Histone H1-mediated epigenetic regulation controls germline stem cell self-renewal by modulating H4K16 acetylation

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Epigenetics plays critical roles in controlling stem cell self-renewal and differentiation. Histone H1 is one of the most critical chromatin regulators, but its role in adult stem cell regulation remains unclear. Here we report that H1 is intrinsically required in the regulation of germline stem cells (GSCs) in the Drosophila ovary. The loss of H1 from GSCs causes their premature differentiation through activation of the key GSC differentiation factor bam. Interestingly, the acetylated H4 lysine 16 (H4K16ac) is selectively augmented in the H1-depleted GSCs. Furthermore, overexpression of mof reduces H1 association on chromatin. In contrast, the knocking down of mof significantly rescues the GSC loss phenotype. Taken together, these results suggest that H1 functions intrinsically to promote GSC self-renewal by antagonizing MOF function. Since H1 and H4K16 acetylation are highly conserved from fly to human, the findings from this study might be applicable to stem cells in other systems.
**Results**

**H1 is intrinsically required for the maintenance of GSCs.**

To overcome the challenge of genetic analysis of H1 due to its multiple gene copies in the *Drosophila* genome, we took advantage of the transgenic RNA interference (RNAi) method by targeting the shared coding sequence of the H1 genes. We generated a transgenic RNAi line (H1 KD) using a recently developed approach that allowed for knocking-down of H1 in both spatial and temporal manners. In combination with a *nos*-GAL4 (*nos*-GAL4) line that specifically expresses GAL4 in germ cells (Supplementary Fig. 1c,d), all H1KD females (*nos*-GAL4 > H1 KD) failed to lay any eggs, indicating that H1 is required for oogenesis. As neither the heterozygous *nos*-GAL4 flies (*nos*-GAL4/+) nor the *nos*-GAL4-driven green fluorescent protein (GFP) RNAi flies (*nos*-GAL4 > GFP-KD) showed any defects in GSC maintenance (Supplementary Fig. 1e–i), we used these two genotypes interchangeably as controls for subsequent experiments. In each control germarium from 3-day-old adults, there were two to three round spectroscome-containing GSCs, recognized by 1B1 antibody staining (Fig. 1a,d). In contrast, 92% of *nos*-GAL4 > H1 KD germline retained only one or zero stem cells immediately adjacent to the cap cells (Fig. 1b,d). We validated the efficiency of H1 depletion using quantitative reverse transcription–PCR (qRT–PCR), immunostaining and western blot (Fig. 1e–g, Supplementary Fig. 3a,b). As shown in Fig. 1e, the H1 transcript level in *nos*-GAL4 > H1 KD ovaries had reduced to 7.4% of that in the controls. The qRT–PCR result was further confirmed using another set of primers (Supplementary Fig. 3b), and was consistent with the western blot assay (Supplementary Fig. 3a). By immunostaining with an H1 specific antibody, H1 protein was shown to be efficiently and specifically depleted in the germline of *nos*-GAL4 > H1 KD flies, but not in the somatic cells (Fig. 1f,g). To further validate that the GSC loss phenotype is indeed caused by H1 depletion, we generated two additional transgenic RNAi lines targeting different sections of the coding regions of H1 and observed similar GSC loss phenotypes when they are expressed using *nos*-GAL4 (Supplementary Fig. 2d–h). Furthermore, the expression of H1 cDNA that is not sensitive to RNAi significantly rescued the GSC loss phenotype caused by H1KD (Fig. 1c,d and Supplementary Fig. 2a–c). Taken together, these observations demonstrate that H1 is required intrinsically for GSC maintenance.

