-lacZ* quantification results, respectively. (**E**-**F**) GSCs show significantly lower E-cadherin accumulation at the GSC-niche junction (arrowheads) in the presence of DNA damage (3d AHS, **E'**) than the control GSCs (no HS, **E**). **F**: E-cadherin quantification results. (**G**-**I'**) Control germaria contain 3 GSCs without DNA damage (no HS, **G**), but 1 GSC in the presence of DNA damage (3d AHS, **G'**). Tkv*/Sax*-expressing germaria contain 3 GSCs without DNA damage (no HS, **I**) and 2 GSCs in the presence of DNA damage (3d AHS,

I'). **H**: GSC quantification results. (**J**-**K**) Germline-specific E-cadherin overexpression fails to rescue the DNA damage-induced GSC loss. In the presence of DNA damage (3d AHS), both the control germarium (**J**) and E-cadherin-overexpressing germarium (**J'**) contain 1 GSC. **K**: GSC quantification results. (**L**) GSC quantification results show that germline-specific E-cadherin overexpression can enhance the rescue effect of constitutive BMP signaling on the DNA damage-induced GSC loss. Scale bars: 25μm (**A**, **A'**, **C**, **C'**, **G**, **G'** and **I**-**J'**) and 10μm (**E**, **E'**).

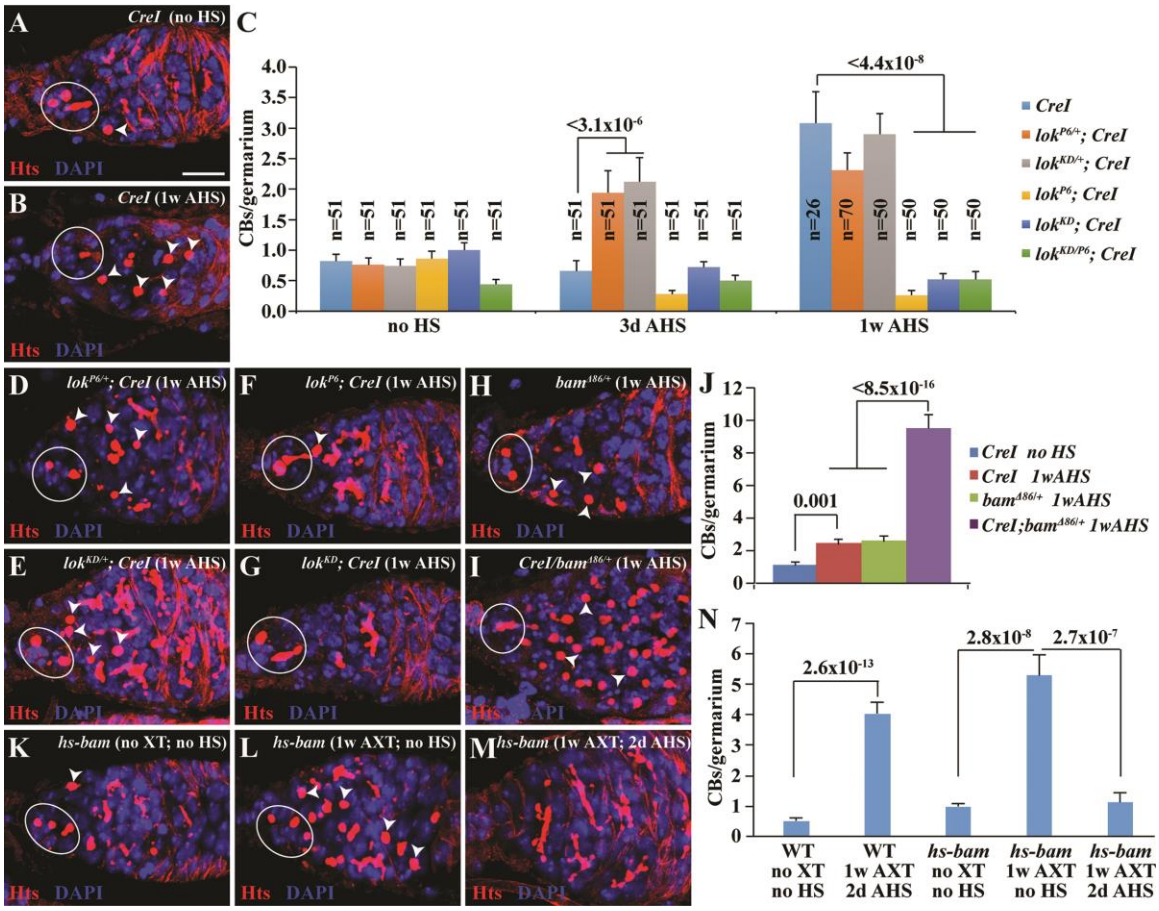


Figure 6. DNA damage slows down GSC progeny differentiation by decreasing Bam function in a CHK2-dependent manner. Ovals indicate GSCs and CPCs, whereas arrowheads indicate CBs. (**A**-**G**) DNA damaged germarium (**B**) contains excess CBs 1w AHS in comparison with the control germarium carrying 1 CB (**A**). DNA-damaged *lok*^{P6} (**D**) and *lok*^{KD} (**E**) heterozygous germaria contain excess CBs 1w AHS, but DNA-damaged *lok*^{P6} (**F**) and *lok*^{KD} (**G**) homozygous germaria contain one or no CB 1w AHS. **C**: CB quantification results. (**H**-**J**) DNA damaged *bam* heterozygous germarium (**I**) accumulates more CBs than the control *bam* heterozygous germarium (**H**). **J:** CB quantification results. (**K**-**N**) Forced *bam* expression (**M**) can drastically decrease the accumulation of CBs in the X-ray-damaged germarium (**L**) in comparison with the control germarium (**K**). **N:** CB quantification results. Scale bars: 10 μm.

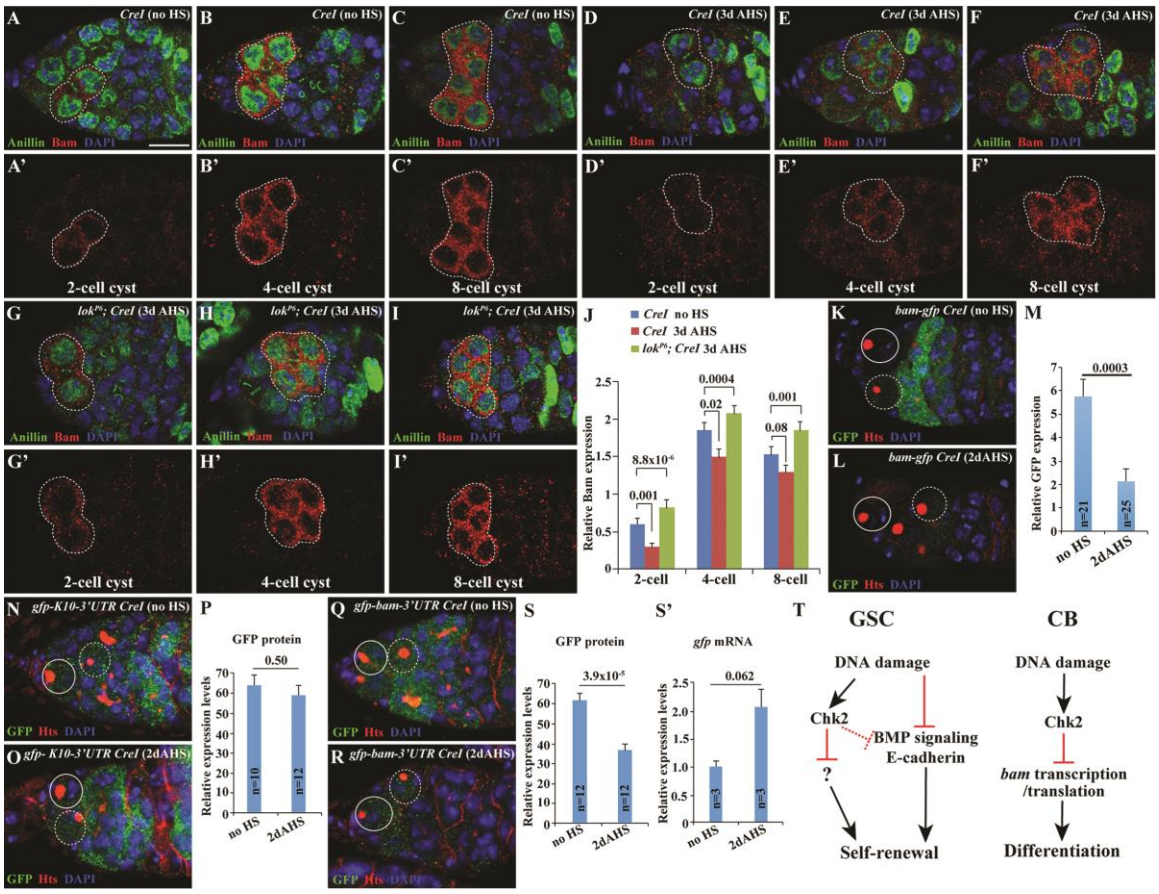


Figure 7. DNA damage decreases Bam protein expression in mitotic cysts. (**A-J**) In comparison with control 2-cell (**A**), 4-cell (**B**) and 8-cell (**C**) cysts (broken lines), DNA-damaged (3d AHS) 2-cell (**D**), 4-cell (**E**) and 8-cell (**F**) cysts (broken lines) significantly decrease Bam protein expression, but DNA-damaged (3d AHS) homozygous *lok* mutant 2-cell (**G**), 4-cell (**H**) and 8-cell (**I**) cysts (broken lines) restore normal Bam protein expression. **J**: Quantitative results on Bam protein expression. (**K-M**) *bam-gfp* expression is significantly decreased in a DNA damaged CB (broken circle, **L**) in comparison with a control CB (broken circle, **K**). **M:** GFP intensity quantification results. (**N-P**) *Pnos-gfp-K10-3'UTR* expression remains unchanged in control (broken circle, **N**) and DNA

