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Histone H1 defect in escort cells triggers germline tumor in *Drosophila* ovary

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ABSTRACT

Drosophila ovary is recognized as one of the best model systems to study stem cell biology in *vivo*. We had previously identified an autonomous role of the histone H1 in germline stem cell (GSC) maintenance. Here, we found that histone H1 depletion in escort cells (ECs) resulted in an increase of spectrosome-containing cells (SCCs), an ovary tumor-like phenotype. Further analysis showed that the Dpp pathway is excessively activated in these SCC cells, while the expression of *bam* is attenuated. In the H1-depleted ECs, both transposon activity and DNA damage had increased dramatically, followed by EC apoptosis, which is consistent with the role of H1 in other somatic cells. Surprisingly, H1-depleted ECs acquired cap cell characteristics including *dpp* expression, and the resulting abnormal Dpp level inhibits SCC further differentiation. Most interestingly, double knockdown of H1 and *dpp* in ECs can reduce the number of SCCs to the normal level, indicating that the additional Dpp secreted by ECs contributes to the germline tumor. Taken together, our findings indicate that histone H1 is an important epigenetic factor in controlling EC characteristics and a key suppressor of germline tumor.

1. Introduction

Stem cells possess unique abilities to self-renew and differentiate into diverse cell types to maintain tissue and organ homeostasis. Abnormal behaviors of stem cells are the leading cause of developmental diseases and tumors (Reya et al., 2001). In addition to intrinsic factors, previous studies have revealed that the microenvironment of stem cells, also called the stem cell niche, plays a pivotal role in guarding stem cell self-renewal and differentiation through intercellular signaling pathways (Fuchs et al., 2004; Morrison and Spradling, 2008; Xie and Spradling, 2000). The stem cell niche contains several types of somatic cell, and each type of cell functions distinctively (Fuller and Spradling, 2007; Li and Xie, 2005). Surrounded by a somatic cell-formed niche, GSCs receive maintenance signals from the niche, and self-renew and generate large numbers of differentiating daughter cells at the same time (Kirilly and Xie, 2007). However, the molecular mechanism underlining the function of different types of somatic cells in the niche remains elusive. Equipped with powerful genetic tools (Ni et al., 2008; Ren et al., 2013), *Drosophila* ovary is an attractive model system to study stem cell biology and the communications between

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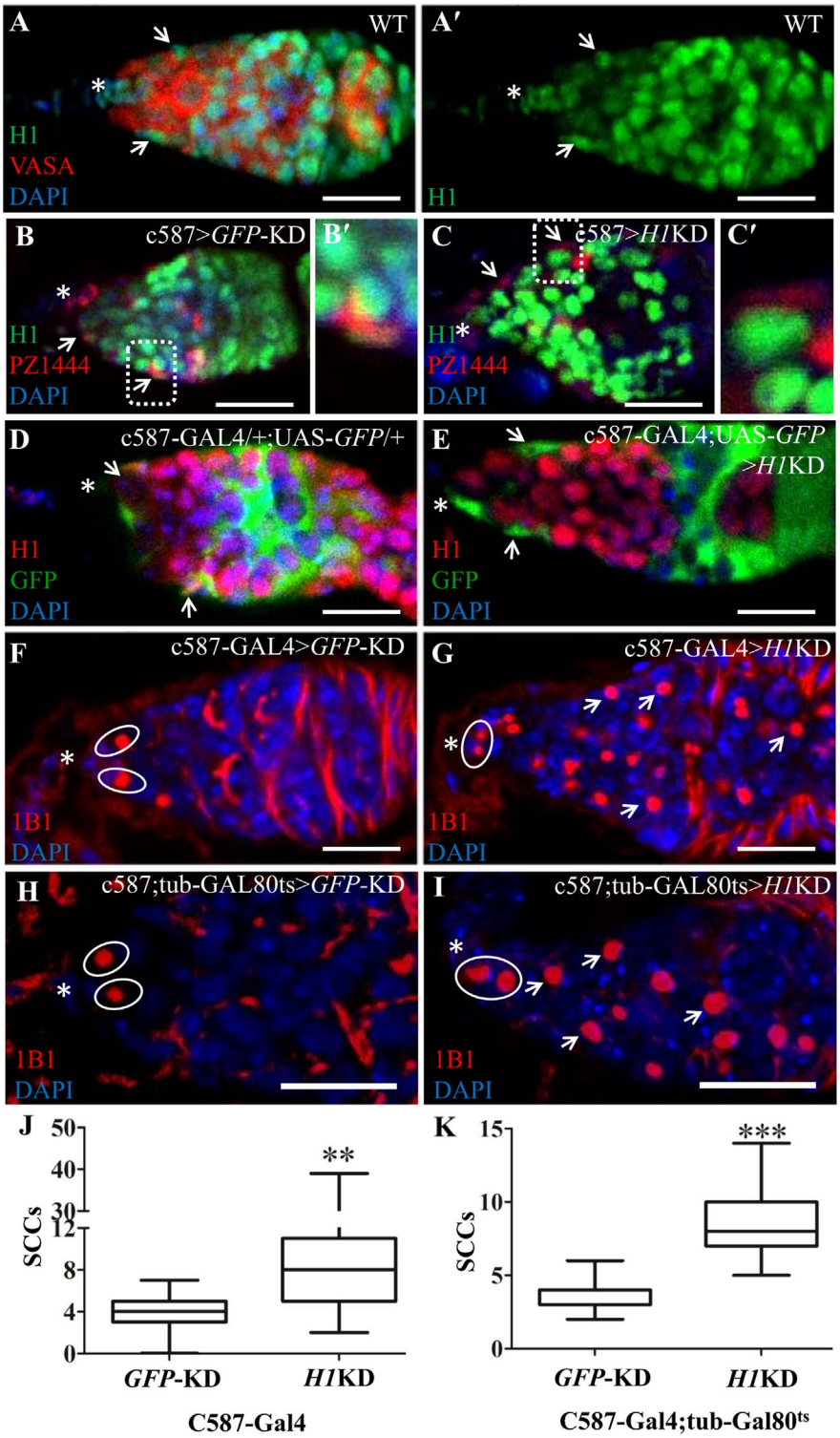