As dBIG1 is also expressed in the *Drosophila* germline, the question is raised as to whether dBIG1 is involved in the GSC maintenance phenotype we observed with H1 knockdown. We performed qRT–PCR using *nos*-GAL4 > H1 KD fly ovaries, and did not detect a significant decrease in dBIG1 expression (Supplementary Fig. 3a,b and Supplementary Fig. 3h,i). Furthermore, we constructed two independent dBIG1 transgenic RNAi fly lines, which efficiently knocked down dBIG1 expression when driven by *nos*-GAL4 (Supplementary Fig. 3c), but did not observe any GSC maintenance phenotypes (Supplementary Fig. 3d–f). In addition, antibody staining showed that both dBIG1 and H1 were expressed in the germline but in distinct patterns, as dBIG1 was mostly expressed at later stages of oogenesis, and in the oocyte (Supplementary Fig. 3h) and nurse cells proximal to the oocyte, whereas H1 was expressed in early-stage germline cells, but not in the oocyte and at a decreased level in nurse cells neighbouring the oocyte (Supplementary Fig. 3g). These results suggest that H1’s roles in GSC maintenance do not involve dBIG1.

To determine further if H1 is required in adult GSCs for their maintenance, we generated *H1KD clones in GSCs using a conditional germline FLP-out system* (Supplementary Fig. 1b). This approach allowed us to deplete H1 in adult germline cells marked by GFP after clone induction (ACI; Fig. 1i). In the

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**Chromatin** is a complex of DNA and proteins within the eukaryotic cell nucleus. The nucleosome is the basic unit of chromatin and consists of five highly positively charged histones called linker histone H1 (H1), H2A, H2B, H3 and H4. The core of the nucleosome octamer contains one tetramer of H3 and H4 and two dimers of H2A and H2B, which undergo a variety of post-translational modifications. These modifications collectively influence the local chromatin structure and correlate with gene transcription, and are tightly associated with stem cell self-renewal, differentiation and proliferation. H1 is also believed to be critical for chromatin remodelling through the condensation of nucleosomes. In mammals, H1 controls chromatin dynamics during early embryogenesis, with just a 50% reduction in H1 variants causing embryonic lethality with a broad array of phenotypes. In addition, the depletion of H1 variants could directly block the differentiation of mouse embryonic stem cells. However, the role of H1 in the regulation of adult stem cells remains to be determined.

One of the major challenges in elucidating the developmental roles of H1 in vivo is the high degree of heterogeneity, as multiple H1 variants with redundant functions exist in most species. With only one version of H1 expressed post-embryonically and well-defined stem cells, the *Drosophila* ovary is a particularly attractive system to study the functions of H1 in adult stem cell regulation (Supplementary Fig. 1a). In the *Drosophila* ovary, two or three germline stem cells (GSCs) are located at the anterior end of the gerarium, which is situated at the tip of each ovariole. They physically interact with cap cells anteriorly and escort cells (ECs) laterally. GSCs can be recognized by their proximity to the cap cells and the presence of a spherical organelle known as the spectroscome. The immediate differentiating GSC daughters, cystoblasts, also carry a spectroscome, but are distant from cap cells. Further differentiated germ cell cysts contain a branched fusome, which is an identical organelle to the spectroscome with a different morphology. Therefore, GSCs and their differentiated daughters can be followed and studied.

GSC self-renewal is known to be co-ordinately regulated by both extrinsic signals from niche cells and intrinsic factors. In the adult *Drosophila* ovary, cap cells and anterior ECs form a self-renewal niche. GSCs receive extrinsic signals such as the chromatin-remodelling factors, ISWI and Domino, have also shown to act intrinsically to promote GSC self-renewal by preventing differentiation, indicating that epigenetic regulation is important intrinsically for controlling GSC self-renewal. In addition, Brc-containing protein complex and Enok are also required intrinsically to maintain GSC self-renewal. Another potential chromatin regulator Stonewall is also involved in the regulation of GSC fate. Various other intrinsic factors, including Mad, Piwi, Scrawny and Eggless, are also important to maintain GSC self-renewal.