damaged (broken circle, **O**) CBs. **P:** GFP intensity quantification results. (**Q-S'**) *Pnos-gfp-bam3'UTR* expression is significantly decreased in a DNA damaged CB (broken circle, **R**) in comparison with a control CB (broken circle, **Q**). **S** and **S':** GFP intensity and *gfp* mRNA quantification results, respectively. (**T**) A working model explaining how DNA damage affects GSC self-renewal and progeny differentiation mechanistically. Scale bars: 10 μm.

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

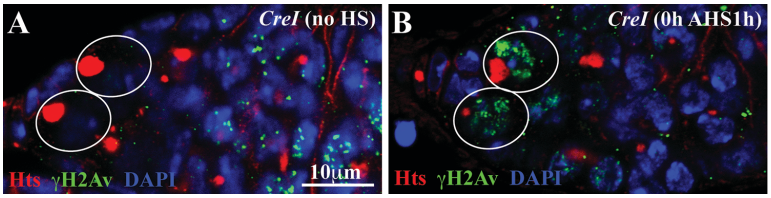
The 286th residue Asp in the transcript CHK2-PB, which is responsible for CHK2 kinase activity, was mutated into Ala to generate *lok^{KD}* by modifying the codon GAC to GCC. The sgRNA target site was "GTCAGGCTTAAGGTCACGAT**GGG**" (PAM in bold). To target this genomic site, we constructed the *lok^{KD}* 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

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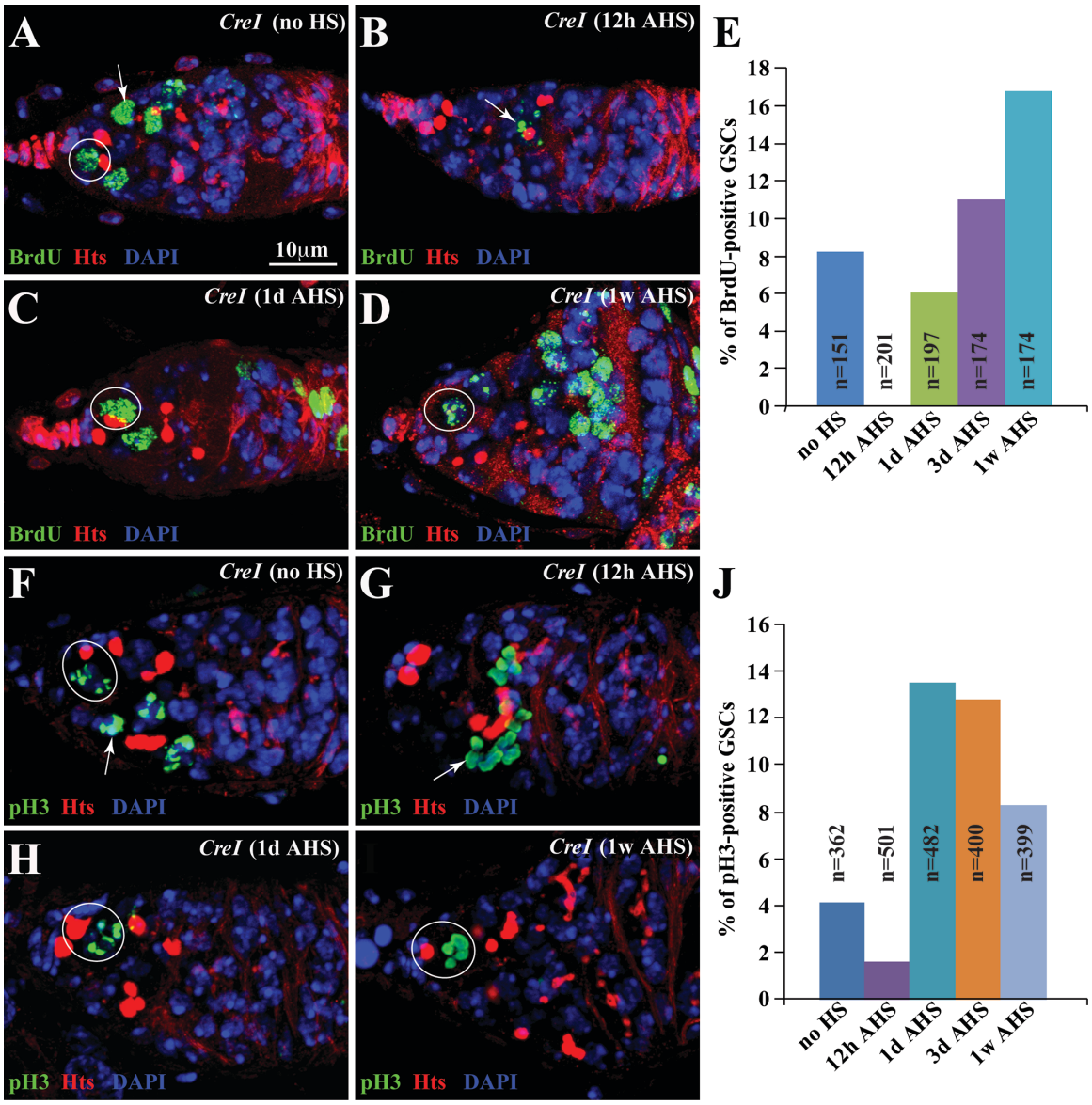
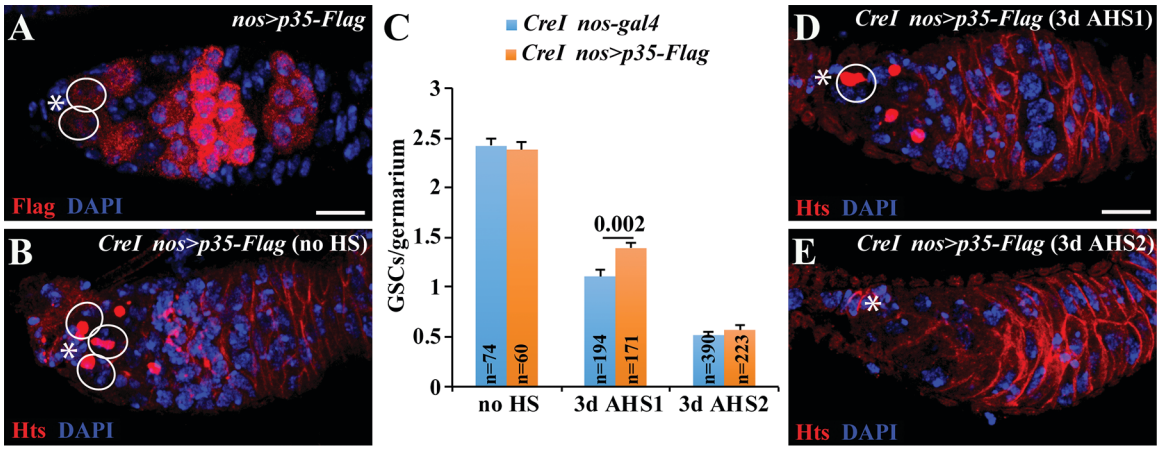