Fig. 1. Histone H1 is required in ECs for regulating germ cell number. (A-G) Germaria are from 3-day-old adult flies, and are stained with anti-Vasa (red in A) to label the germ cells, anti-GFP (green in D-E) to indicate the ECs, 1B1 (red in F-G) to mark spectrosomes/fusomes, and 4,6-diamidino-2-phenylindole (DAPI, blue) to show the nuclei. Asterisks indicate the niche cap cells in this and all subsequent figures. (A-A′) H1 expression in wild-type germarium. (A) H1 is highly expressed in ECs (arrow). (A′) H1 fluorescence channel of (A). (B-C) In c587-Gal4 > *GFP*-KD germarium (B), ECs (arrows; marked by *PZ1444*) express high levels of H1. In c587 > *H1*KD germarium (B′), zoom in of (B) showing overlap of PZ1444 and H1. (C), H1 is knocked down to a non-detectable level specifically in ECs. (C′), zoom in of (C). (D-E) In the germarium of c587-Gal4; UAS-GFP ovary (D), H1 is highly expressed in GFP-labeled ECs (arrows), whereas H1 fluorescence is almost undetectable in the ECs of c587-Gal4 > *H1*KD germarium (E), which demonstrates that H1 is specifically knocked down in c587-Gal4 > *H1*KD ECs. (F-G) The c587 > *GFP*-KD control germarium (F) has two GSCs and one cystoblast (ovals) indicated by the round spectrosomes, while the c587 > *H1*KD germarium (G) has two GSCs (ovals) and a cluster of undifferentiated SCCs (arrows). (H-I) Germaria are from adult flies raised at 29 °C for 3 weeks. Two germline stem cells (ovals) indicated by the spherical spectrosomes are located at the anterior part of the control (c587;tub-gal80^{ts} > *GFP*-KD) germarium (H). Extra spectrosome-containing cells (SCCs, arrows) aggregate away from the cap cells in the H1 knockdown (c587;tub-gal80^{ts} > *H1*KD) germarium (I). (J) Quantification results show that each control germarium contains 3.8 SCCs on average, while the *H1*KD germarium has 10.4 SCCs on average. Data are shown as means ± s.e., n=45, 60 for the two groups, respectively; p-value=0.0032. (K) Quantification results show that each control germarium contains 3.3 SCCs on average, while the c587-Gal4; tub-Gal80^{ts} > *H1*KD germarium has 8.6 SCCs on average. Data are shown as means ± s.e., n=42, 54 for the two groups, respectively; p-value < 0.0001. Scale bars: 10 μm. The anterior is to the left in this and all subsequent figures.

niche cells and GSCs (Chen and McKearin, 2005; Lin, 2002; Losick et al., 2011).

Each *Drosophila* female has two ovaries, and each ovary consists of 16–20 ovarioles which are assembled lines of egg chambers. The most anterior structure of each ovariole is called a germarium (Lin, 1997). Two or three GSCs are located at the tip of the germarium, physically associated with cap cells (CCs) anteriorly and escort cells (ECs) laterally, and can be easily identified by the spectrosome stained with the 1B1 antibody (de Cuevas et al., 1997; Lin et al., 1994). While intrinsic factors control GSC proliferation and differentiation, GSCs are also non-autonomously regulated by the somatic cells that form the GSC microenvironment, also known as a GSC niche. The GSC niche mainly consists of CCs and ECs (Xie and Spradling, 2000). Cap cells are the major source of the bone morphogenetic protein Decapentaplegic (Dpp), which is the signaling ligand that is important for maintaining GSC stemness and preventing differentiation, and can be identified by antibodies against laminC and Hedgehog (Hh) (Lu et al., 2015; Xie and Spradling, 1998, 2000). The number of cap cells is developmentally regulated by the Notch signaling pathway, and there are normally five to seven cap cells, which determine the size and function of the niche (Song et al., 2007; Ward et al., 2006). Abnormal increase of cap cell number could elevate the Dpp signal level in the niche and thus cause augmentation of GSCs (Song et al., 2007). ECs wrap around GSCs and accompany differentiated germ cells to the middle region of the germarium, and can be identified by c587-GFP and the enhancer trap line PZ1444 (Decotto and Spradling, 2005; Xie, 2013). ECs have been proposed to restrict BMP signaling within a one-cell-diameter range and to support the differentiation of the GSC lineage. Previous studies showed that transcriptional repressors, such as *lsd1*, *eggless* and *piwi*, are required in ECs to repress tumor-like growth (Eliazer et al., 2011; Jin et al., 2013; Ma et al., 2014; Wang et al., 2011).

Histone H1 is generally considered as a transcriptional repressor, as it is supposed to anchor onto the nucleosome at the DNA entry and exit points to promote the higher order chromatin structure (Thomas, 1999). The highly condensed chromatin can block transcription activators from touching on promoter regions, and thus H1 functions as a repressor (Croston et al., 1991; Misteli et al., 2000). Previous studies have shown that H1 is required for restriction of transposon activities, and prevention of DNA damage and apoptosis in both somatic and embryonic cells (Konishi et al., 2003; Vujatovic et al., 2012). In *Drosophila*, H1 functions intrinsically to promote GSC self-renewal by repressing Bam, and the depletion of H1 in ECs can generate excessive GSC-like cells, a tumor-like phenotype (Sun et al., 2015). Ovarian cancer is a highly lethal disease among all gynecologic malignancies and is the fifth leading cause of cancer-related death in women. Interestingly, the expression of H1 is 40% lower in cells of human malignant adenocarcinomas than in benign lesions, so H1 has been proposed as a potential biomarker for the diagnosis of ovarian cancer (Medrzycki et al., 2012; Scaffidi, 2016). The highly conserved role of H1 between insects and mammals enables us to dissect the mechanism underlining H1 function in *Drosophila*.

In this study, we found that histone H1 is required in ECs to control the proper differentiation of GSCs. Specifically, knockdown of H1 in ECs resulted in a tumor-like phenotype. Further analysis showed that the accumulated spectrosome-containing cells (SCC) have attenuated expression of bam, a key differentiation factor. Consistent with its role in other somatic cells, H1 functions as a transcriptional repressor to silence the transposons in ECs. After H1 depletion in ECs, DNA damage marked by pH2Av increased dramatically, followed by EC apoptosis. Interestingly, H1-depleted ECs acquired cap cell characteristics, and an elevated Dpp signal inhibited the SCC to further differentiate as a result. Finally, double knockdown of H1 and *dpp* in ECs can rescue the spectrosome-containing cell tumor phenotype. Taken together, our findings support the idea that histone H1 is an important epigenetic factor in controlling EC characteristics and acts as a suppressor of *Drosophila* SCC tumors.

2. Results

2.1. H1 depletion in ECs results in a germline tumor phenotype

H1 is highly expressed in both CCs and ECs (Fig. 1A and A′), two groups of cells that together form the somatic niche for GSCs. PZ1444 is an enhancer trap line that directs lacZ expression in CCs and ECs. H1 is expressed in PZ1444 lacZ positive cells, but at a higher level in the lateral ECs than the anterior CCs (Fig. 1B). To modulate H1 expression, we generated UAS-*H1*shRNA transgenic lines, as multi-copies of H1 genes exist in the fly genome, which prevents us from generating an H1 mutant through the well-established CRISPR approach (Ren et al., 2013, 2014a, 2014b). Flies with H1 RNAi driven by bab1-Gal4 in cap cells cannot live to adulthood, even at 18 °C, possibly because bab1-Gal4 also drives H1 depletion during embryogenesis. We therefore focused on the impact of H1 loss in ECs.

The driver c587-Gal4 is expressed in ECs and somatic follicle progenitor cells, and has been widely used to trigger RNAi in ECs. To check the co-localization of PZ1444 and c587 > GFP, we immunostained both the c587 > GFP and PZ1444 lacZ (Supplementary Fig. 1A). Quantification results showed that approximately 90% of PZ1444 positive cells are escort cells as they are c587 > GFP and PZ1444 double positive cells (Supplementary Fig. 1B and C). This result suggested that c587 is highly expressed in PZ1444 positive escort cells. To test the efficiency of the H1 RNAi in ECs, we immunostained the *Drosophila* ovary with an anti-H1 antibody. We again used the PZ1444 enhancer trap line to mark CCs and ECs (Margolis and Spradling, 1995; Xie and Spradling, 2000). In control c587-Gal4 > *GFP*-KD germarium, H1 fluorescence is abundant in ECs marked by PZ1444 (Fig. 1B). In contrast, H1 fluorescence was reduced to an undetectable level in c587-Gal4 > *H1*KD ECs (Fig. 1C), indicating the high efficiency of the H1 KD. Consistently, against the c587-Gal4/+; UAS-GFP/+ genetic background, H1 is highly expressed in the ECs in the control germarium(arrows in Fig. 1D), while the c587-Gal4 > *H1*KD germarium shows no H1 fluorescence in GFP-positive ECs (arrows in Fig. 1E) but abundant H1 in the germline (red in Fig. 1E), further indicating that c587-Gal4 H1 RNAi was very efficient and specific. We then looked at the germline defects by immunostaining. While each of the c587-Gal4 > *GFP*-KD control germaria had 3.8 SCCs, the c587-Gal4 > *H1*KD germaria had 10.4 SCCs on average at 29 °C,3 days after eclosion, which resembles a tumor phenotype (Fig. 1F, G and J).