In this study, we have first revealed a critical role of H1 in maintaining GSCs by preventing Bam activation. Interestingly, we find that the H4K16ac level is selectively upregulated in the H1 knockdown GSCs, and that the association of H1 on chromatin is antagonized by males absent on the first (MOF), a histone acetyltransferase specific for H4K16. Most interestingly, the knocking down of mof significantly suppresses the GSC loss phenotype induced by the depletion of H1. Taken together, we find that a balance between H1 and H4K16ac in the chromatin is antagonized by males absent on the first (MOF), a histone acetyltransferase specific for H4K16. Most interestingly, the knocking down of mof significantly suppresses the GSC loss phenotype induced by the depletion of H1. Taken together, we find that a balance between H1 and H4K16ac in the chromatin is required for the maintenance of GSCs.
controls, the percentage of germla with GFP-positive GSCs remained at a similar level 3 weeks ACI compared with that at 2 days ACI (Fig. 1h). However, the percentage of germla with GFP-marked GSCs remarkably declined to 19% 3 weeks ACI (Fig. 1h). These results support the idea that H1 is intrinsically required for the maintenance of adult GSCs.

**Figure 1 | H1 is required for GSC maintenance intrinsically.** (a–c) Germla are from 3-day-old adult flies, and are stained with 1B1 (red) to reveal spectrosomes/fusomes, anti-Vasa (green) to show the germ cells, 4,6-diamidino-2-phenylindole (DAPI, blue) to show the nuclei and anti-LaminC (also red) to show the nuclear lamina of cap cells and terminal filament cells. Germla from controls (a) have two GSCs (circles), whereas those from nos-GAL4 > H1 KD (b) have no GSC (arrow). (c) H1 OE rescues the loss of GSCs. (d) Column chart showing that nos-GAL4 > H1 KD germla contain fewer GSCs than controls, and that H1 OE can rescue the H1 KD GSC loss phenotype. n = 99, 97 and 90 for the three groups, respectively. (e) qRT-PCR results show that the transcription of H1 in nos-GAL4 > H1 KD ovaries is reduced to 7.4% compared with that in the control. Data are shown as mean ± s.d. of three independent experiments. (f,g) H1 (green) is reduced to undetectable level in nos-GAL4 > H1 KD germline cells (g) compared with that in the control (f), in 3-day-old adults. (h) The percentage of germla carrying H1 KD GSC clones dramatically decreases with time compared with those carrying wild-type GSC clones. Results are from three experiments and the error bars show the 95% confidential intervals. (i) H1 is eliminated in GFP-marked H1 KD GSCs (broken circles) compared with that in GFP-negative GSCs (circles) 7 days ACI. Scale bars, 10 μm. Anterior is to the left in this and all subsequent images of germla. Asterisks indicate the niche cap cells in this and all subsequent images. d, day.

Intrinsic depletion of H1 leads to bam activation in GSCs. At the cellular level, the H1 depletion-induced GSC loss phenotype could be caused by apoptosis, premature differentiation, or both. Previous studies showed that H1 depletion can trigger genomic instability and apoptosis in somatic cells *in vivo* and in cultured embryonic cells. However, we did not observe apoptotic
GSCs in the H1KD germaria by anti-cleaved caspase-3 immunostaining (Supplementary Fig. 4a). Furthermore, H1 knockdown cysts have been detected in egg chambers (Fig. 1i and Supplementary Fig. 3i), which is consistent with the apoptosis assay and suggests that H1 is not required after the germline cells have differentiated. In addition, the GSC loss phenotype was not rescued upon expression of the caspase inhibitor p35 (Supplementary Fig. 4b–d). These results suggest that the GSC loss phenotype caused by H1 depletion is unlikely due to apoptosis.