Figure S2. GSCs resume their proliferation one day after DNA damage. Ovals indicate GSCs, whereas arrows highlight mitotic cysts. (**A-E**) GSCs in the *hs-I-CreI* germarium are negative for BrdU labeling 12h AHS (**B**) and then become positive again 1d AHS (**C**) and 1w AHS (**D**) as in the control (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.



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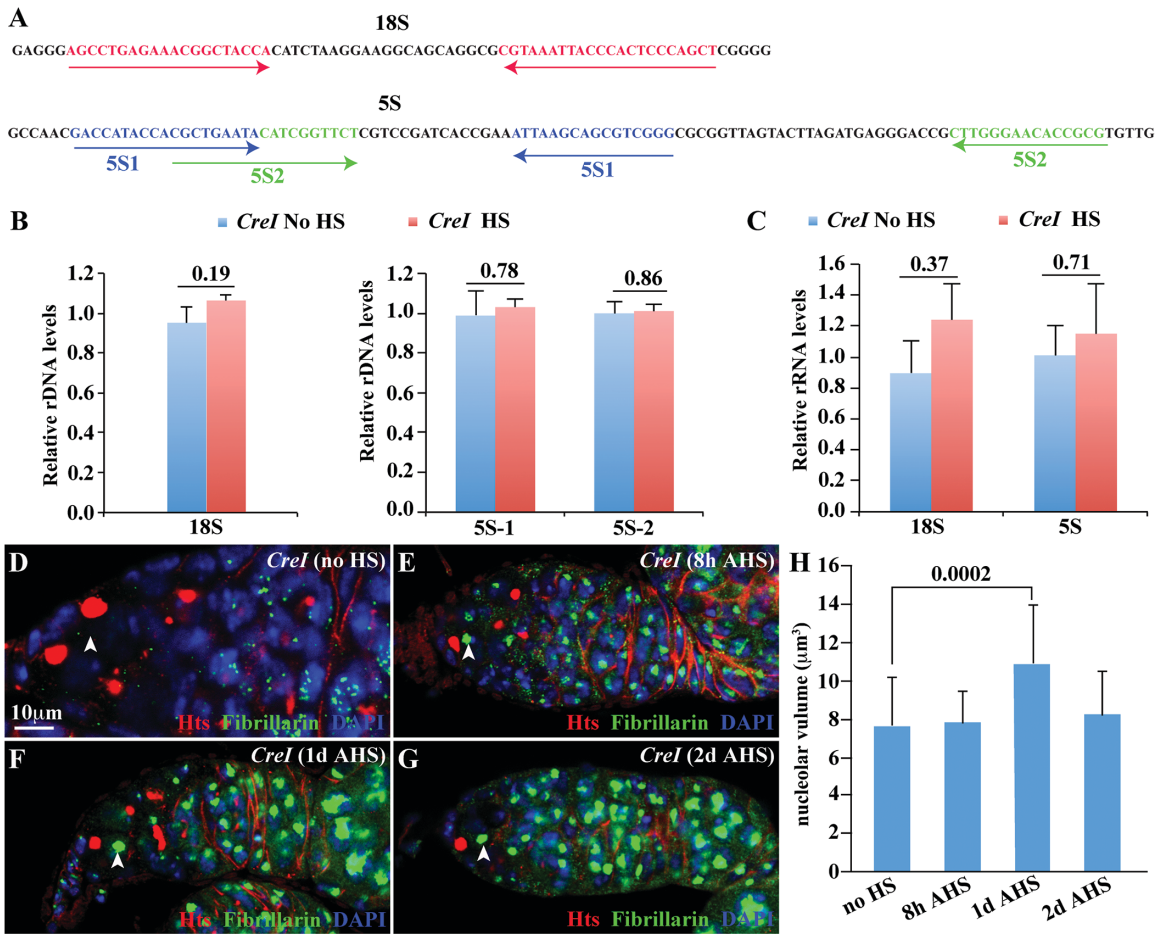