To determine whether the tumor-like phenotype originated in the adult flies or during the larval stage, we performed a temperature-shift experiment combining c587-gal4 and tublin-gal80^{ts} to knockdown H1. As gal80^{ts} prevents the expression of the shRNA at 18 °C, we raised the parental flies at 18 °C, and later transferred the adult progeny to the restricted temperature of 29 °C. Three weeks later, we found that on average, each c587-Gal4; tub-Gal80^{ts}; *H1*KD germarium contained 8.6 SCCs while the control *GFP*-KD germaria contained 3.3 SCCs (Fig. 1H, I and K). These results together indicate that the SCC hyper-proliferation phenotype is caused by H1 knockdown in adult ECs rather than during fly development.

To avoid the potential off-target effect of shRNA-mediated knockdown, we had assayed another histone H1 RNAi line, with an shRNA targeting a different region of the H1 transcript. We observed the same phenotype when H1 was knocked down by this new shRNA. 78% of the *H1*KD germaria had more than 10 SCCs, while none of the *GFP*-KD control germaria had more than 10 SCCs. And each H1 knockdown germarium has 17 SCCs on average, while each of the control GFP knockdown germaria has only about 4 SCCs on average (Supplementary Fig. 2A–D). These results demonstrate that the hyper-proliferation of SCCs is specifically caused by the depletion of H1. Importantly, the SCC tumor phenotype can be significantly rescued by overexpressing an RNAi-insensitive H1, as only 5% of c587-Gal4 > *H1*-OE; *H1*KD germaria had more than 10 SCCs compared to that 38%

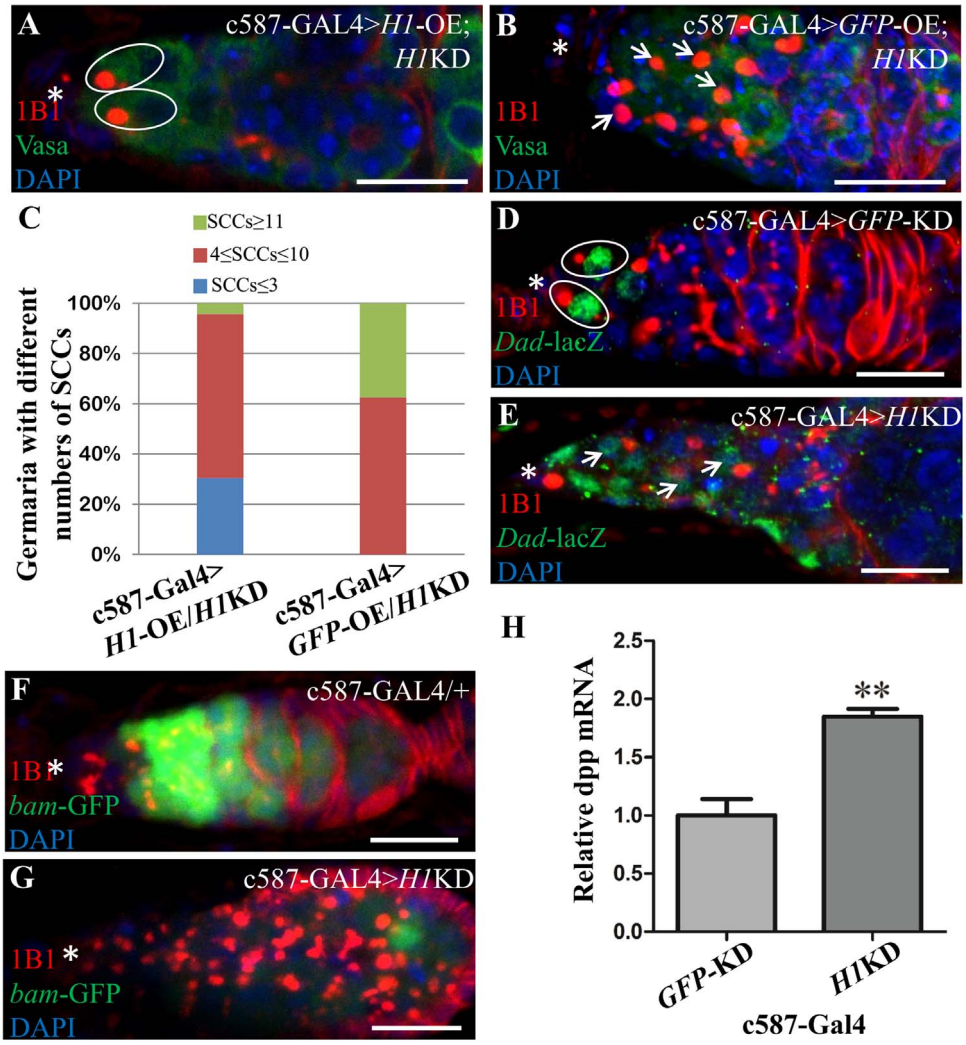


Fig. 2. The SCCs accumulated after H1 depletion are CB-like and have upregulated *dpp*-signaling. (A-B) Over-expression of H1 can effectively rescue the differentiation defects caused by H1 knockdown in ECs (A). GSCs are highlighted by ovals. However, over-expression of GFP (B) has no effect on the differentiation defects. SCCs are indicated by arrows. (C) Quantification of the results in (A-B) showing that H1-OE can rescue the differentiation defect caused by *H1*KD in ECs. n=60 and 56 for the two groups, respectively; p-value=0.0188. (D-G) Germaria are from 3-day-old adult flies, and are stained with anti-LacZ (green in D-E) to show the Dad-positive cells, and anti-GFP (green in F-G) to indicate the expression pattern of Bam in cysts and SCCs. (D-E) In c587 > *GFP*-KD control germarium (D), only GSCs (ovals) are positive for Dad-LacZ. In contrast, SCCs (arrows) outside the GSC niche are positive for Dad-LacZ in the c587 > *H1*KD germarium (E). (F-G) Differentiated cysts express high levels of bam-GFP in the control germarium (F). Many undifferentiated SCCs are accumulated in c587 > *H1*KD germarium (G), and most of them express low levels of bam-GFP. (H) qRT-PCR results shows that the transcriptional level of *dpp* increased by 1.9 fold when H1 was knocked down in ECs. Data are shown as means ± s.e. of three independent experiments; p-value=0.0056.

of control c587-Gal4 > *GFP*-OE; *H1KD* germaria had more than 10 SCCs. In addition, 30.4% of the H1-OE rescued germaria had 3 or fewer SCCs, compared with 0% of the GFP-OE negative control (Fig. 2A–C). These results further support that the H1 RNAi is specific and suggest that H1 is required in ECs to non-autonomously control spectrosome-containing cell number.