To test whether H1 depletion causes the premature differentiation of GSCs, we analysed the transcription activation of bam in the H1-depleted GSCs, using bamP-GFP as a reporter. As demonstrated earlier, ectopic expression of bam can trigger GSC differentiation, and therefore result in GSC loss35. In control germaria, bamP-GFP accumulated primarily in late cystoblasts and differentiating germline cells and was undetectable in the GSCs (Fig. 2a). However, in H1KD germaria, bamP-GFP expression was not only observed in differentiating germline cells but also in GSCs and germline cysts attached to the niche (Fig. 2b,c), suggesting that premature differentiation of GSCs likely contributes to their loss when H1 is depleted. In addition, qRT–PCR experiments showed that the bam expression level in nos-GAL4 > H1KD ovaries had increased to 1.5-fold of that in the control ovaries (Fig. 2d). Furthermore, we examined the endogenous protein level of Bam in the H1KD mosaic germlarium using immunostaining. In contrast to control GSCs, the intensity of the Bam signal was increased in H1KD GSCs (Fig. 2e,f), further supporting that the ectopic expression of bam after H1 depletion is associated with GSC loss.

Next, we asked whether the upregulation of bam in H1KD GSCs is caused by compromised Dpp signalling. We examined the expression levels of Dad-lacZ and pMad, two reporters of GSCs is caused by compromised Dpp signalling. We examined various histone modifications in H1KD germaria, using bamP-GFP expression was not only observed in differentiating germline cells but also in GSCs and germline cysts attached to the niche (Fig. 2b,c), suggesting that premature differentiation of GSCs likely contributes to their loss when H1 is depleted. In addition, qRT–PCR experiments showed that the bam expression level in nos-GAL4 > H1KD ovaries had increased to 1.5-fold of that in the control ovaries (Fig. 2d). Furthermore, we examined the endogenous protein level of Bam in the H1KD mosaic germlarium using immunostaining. In contrast to control GSCs, the intensity of the Bam signal was increased in H1KD GSCs (Fig. 2e,f), further supporting that the ectopic expression of bam after H1 depletion is associated with GSC loss.

To test further whether Bam upregulation is the major cause of the stem cell phenotypes in H1KD germaria, we introduced one copy of bam186, a mutant allele of bam, into the H1KD GSCs. The bam186 heterozygous ovaries show a moderate accumulation of GSC-like cells35. As expected, the presence of one copy of bam186 significantly rescued the loss of GSCs in nos-GAL4 > H1KD ovaries, with 40% of germaria carrying two GSCs compared with 6.5% of those with two wild-type bam alleles (Fig. 2g–i). Taken together, these results support the notion that H1-induced activation of bam expression is largely responsible for the loss of GSCs in H1KD ovaries.

**H1KD selectively results in hyperacetylation on H4K16.** Loss of H1 has been shown to alter several histone modifications, including the methylation of H3K4, H3K9 and H3K27, in specific genes during embryonic stem cell differentiation in vivo12. We examined various histone modifications in H1KD germaria driven by nos-GAL4, including the transcriptional repression ones (H3K9me2 and H3K27me3) and the transcriptional activation ones (H3K4me2, H4K5ac, H4K8ac, H4K16ac and H4K12ac). Interestingly, the level of H4K16ac, a hallmark of hyperactive chromatin, was distinctly upregulated in the germline cells upon depletion of H1 (Fig. 3a,b), but the other modifications remained unchanged (Supplementary Fig. 5a–l). Using a mosaic analysis in GSCs, we confirmed the upregulation of H4K16ac signals in H1KD GSCs (Fig. 3c,d). The augmented H4K16ac is not due to upregulation of mof, a specific H4K16 acetyltransferase27,28, as the expression level of mof was not affected upon H1 depletion (Supplementary Fig. 4g). In addition, the increased H4K16ac was not due to upregulation of H4 level, as the levels of H4K5ac, H4K8ac and H4K12ac were not altered in GSCs compared with those in the control. Therefore, these results suggest that H1 is required to maintain appropriate levels of H4K16ac in GSCs.