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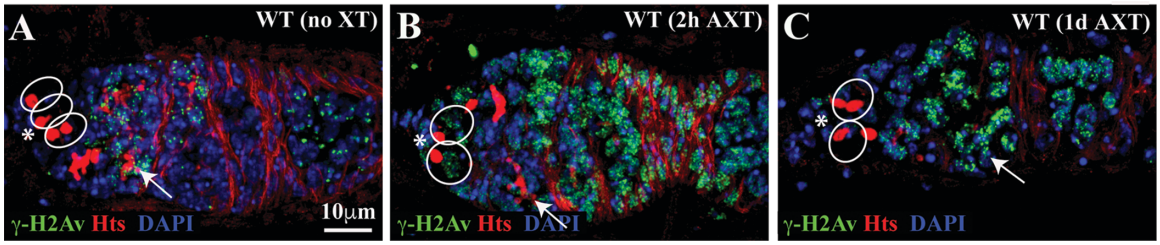

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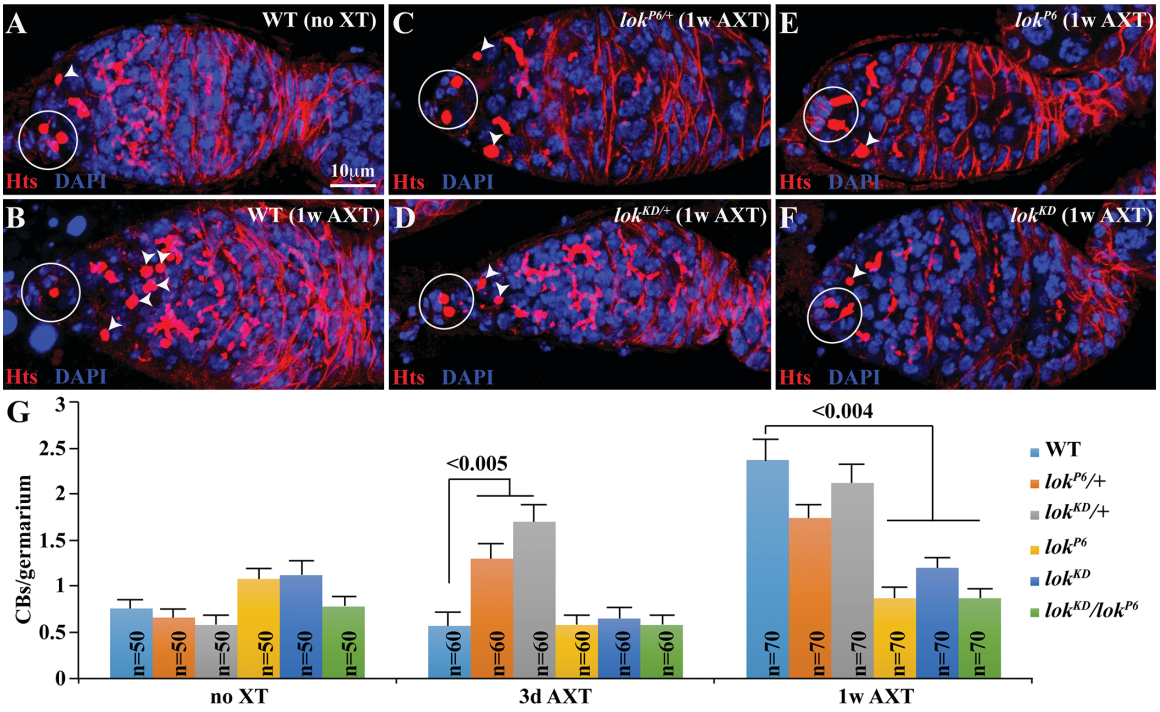