2.2. The SCCs accumulated after H1 depletion are CB-like and have upregulated dpp signaling

Both GSCs and cystoblasts (CB) carry round spectrosomes. A cystoblast is a differentiated daughter cell of GSC, and it divides four times incompletely to produce 16 daughter cystocytes with a shared cytoplasm and a branched fusome that derived from the spectrosome (de Cuevas et al., 1997). To further determine whether these SCCs accumulated in the germaria are GSC-like, CB-like, or even further differentiated, we evaluated the expression levels of Dad-lacZ and bamP-GFP in germaria with *H1*KD in ECs (Song et al., 2004). Dad-lacZ is a Dpp signaling marker and is only expressed in the GSCs and the early cystoblasts, while bamP-GFP is expressed in the differentiating cysts (Chen and McKearin, 2003a, 2003b; McKearin and Ohlstein, 1995; Tsuneizumi et al., 1997). In the c587-Gal4 > *H1*KD germaria, we observed that most of the SCCs away from the cap cells retained Dad-lacZ expression and repressed bamP-GFP expression like the control CBs (Fig. 2D–G). Taken together, these immunostaining results indicate that the excessive SCCs are CB-like cells and that their failure to further differentiate is most likely due to upregulated Dpp/BMP signaling in those SCCs. In addition, we have detected the expression of *dpp* at the transcription level in the whole ovary of *Drosophila* by using quantitative real-time PCR (qRT-PCR). Results of qRT-PCR showed that the transcriptional level of *dpp* increased by two fold (Fig. 2H). This result further suggests that the upregulated Dpp/BMP signaling in those SCCs results from the elevated Dpp secreted to the space around them.

2.3. Histone H1 is required in escort cells for suppressing the transcription of transposons, and for preventing DNA damage and apoptosis

H1 is required to form higher order chromatin structure and thus

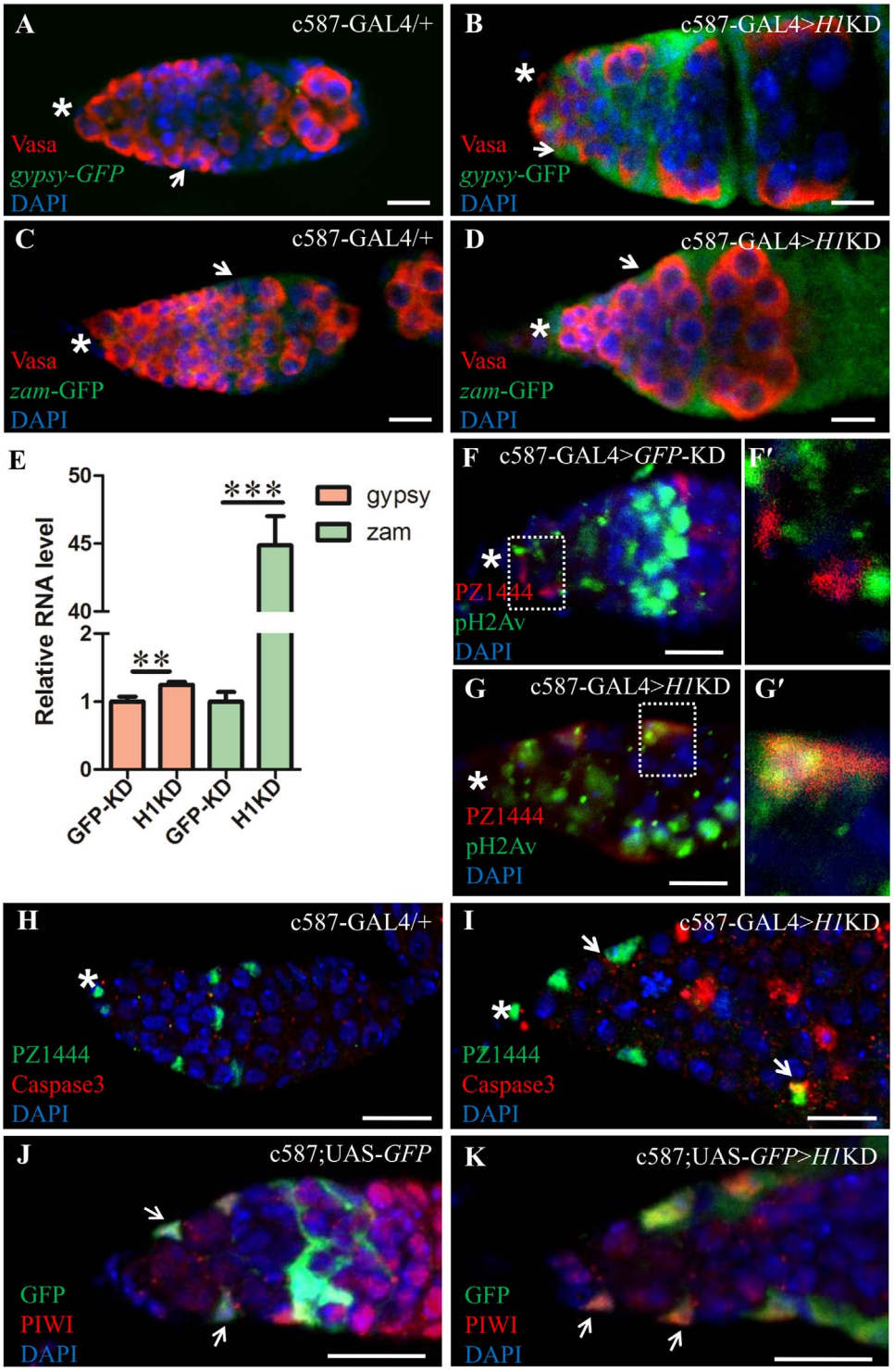


Fig. 3. Depletion of H1 leads to elevated transposable element activity and DNA damage and further apoptosis in ECs. (A-B) c587-meditated *H1*KD (B) elevates the expression of *gypsy-GFP* in ECs (arrows) in comparison with the control germarium (A) in which *gypsy-GFP* signal is not detected in ECs. (C-D) Similar to the expression pattern of *gypsy-GFP*, no *zam-GFP* signal is detected in the ECs (arrow) of control germarium (C), while *zam-GFP* is expressed strongly in ECs (arrows) in *H1*KD germarium (D). (E) qRT-PCR results shows that the transcriptional levels of *gypsy* and *zam* increased by 1.2 fold and 45 fold respectively when H1 was knocked down in ECs. Data are shown as means ± s.e. of three independent experiments; p-value of gypsy group is 0.04, *zam* group is < 0.0001. (F-G) H1 knockdown driven by c587-Gal4 (G) causes the accumulation of pH2Av in *PZ1444*-positive ECs (dashed rectangle) in comparison with the control germarium (F) in which *PZ1444*-positive ECs (dashed rectangle) are pH2Av-negative. (F′) and (G′) are zoomed-in figures of the rectangle areas in (F) and (G), respectively, highlighting *PZ1444*-positive ECs. (H-I) The fluorescence signal of cleaved Caspase-3 (red) is undetectable in the *PZ1444*-positive ECs of c587-Gal4 control germarium (H). Whereas, cleaved Caspase-3 fluorescence is detected in ECs (arrows) labeled by *PZ1444* in the *H1*KD germaria (I). (J-K) Compared with that in the c587; UAS-GFP germarium (J), the expression level of *piwi* does not decrease when H1 is knocked down in the ECs (K). These results suggest that the increased activities of transposons are not caused by lack of Piwi.