MOF antagonizes the association of H1 on chromatin. As a previous study showed opposite binding trends of H1 and H4K16ac on chromatin29, we wondered whether H1 affects H4K16ac on the chromatin level. The H1-RNAi line that we used could also induce H1KD in somatic tissues (Supplementary Fig. 6a,b), we thus generated H1KD in the salivary gland and found that H1KD leads to the spread of H4K16ac from the X chromosome to autosomes (Fig. 4a,b). To further test whether H4K16 acetylation affects the H1 level on chromatin in vivo, we increased H4K16ac by overexpressing mof, the H4K16 acetyltransferase. As expected, overexpression of mof resulted in an increase of H4K16ac on chromosomes (Fig. 4c). Importantly, mof overexpression restricted H1 association on chromatin and triggered chromatin decondensation in salivary gland cells (Fig. 4d). These results show that the overexpression of mof behaves similarly as H1KD to increase H4K16ac, and prevents H1 association with the chromatin in vivo. We have also tested an H4K16A mutant gene with the 16th lysine changed to alanine, which makes the residue incapable of being acetylated40. Overexpression of this transgene in the salivary gland showed that H4K16A co-localizes with H1 on the polytene chromosome, in contrast to the antagonizing pattern of H1 and H4K16ac (Supplementary Fig. 7). These in vivo results suggest the importance of the 16th lysine in the antagonizing relationship between H1 and H4K16ac, thus further support a model of mutual repression between MOF and H1 on H4K16ac.

Antagonism between H1 and MOF in the maintenance of GSC. To determine whether elevated H4K16ac affects GSC maintenance, we used nos-GAL4 to overexpress mof to increase H4K16ac in germ cells, including GSCs. As shown in Fig. 5a–c, MOF overexpression indeed caused the GSC loss phenotype, similar to H1KD. Overexpression of MOF in GSCs using the mosaic assay also caused increased H4K16ac expression (Supplementary Fig. 6c) as expected. Similar to the case of MOF overexpression polytene chromosomes, where dissociation of H1 from chromatin could be observed (Fig. 4e), the level of nuclear H1 in the GSC was also reduced (Supplementary Fig. 6d). This observation is consistent with a previous study, which showed that knocking down of mof in the mouse embryo leads to an increased level of H1 (ref. 41). Interestingly, MOF-overexpressing GSC clones also had moderately elevated H1 levels compared with neighbouring control GSCs (Supplementary Fig. 6e), reminiscent of the similar defect in H1KD clones (Fig. 2e). To determine whether the lack of H4K16 acetylation affects GSC differentiation, we also used nos-GAL4 to overexpress H4K16R, a lysine to arginine mutant that mimics non-acetylated H4K16, specifically in the developing germ cells, including GSCs. Strikingly, ectopic expression of H4K16R significantly increased the spectrosome-containing undifferentiated single-germ cells (Fig. 5d). These results suggest that H4K16ac is critical for the balance between GSC self-renewal and differentiation.

To test the notion that H1 and MOF control the H4K16ac levels for balancing self-renewal and differentiation of GSCs, we simultaneously depleted both H1 and MOF in germ cells, including GSCs, using nos-GAL4. Double H1 and MOF
knockdown (KD) ovaries displayed a significant rescue of the GSC loss phenotype caused by H1 KD (Fig. 5e–h and Supplementary Fig. 8a–c). Consequently, females with both H1 and MOF depleted in GSCs laid significantly more eggs than the H1 and GFP double KD females (Fig. 5i). These results demonstrate the antagonistic relationship between MOF and H1 in the regulation of GSC self-renewal and differentiation.

Discussion
In this study, we used Drosophila ovarian GSCs as a model system to reveal that H1 and MOF antagonize each other’s function to control H4K16ac levels, which are critical for GSC self-renewal and differentiation. The loss of H1 in GSCs resulted in their premature differentiation, and the derepression of bam, a critical regulator of GSC differentiation. In addition, our study shows a