Figure S6. CHK2 inactivation can rescue the germ cell differentiation defect caused by X-ray-induced DNA damage. Circles and arrowheads indicate CPCs/GSCs and CBs, respectively. (**A**, **B**) X-ray-treated germarium (1w AXT, **B**) contains excess CBs in comparison with the control untreated germarium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

Supplemental Information

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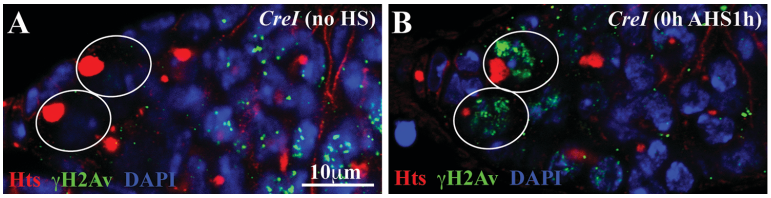
Supplemental figures



C

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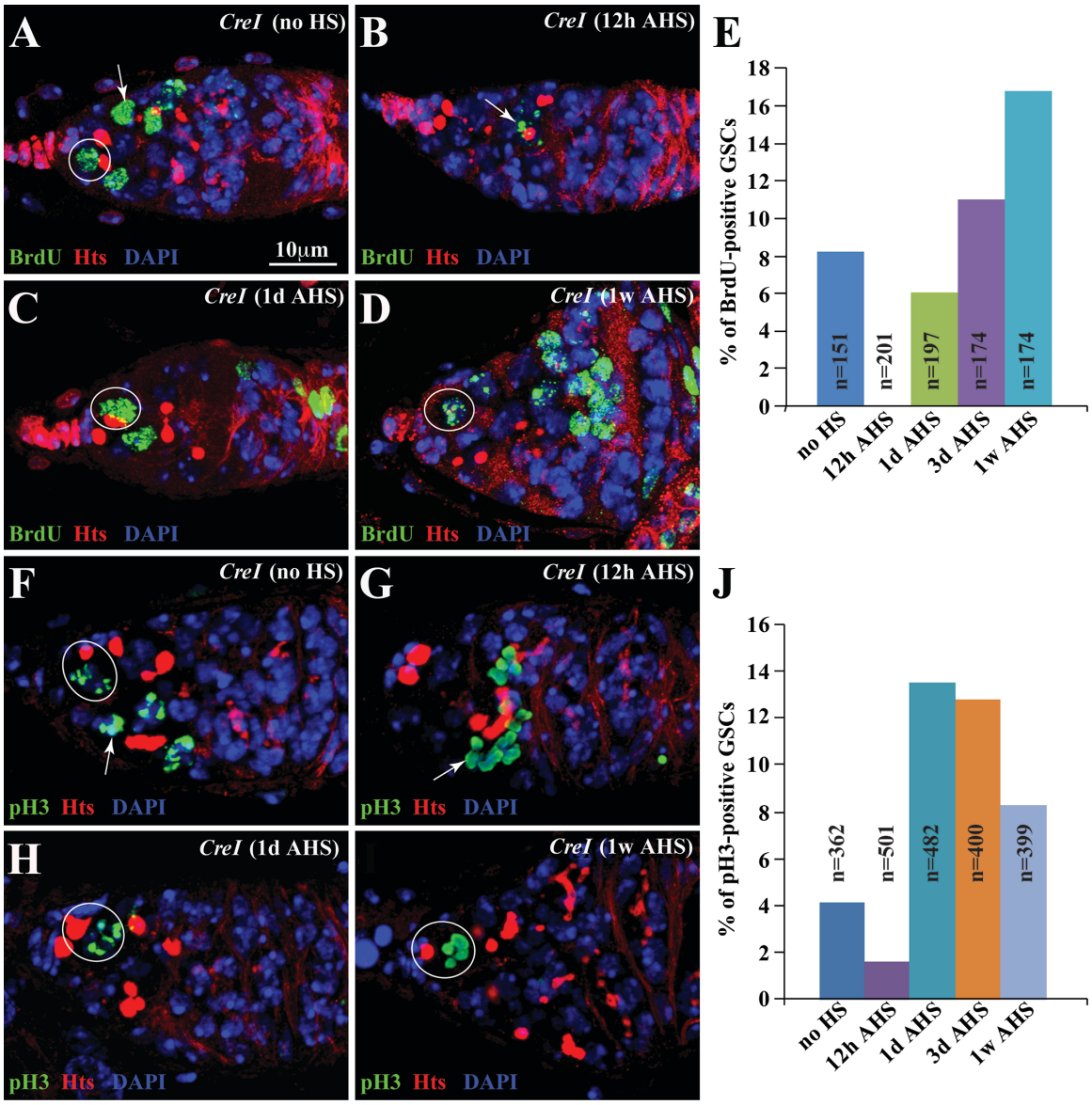