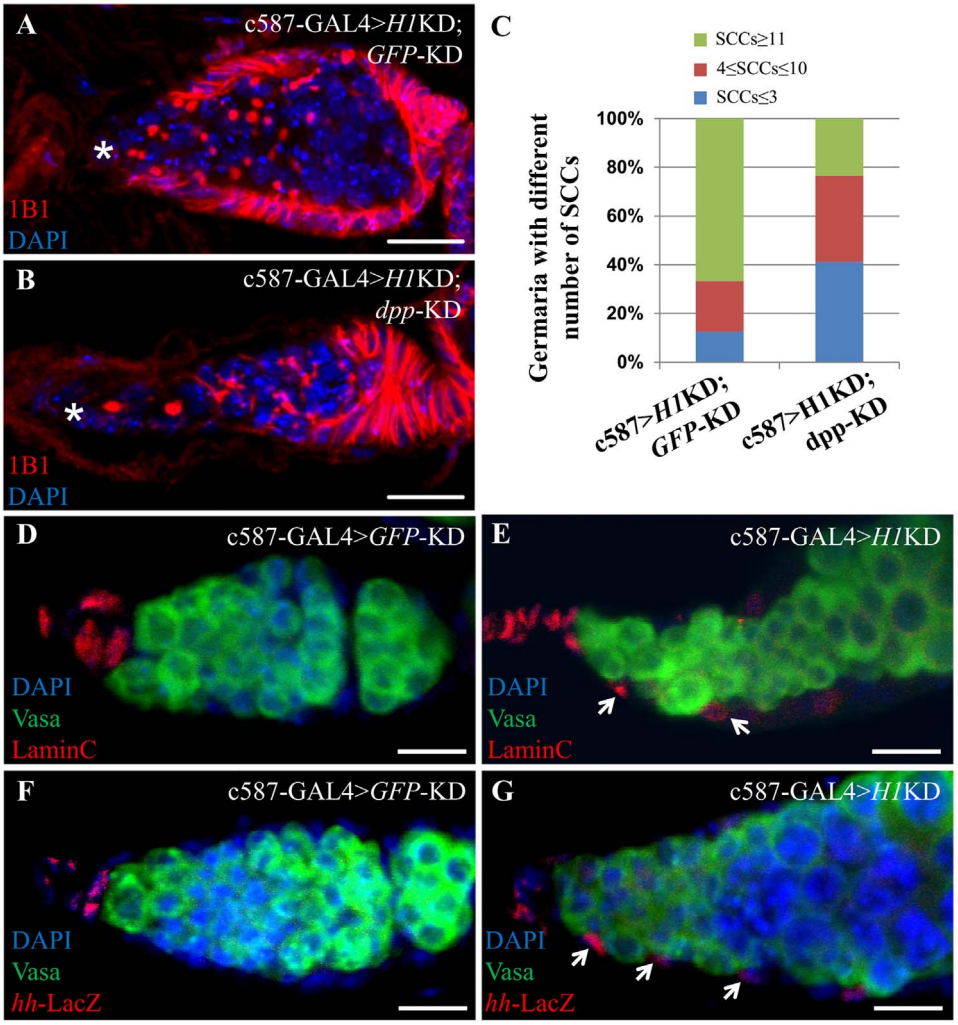


Fig. 4. Escort cells adopt cap cell features after H1 knockdown. (A-C) Knocking-down of *GFP* in the ECs in addition to H1 does not rescue the differentiation defects of germ cells (A). c587-mediated *dpp* knock-down can significantly rescue the germ cell differentiation defects resulting from depletion of H1 in ECs (B). (C) Quantification of results in (A-B) showing that reduction of *dpp* in ECs can significantly rescue the germ cell differentiation defects caused by *H1*KD. n=63 and 67 for the two groups, respectively; p-value=0.0329. (D-G)The anti-LaminC antibody (red in D-E; nuclear lamina) and the anti-LacZ (*hh-LacZ*, red in F-G) antibody are both markers for the CCs and TF cells. 4,6-diamidino-2-phenylindole (DAPI, blue) shows the nuclei. (D-E) The expression of Lamin C is restricted to CCs and TF cells in c587 > *GFP*-KD control germarium (D). In c587 > *H1*KD germarium (E), Lamin C is also expressed in ECs (arrows). (F-G) Instead of being restricted in the CCs and TF cells in controls (F), *hh-LacZ* is also expressed in ECs (arrows) in the c587-meditated H1 knock-down germarium (G). Scale bars: 10 μm.

generally functions as a repressor to silence gene transcription, which includes transcription of transposons (Lu et al., 2009; Ni et al., 2006; Vujatovic et al., 2012). To monitor the changes of transposons in H1-depleted ECs, we constructed two transposon reporters by using *gypsy* and *zam* regulating sequences (Malone et al., 2009). In contrast to those in the control c587-Gal4/+ line, the transcription levels of the transposon reporters, *gypsy-GFP* and *zam-GFP*, were dramatically elevated in ECs in the c587-Gal4 > *H1*KD line (Fig. 3A–D). In addition, qRT-PCR results showed that the transposon levels of *gypsy* and *zam* increased about 2 fold and 45 fold in the H1 knockdown germaria, respectively, compared with those in the controls (Fig. 3E). These results suggest that H1 is a repressor of transposons in ECs.

Transposons have the ability to randomly break double-stranded DNA, and thus cause DNA damage. To figure out if the elevated transposon levels could cause DNA damage in the *H1*KD ECs, we immunostained phosphorylated H2Av (pH2Av), a sensitive marker for DNA lesions (Gong et al., 2005). As expected, pH2Av could not be detected in the control *GFP*-KD ECs marked by PZ1444 (Fig. 3F and F′), while in *H1*KD ECs abundant phosphorylated H2Av was accumulated in nuclei (Fig. 3G and G′). Quantitative results also show that the number of pH2Av and PZ1444 double positive cells are higher in the c587 > *H1*KD germaria than in the control c587 > *GFP*-KD germaria (Supplementary Fig. 3A). These results indicate that the *H1*KD in ECs resulted in elevated transposon levels and DNA damage.

In multicellular organisms, DNA damage can induce apoptosis to eliminate the damaged cells. Caspase-3 activation is a marker of apoptosis, as it is required for DNA fragmentation and morphological changes associated with the apoptotic process (Ohsawa et al., 2008). To examine whether *H1*KD can induce EC apoptosis, we performed an immunostaining experiment using antibody against cleaved caspase-3 (Fan and Bergmann, 2010). In the control c587-Gal4/+ germaria, all ECs marked by PZ1444 were caspase-3 negative (Fig. 3H), while in *H1*KD germaria, caspase 3 could be detected in some ECs (Fig. 3I). Quantitative results also show that the number of caspase 3 and PZ1444 double positive cells are higher in the c587 > *H1*KD germaria than in the control c587 > *GFP*-KD germaria (Supplementary Fig. 3B). These results indicate that transposon-induced DNA damage can trigger EC apoptosis. Taken together, these results show that H1 represses the transcription of transposons and that *H1*KD can trigger DNA damage and further apoptosis in ECs.