Figure 2 | The loss of H1 KD GSCs is due to premature differentiation triggered by bam upregulation. (a–c) bamP-GFP expression (green) is repressed in GSCs of control flies (a), but expands to the GSCs in nos-GAL4 > H1 KD germlia (arrowhead in b). Note that the bamP-GFP-positive germline cells (arrow) neighbouring the cap cells (red nuclear lamina) in c share a branched fusome (red), indicating that premature differentiation of the GSCs occurs in H1 KD germlia. Arrowheads in a point to GSCs. (d) qRT-PCR results show that the transcription of bam in nos-GAL4 > H1 KD ovaries has increased to 1.5-fold compared with that in the control (n = 3, mean ± s.d.). (e) Bam (red) is increased in the GFP-marked H1 KD GSCs (broken circles) compared with that in the GFP-negative control GSCs (circles) 7 days ACI. (f) Quantification of results in e showing that the relative Bam intensity in GFP-marked H1 KD GSCs is about twofold higher than that in GFP-negative control GSCs (n = 5, mean ± s.d.). The intensity of Bam in control GSCs is set to 1. Data are evaluated with Student’s t-test. (g,h) The bam186 heterozygous mutation (h) can suppress GSC loss in nos-GAL4 > H1 KD females (g). (i) Quantification of results in g,h showing that the bam186 heterozygous mutation can rescue the GSC reduction in H1 KD germlia. n = 61 and n = 25 for nos-GAL4 > H1 KD and nos-GAL4 > H1 KD; bam+/− groups, respectively. Scale bars, 10 μm. DAPI, 4,6-diamidino-2-phenylindole.
causal relationship between the knockdown of linker H1 and hyper acetylation on H4K16. Furthermore, this H4K16 hyper acetylation in the germline are increased in Figure 3 | H1 selectively modulates H4K16ac level. (a,b) H4K16ac levels (red) in the germline are increased in nos-GAL4–H1KD flies (b) compared with those in controls (a). Anti-Vasa antibody (green; cytoplasm) marks the germline cells, and anti-LaminC (also green; nuclear lamina) marks the gap cells and terminal filament. (c) H4K16ac level (red) in the GFP-positive (nucleus) H1KD GSC (broken circles) is markedly elevated compared with that in the GFP-negative control GSC (circles). Anti-LaminC (also green; nuclear lamina) marks the gap cells and terminal filaments. (d) Quantification of results in c showing that the relative H4K16ac fluorescent intensity in the GFP-positive H1KD clone is more than twofold higher compared with that in the GFP-negative control GSCs (n = 5, mean ± s.d.). The intensity of H4K16ac in control GSCs is set to 1. Data are evaluated with Student’s t-test. Scale bars, 10 μm. DAPI, 4,6-diamidino-2-phenylindole.

supported by experiments showing that double depletion of H1 and MOF in GSCs can rescue the H1KD phenotype.

Based on our study, we propose a model to explain the functions of H1 in regulating renewal and differentiation of GSCs in Drosophila (Supplementary Fig. 8d). We propose that H1 supports GSC maintenance by suppressing regulators of differentiation, such as bam (Fig. 2). This may require at least a certain level of H1 binding on chromatin, which keeps the genes required for differentiation in an inactive state or at a ‘basal level’ of transcription, without disrupting stem cell identity. We further propose that transcriptional activator MOF balances the function of H1 in GSCs by antagonizing the repressive role of H1. This model is consistent with recent studies showing that MOF resides in a complex with several activators of transcription48, and that the loss of MOF in mouse embryonic stem cells resulted in condensed chromatin and differentiation49. Interestingly, when H1 is specifically knocked down in the ECs in the germline, which form part of the niche for the differentiation of the GSCs and cystoblasts, we have observed an increase of GSC-like, spermatagonia-containing cells in the germline, which resemble an ovarian tumour. Strikingly, this phenotype can also be suppressed by the concomitant knocking down of mof, which also supports our model (Supplementary Fig. 9). Taken together, our results support that linker H1 is a regulator with a critical function in the regulation of GSC self-renewal, and MOF antagonizes this function through H4K16 acetylation. It will be interesting to test whether this mechanism is conserved in mammals, and whether this novel mechanism can explain how dysregulated H1 contributes ovarian tumorigenesis in humans in the future.