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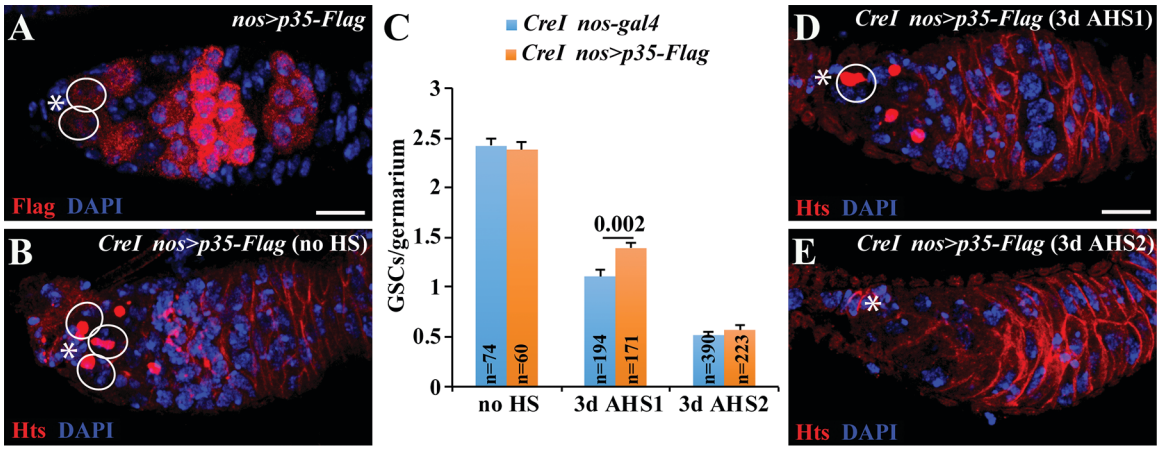

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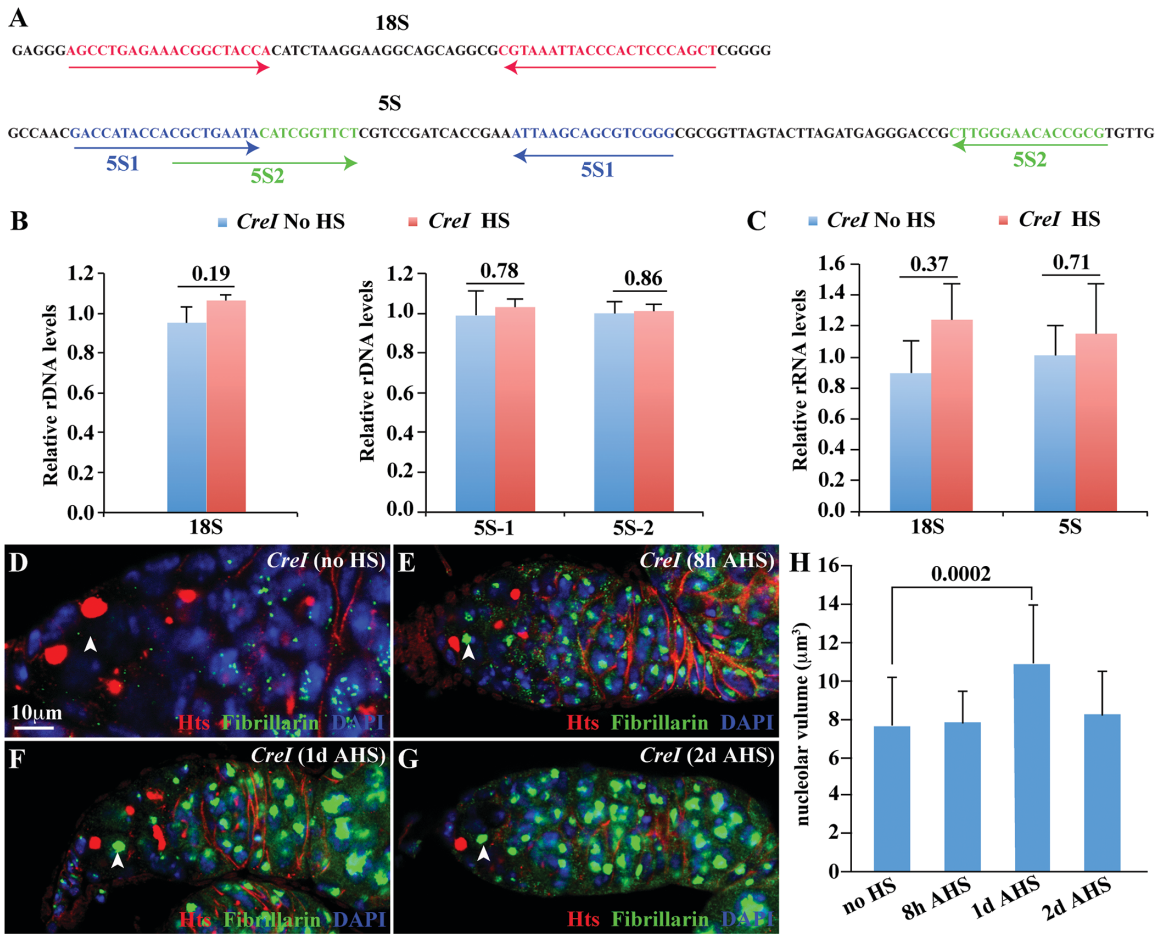