It has been well established that Piwi is a crucial factor in repressing the activity of transposable elements (Khurana and Theurkauf, 2010).

We tested the expression of Piwi to verify whether the elevated activities of transposons resulted from a decrease of Piwi in the *H1*KD germaria. Immunostaining results reveal that the expression of Piwi is not reduced in ECs of *H1*KD germarium compared with that in the control ECs. On the contrary, Piwi expression rises a little in *H1*KD ECs (Fig. 3J and K). These results demonstrate that the hyper-proliferation phenotype of SCCs in *H1*KD germaria is not due to the lack of Piwi. In consistent with previous studies (Iwasaki et al., 2016), knocking down of *piwi* in the ECs shows an accumulation of SCCs, which resembles the *H1*KD phenotype. In addition, the *piwi*-knock-down SCC hyper-proliferation can be partially rescued by a simultaneous over-expression of H1 (Supplementary Fig. 4A–C). These results support that H1 could be acting downstream of *Piwi* to regulate germline differentiation rather than upstream of *Piwi*.

As ECs have been proposed to restrict BMP signaling to support the differentiation of the GSC lineage (Decotto and Spradling, 2005; Xie, 2013), the *H1*KD triggered EC apoptosis might play important roles in the germline SCC hyper-proliferation. To test this, we over-expressed p35, a suppressor of apoptosis together with *H1*KD driven by c587-GAL4. After p35 overexpression, Caspase3 level in *H1*KD ECs are significantly reduced (Supplementary Fig. 5A and B) and the ovary size was restored (Supplementary Fig. 5C and D), which suggests the *H1*KD triggered EC apoptosis was suppressed. However, we can still detect the hyper-proliferation of SCCs in the c587 > H1KD;UAS-p35 germaria (Supplementary Fig. 5E–G). These results suggest that the germline tumor phenotype we observed was mainly caused by c587 > *H1*KD rather than caspase 3 dependent apoptosis.

2.4. Escort cells with H1 depletion acquire cap cell characteristics

It is known that CCs are the major source for producing Dpp to maintain GSCs in germaria (Xie and Spradling, 1998, 2000). ECs have been shown to control GSC differentiation mainly by preventing Dpp diffusion outside the GSC niche, while only anterior ECs act as a source of Dpp for GSCs (Xie and Spradling, 2000). In wild-type germaria, Dad-lacZ is only expressed in GSCs and CBs, while in the c587-Gal4 > *H1*KD germarium, Dad-lacZ is highly expressed in germ cells a few cells away from the GSC niche, indicating that the Dpp might come from ECs far away from the GSC niche.

To determine whether the elevated Dpp in *H1*KD germaria is secreted from ECs, we constructed a transgenic RNAi line against both H1 and Dpp (Ni et al., 2008; Perrimon et al., 2010). In contrast to the control H1 and GFP double RNAi line driven by c587-Gal4, which demonstrates an ovary tumor-like phenotype with less than 20% of the germaria having normal numbers of SCCs, which is similar to the c587-Gal4 > *H1*KD line (Fig. 4A and C), H1 and Dpp double RNAi in ECs significantly rescued the SCC tumor phenotype as more than 40% of the germaria had normal numbers of SCCs (Fig. 4B and C). These results indicated that Dpp knockdown in ECs could rescue the germline tumor phenotype caused by c587 > *H1*KD. Furthermore, in immuno staining experiments using a *dpp*-lacZ enhancer trap line (Li et al., 2016), we detected *dpp*-lacZ expression in escort cells in c587 > *H1*KD germaria, while *dpp*-lacZ signal could only be detected in terminal filament cells and CCs in control c587 > *GFP*-KD germaria (Supplementary Fig. 6A and B). Altogether, these results indicate that the *H1*KD ECs secreted Dpp like CCs which contributed to the augmentation of SCCs in *Drosophila* ovary. On the other hand, *dpp* RNAi in ECs did not suppress the *H1*KD triggered zam-GFP activation (Supplementary Fig. 7A and B), increase of H2Av cells (Supplementary Fig. 7C–E), increased Caspase3 expression (Supplementary Fig. 8A–C), or ovary size, which suggests that the decreased *dpp* level did not rescue the SCC hyper-proliferation phenotype through the suppression of Caspase3-dependent apoptosis.

As CCs are usually the major Dpp providers, we wondered whether *H1*KD ECs have acquired similar characteristics to CCs, besides Dpp signaling. We performed immunostaining experiments using an anti-body against Lamin C, a frequently used marker that is expressed in CCs but not in ECs. In control germaria, Lamin C is only expressed in terminal filaments (TFs) and CCs (Fig. 4D), which is consistent with previous reports, whereas *H1*KD ECs also express Lamin C (Fig. 4E), indicating that H1-depleted ECs have acquired CC-like features. In addition, we have tested another CC marker, *Hh*-lacZ, an enhancer trap line with *lacZ* inserted in the *Hh* locus that recapitulates the same pattern of endogenous *Hh* (Dominguez and Hafen, 1997; Lu et al., 2015). Again, compared with that in the controls, the *Hh*-lacZ is also expressed in the H1-depleted ECs far away from CCs (Fig. 4F and G), further supporting that H1 is involved in the control of EC characteristics.

In summary, H1 is involved in the fate decision between ECs and CCs. The H1-depleted ECs possess the ability to secret Dpp, which in turn causes the activation of BMP signaling in the germline, contributing to the tumor-like phenotype.

3. Discussion

Stem cells and their relevant niches form functional units that ensure gamete production, adult organ homeostasis, and regeneration of tissues following injuries or diseases. Self-renewal and differentiation properties are the foundation for stem cells to execute their functions, and are regulated through a constellation of intrinsic and extrinsic molecular mechanisms. Self-renewal of GSCs is mainly regulated by BMP signaling in a non-autonomous manner, while differentiation is controlled by the intrinsic factor, bam. In this study, we found that the depletion of H1 allow ECs to acquire CC characteristics including the ability to produce Dpp, resulting in a SCC tumor phenotype (Fig. 5A and B). Interestingly, we also observed hyper-proliferation of follicle cell-like cells in an outlier group of *H1*KD germaria, which might result from excessive Dpp or Hh secreted by the mutant ECs (Supplementary Fig. 11). These results are consistent with previous reports that H1 expression is correlated with ovarian tumor in human clinical samples, such that H1 has been proposed as a potential biomarker for diagnosis of this disease (Medrzycki et al., 2012; Scaffidi, 2016).

In addition to our results with H1, previous results showed that specifically removing *eggless*, *lsd1*, *hp1* and *piwi* in ECs caused similar SCC tumor phenotypes, indicating that these genes might genetically interact and function in the same pathway (Eliazer et al., 2014; Jin et al., 2013; Ma et al., 2014; Wang et al., 2011). Indeed, H1 has been detected in the same complex as HP1 and Piwi based on biochemical approaches (Brower-Toland et al., 2007; Lu et al., 2013). Consistently, in our experiments, H1 overexpression in ECs could rescue the germline tumor phenotype caused by c587 > *piwi*-KD, so it is becoming increasingly clear that epigenetic factors play pivotal roles in regulating the maintenance of stem cells non-autonomously, in addition to their intrinsic roles in stem cells.