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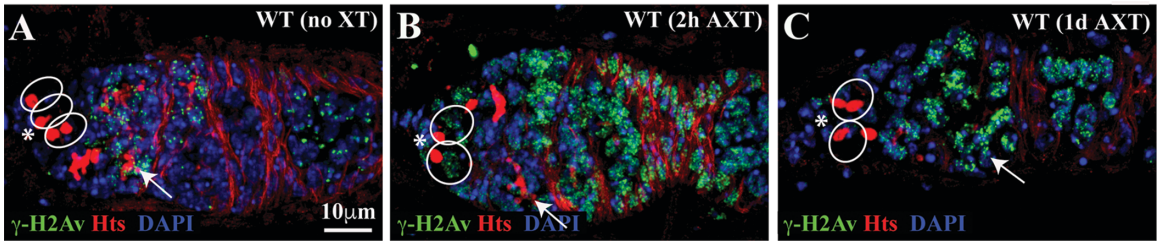

Figure S5. X-ray irradiation can efficiently introduce DNA damage into GSCs and their progeny. Ovals and asterisks indicate GSCs and CPCs, respectively. (**A**) Wild-type (WT) germarium contains γ-H2Av-negaitve GSCs and γ-H2Av-positive meiotic germ cells (arrow) without X-ray treatment (no XT). (**B**) 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.

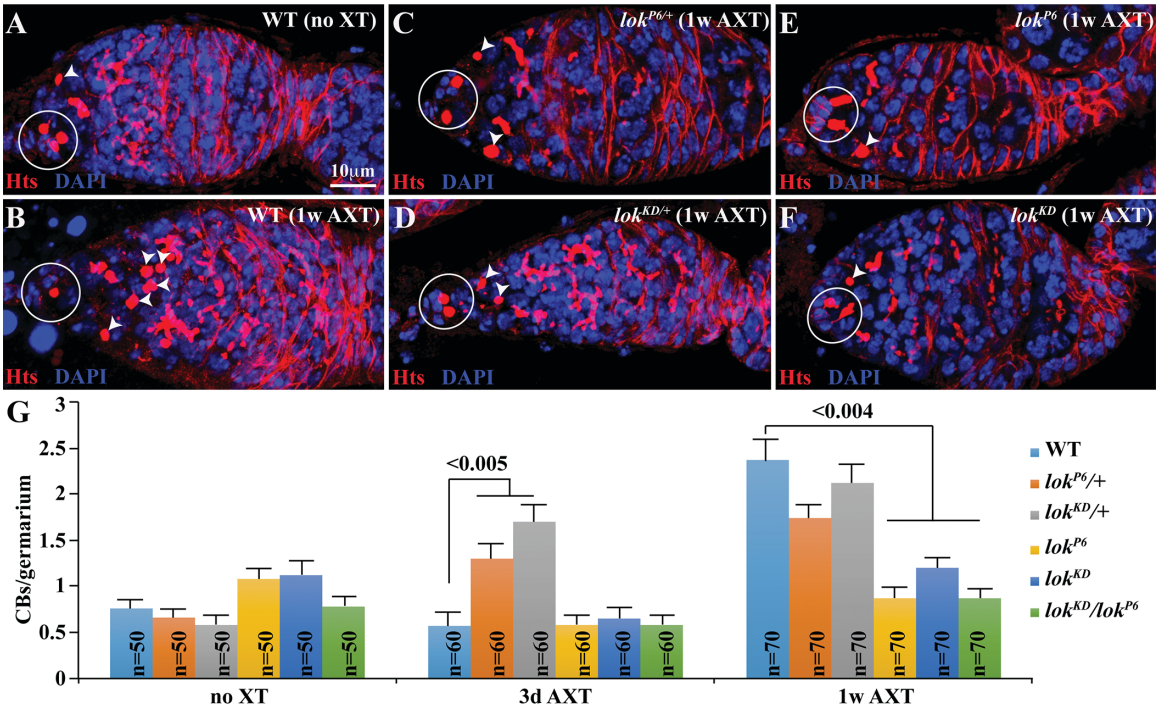

Figure S6. CHK2 inactivation can rescue the germ cell differentiation defect caused by X-ray-induced DNA damage. Circles and arrowheads indicate CPCs/GSCs and CBs, respectively. (**A**, **B**) X-ray-treated germarium (1w AXT, **B**) contains excess CBs in comparison with the control untreated germarium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