After H1 depletion in ECs, the excessive SCCs indicate that CBs receive stronger Dpp signals than those in the wild type. Though the number of CCs is usually in positive relation to GSCs, we did not observe any obvious changes of CC number in the c587-Gal4 > *H1*KD germarium. Surprisingly, *H1*KD ECs were observed to have CC characteristics when we looked at two markers of CCs. Furthermore, the *H1*KD ECs also possess the ability to secret Dpp, another hallmark of CC function. In addition, the *H1*KD ECs also express Dad-lacZ, a germline marker, but not Vasa (Supplementary Fig. 9A and B). Interestingly, the phenomenon that *H1*KD ECs acquired CC characteristics could be further confirmed by the result that Dpp depletion in the ECs can rescue the H1 RNAi triggered germline tumor phenotype.

How is H1 involved in EC acquiring CC characteristics? To test if c587 > *H1*KD caused ectopic expression of Dpp in ECs is the reason for EC characteristic change, we check the Lamin C positive EC number in H1 and Dpp double knockdown germaria. The results showed that there is no significant difference in terms of Lamin C and PZ1444

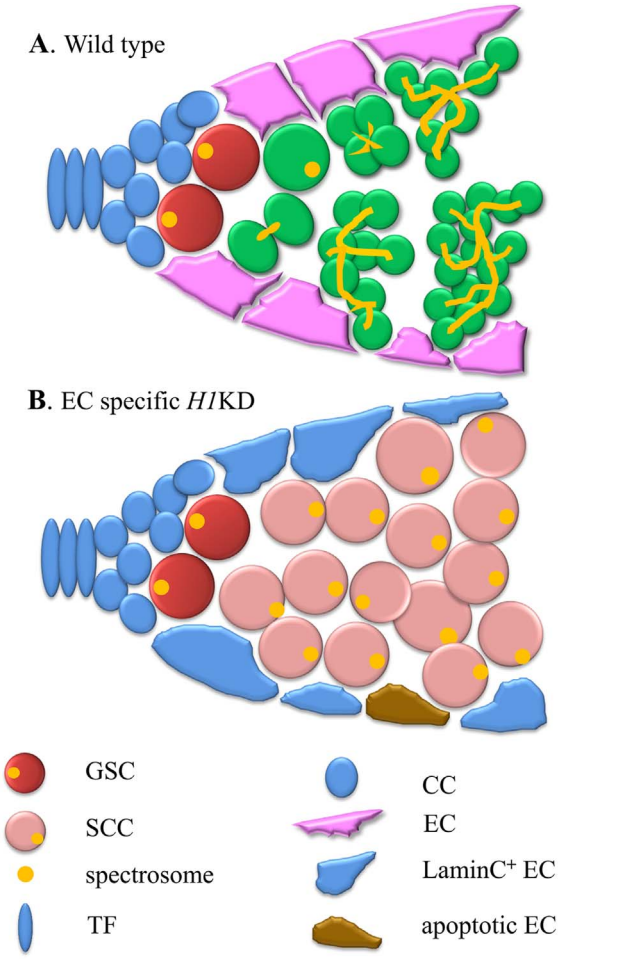


Fig. 5. Cartoon models illustrating H1-mediated regulation of germ cell differentiation in escort cells in germaria. Wild-type germarium. GSCs (red) are located in the anterior (left) part and anchored to the niche, which is mainly composed of terminal filaments (blue) and cap cells (blue). Each GSC generates a new GSC and a cystoblast (lime-green) in a process known as self-renewal. Subsequently, the cystoblast will undergo four synchronous incomplete divisions to produce 16 interconnected cystocytes (green) that are collectively called a cyst (forest-green). One cystocyte of the cyst develops into the oocyte. Escort cells (violet), the differentiation niche, play a pivotal role during the differentiation of germ cells. (B) EC-specific *H1*KD germarium. The differentiation of cystoblast is disrupted when histone H1 is specifically knocked-down in escort cells. Knocking-down of *histone H1* in escort cells results in the disruption of cystoblast differentiation, and a germarium full of undifferentiated CB-like cells (grey-red) that contain spherical spectrosomes. The escort cells in *H1*KD germarium express cap-cell markers such as Lamin C and *hh*-LacZ, as well as *dpp*, which may contribute to the differentiation defects of germ cells. Some of the *H1*KD escort cells would go through apoptosis (brown).

double positive cells between c587 > *H1*KD;*GFP*-KD germaria and c587 > *H1*KD;DPP-KD germaria (Supplementary Fig. 10A–D). This result suggested that Dpp ectopic upregulation is not the reason that ECs acquired the CC characteristics such as LaminC expression. Another possibility is that H1 achieves this by ubiquitously control ling gene transcription. However, based on previous microarray data from *in vivo* and *in vitro* materials with H1 and its partner-HP1 depleted, significant transcript changes only occur in a few hundred genes and these genes do not include *bam* and *dad*, the two reporter genes that we tested (De Lucia et al., 2005). In addition, the activity of the *notch* signaling pathway is not affected significantly after H1 knockdown, based on our qRT-PCR experiments. These results indicate that H1 plays a more specific role in determining EC characteristics. However, it is worth testing whether H1 sits on the promoters of *dpp*, *Lamin C* and *Hh* genes, and directly controls the transcription in ECs by applying chromatin immunoprecipitation.

H1 anchors on the nucleosome and is required to further condense nucleosomes into chromatin. H1 facilitates heterochromatin formation, and thus is believed to be a factor in the suppression of gene transcription, including transcription of transposons. In the transposon activity assay, we found that H1 depletion in ECs triggered a significant over-expression of *gyspy*-GFP and *zam*-GFP (Fig. 3A–D), two transposon reporters that we had generated. This result demonstrates that H1 is a transposon repressor, which is consistent with its role in other somatic cells. DNA damage is evident in these transposon-activated ECs, as is apoptosis, which is likely due to the excessive DNA damage. However, our results show that EC apoptosis does not play an important role in the *H1*KD triggered SCC hyper-proliferation, since p35 over-expression decreases Caspase3 expression but not SCC accumulation in *H1*KD germarium, and *dpp* depletion rescues SCC hyper-proliferation without rescuing excessive Caspase3 expression or decreased EC numbers. Our results do not completely rule out the possibility that the Caspase3-independent apoptosis of ECs is involved in the SCC hyper-proliferation, but the fact that p35 over-expression also restores the normal ovary size suggests that this possibility is very unlikely. DNA damage results in cell stress and triggers the JNK signaling pathway, which has been known to induce the BMP pathway, it is possible that *H1*KD regulates *dpp* signaling indirectly. It deserves further investigation to test whether elevating the JNK signal by introducing *egr* or other JNK pathway members can cause activation of the BMP pathway, and lead to phenotypes similar to *H1*KD.

4. Materials and methods

4.1. Drosophila *stocks and culture*

The following fly stocks were used in this study: *UAS-H1KD* RNAi line (TH00592.N, TH00803.S, TH00804.S), *UAS-GFP* RNAi line (TH00781.N), *UAS-Dpp* RNAi line (SH00007.N), *UAS-GFP* (TH10512.N), *P[bamP-GFP]* (Chen and McKearin, 2003a, 2003b), *Dad-LacZ* (Tsuneizumi et al., 1997), PZ1444 (Margolis and Spradling, 1995), and c587-Gal4 (Bloomington #25421). The sequences of the sense oligos of the transgenic RNAi lines are:

TH00412.N: 5′-CCCGGTCATGCTGCAGACGAA-3′.
TH00592.N: 5′- ACCAGCGACAGTTGAGAAGAA -3′.
TH00803.N: 5′-TTAGAGGCTACCTTCTTGCTT-3′.
TH00804.N: 5′-TTGCCTTTGACGCTTTCGCTA-3′.
TH00781.N: 5′-CCCGAAGGTTATGTACAGGAA-3′.
SH00007.N: 5′-TCGTTCAGTGATAGTGATAAA-3′.

Drosophila stocks were raised at 25 °C with 60% humidity on standard cornmeal/sugar/agar media unless otherwise specified. To enhance the efficiency of RNAi-mediated knockdown, flies were crossed and cultured at 29 °C with 60% humidity.

4.2. *Generation of transgenic flies*

By using a previously described method, we cloned the coding sequences of H1 and GFP into Valium20 vector (Ni et al., 2008, 2011). Transgenes expressing H1 and GFP proteins were constructed as follows. The coding sequence of H1 was amplified (forward primer: 5′-GGTCTAGAATGGCCATGTCTGATTCTGCAGTTGCA-3′; reverse primer: 5′-AATCTAGATTACTTTTTGGCAGCCGTAGTC-3′). The PCR product of H1 was digested with *Xba*I and cloned into the Valium20 vector (Ni et al., 2008). The coding sequence of GFP was amplified (forward primer 5′-CTAGCTAGCACCATGGTGAGCAAGGGCGAGG-3′; reverse primer: 5′-CGGAATTCTTACTTGTACAGCTCGTCCA-3′). The PCR product of GFP was digested with *Nhe*I and *Eco*RI and also cloned into theValium20 vector.

To generate the constructs of *gypsy*-eGFP and *zam*-eGFP, we firstly digested the Valium20 vector with *Spe*I and *Sac*I, and then successively

cloned the sequences of *SV40*, *eGFP*, *gypsy*/*zam* LTR into this modified vector. The sequences of *SV40*, *eGFP*, *gypsy*/*zam* LTR are amplified by the following primers respectively:

SV40-forward: 5′-GGTCTAGAGGATCTTTGTGAAGGAACC-3′.
SV40-reverse: 5′-GACTAGTTGGAACCAGACATGATAAGA-3′.
eGFP-forward: 5′-CGGGATCCATGGTGAGCAAGGGCGAGG-3′.
eGFP-reverse: 5′-GCTCTAGATTACTTGTACAGCTCGTCCA-3′.
gypsy-forward: 5′-AAGAATTCAGTTAACAACTAACAATGTATTGC TTC-3′.
gypsy-reverse: 5′-AAGGATTCGTTGGTTGGCACACCAC.
zam-forward: 5′-AAGAATTCAGTTACCGACCCATCGGTAC-3′.
zam-reverse: 5′-AAGGATTCAGTTACCTCCGGGGAGTCTT-3′.

The PCR products of *SV40*, *eGFP* and *gypsy*/*zam* LTR were separately digested with corresponding restriction endonucleases (*Xba*I and *Spe*I for *SV40*, *Bam*HI and *Xba*I for *eGFP*, *Eco*RI and *Bam*HI for *gypsy*/*zam* LTR) and then cloned into the modified Valium20 vector. The generation-zero (G0) flies were generated by injecting the constructs into injection stocks (*y sc v nanos-integrase; attP40* or *y sc v nanos-integrase;; attP2*) The G0 flies were then crossed following the standard protocol for generating the transgenic fly lines (Ni et al., 2011).

4.3. Immunostaining of ovaries and fluorescent microscopy

Immunostaining of *Drosophila* ovaries was performed according to a method described previously (Sun et al., 2015). Ovary samples were dissected in ice-bath PBS and fixed in PBS with 4% formaldehyde for 15 min, and then washed with PBT (PBS and 0.1% Triton X-100) five times for 15 min each wash. The ovary samples were blocked in 0.5% goat serum diluted with PBT for 1 h. Then the samples were incubated with appropriate primary antibodies diluted in PBS at 4 °C overnight. On the next day, the ovaries were washed with PBT five times for 15 min each time, incubated with the appropriate secondary antibodies also diluted in PBS for 3 h at room temperature, then washed again with PBT five times for 15 min each time. After washing, the stained ovaries were mounted in Fluoromount mounting media (Sigma, F4680). Images were obtained with a Zeiss LSM780 fitted with an ultraviolet laser.

In this study, the following primary antibodies were used: mouse monoclonal anti-Hts antibody (1B1, Developmental Studies Hybridoma Bank (DSHB), 1:100), rabbit polyclonal anti-Vasa (Santa Cruz Biotechnology, sc30210, 1:200), rabbit polyclonal anti-H1 (anti-H1C; Fang-Lin Sun) (Ni et al., 2006), rabbit polyclonal anti-GFP (Abcam, ab290, 1:1000), mouse monoclonal anti-LaminC antibody LC28.26 (DSHB, 1:100), mouse monoclonal anti-GFP (Roche, 1814460, 1:50), anti-beta-Galactosidase (Cappel, 55976, 1:1000), rabbit polyclonal anti-pS137 H2Av antibody (Rockland Immunochemicals, 1:5000), rabbit polyclonal anti-cleaved Caspase-3 (Cell Signaling, 9661, 1:200). Corresponding secondary antibodies (Jackson ImmunoResearch Laboratories) conjugated with FITC or TRITC were used at 1:200.

4.4. qRT-PCR

Total RNA was extracted from the 3-day-old *Drosophila* ovaries using the AxyPrep Multisource Total RNAMiniPrep Kit (Axygen). A total of1μg RNA was used to create cDNA *via* reverse transcription. This is done with the GoldScript cDNA Kit (Invitrogen) according to the manufacturer's protocol, then with PCR to amplify the target sequence.

Quantitative real time PCR was performed by using SYBR Premix Ex Taq (TAKARA), and the results were analyzed with the iQ5 real-time PCR detection system (Bio-Rad). Results were normalized against act5C. Primer sequences for qRT-PCR experiments are listed below:

act5c-F: 5′-ATACTCCTCCCGACACAAAGC-3′.
act5c-R: 5′-CAGGTAGTCGGTCAAATCGC-3′.
dpp-F: 5′-TGGCGACTTTTCAAACGATTGT-3′.
dpp-R: 5′-CAGCGGAATATGAGCGGCAA-3′.
gypsy-F: 5′-CCAAGGACTTCATCCGCCACC-3′.
gypsy-R: 5′-GCGGGTGCGCTTGTTCGATCC-3′.
zam-F: 5′-ACTTGACCTGGATACACTCACAAC-3′.
zam-R: 5′-GAGTATTACGGCGACTAGGGATAC-3′.
notch-F:5′-CGCTTCCTGCACAAGTGTC-3′.
notch-R:5′-GCGCAGTAGGTTTTGCCATT-3′.
Su(H)-F:5′-CTTGCTGCCGGGTCCTTAC-3′.
Su(H)-R:5′-CTCGCGCATGTACTTCTCCA-3′.
Hairy-F:5′-GACTCTGATTCTGGATGCCAC-3′.
Hairy-R:5′-CTAACCTCGTTCACACAGTCG-3′.

4.5. Statistical analysis

We used the GraghPad Prism (GraphPad Software, Inc.) to do statistical analysis and draw the box-plot graphs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.02.012.

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