**Methods**

**Drosophila husbandry and genotype information.** The following fly stocks were used in this study: UAS-H1KD RNAi lines (TH100868.N, TH100825.N, and TH100826.N), a UAS-mof RNAi line (TH08707.N), UAS-dBtg1 RNAi lines (TH11322.N and TH11332.N), UAS GFP RNAi line (TH00722.N), UAS-GFP (TH10151.N), P[mosP-GAL4::p:sp1]+64, y, hs-FLP, nos < STOP > GAL4–VP16, UAS-GFP/CyO29, P[bam]-GFP22, Dad-LacZ25, bam26 (ref. 47), UAS-p35 (ref. 48), 1824-GAL4 (AB1-GAL4; Bloomington #1824), OK107-GAL4 (w*; (GawB)eyOK107, a gift from Dr Yi Zhong) and s87-Gal4 (Bloomington #25412).

Sense Oligos of the transgenic RNAi lines are:

- TH00782.N: 5′-CCCGAAGGTGATAGCAGGAA-3′
- TH00825.N: 5′-AAAGCAAGAGTTAGCTGCTCT-3′
- TH100826.N: 5′-TAGGGAAACGCTCAGAAGCA-3′
- TH100868.N: 5′-ACACAGGACAGTGGAGAGAA-3′
- TH00870.N: 5′-CTCGACCTCAGCGGTGTCCAA-3′
- TH11322.N: 5′-CGGGGAAATGTTGATGTTTAAA-3′
- TH11323.N: 5′-ATGTTAAGGATCGTCCTATTAAA-3′
All Drosophila stocks were maintained at 25 °C with 60% humidity on standard cornmeal/agar media unless otherwise specified.

**Transgene constructs and production of transgenic flies.** H1, MOE–GFP fusion protein and H4.K16R were constructed using a previously described method and a ValiumP-GFP vector. Transgenes expressing H1 and MOE–GFP fusion proteins were constructed as follows: the H1-coding sequence was amplified (forward primer: 5′-GGTCTCAGATGGGACTGATCTCCAGGATGCTCCAA-3′; reverse primer: 5′-AATCTAGATCTTGTGACCGGATAGTC-3′). The H1 PCR product was digested with XbaI and cloned into a ValiumP-GFP vector. The H4K16R was replaced with arginine (H4K16R) in H4-GFP. The full cDNA of histone H4 was amplified (forward primer: 5′-GGTCTCAGATGGGACTGATCTCCAGGATGCTCCAA-3′; reverse primer: 5′-AATCTAGATCTTGTGACCGGATAGTC-3′). The PCR product was digested with XbaI and cloned into the ValiumP-GFP vector. The H4.K16R gene was then generated by converting the AAG codon into AGG using the AccuPrime Pfx DNA Polymerase Kit (Invitrogen) according to the manufacturer’s instructions. Lysine 16 was replaced with arginine (H4.K16R) in H4-GFP.

**Transgenic fly lines were produced by injecting the constructs into y Sc v nanos-integrase; attP40 stocks following the standard procedure27.**
Figure 4 | MOF antagonizes the association of H1 on chromatin. (a,b) H4K16ac is normally enriched only in the X chromosome in salivary gland cells of male third-instar larvae (a), but has also accumulated on autosomes in 1824-GAL4-driven H1KD (1824-GAL4→H1KD) polytene chromosomes (b). Note the decondensation of chromatin compared with in a. (c) Overexpression of MOF (green) causes a global increase of H4K16ac (red) on all chromosomes that co-localizes with MOF. Polytene chromosomes are from OK107-GAL4-driven UAS-MOF-GFP flies. (d) In MOF-overexpressing salivary gland cells (green), the association of H1 on chromatin is greatly attenuated and the decondensation of chromatin is evident, compared with that in the neighbouring control cells. The right panel is a magnified view of the rectangle area in the left panel. Note the similarities between H1KD (b) and MOF-GFP OE (d) phenotypes. Scale bars, 40 μm in a and c, 20 μm in b, d. 

For the overexpression of MOF and H4K16A in salivary glands, the MOF-GFP- and H4::GFP-coding sequences were subcloned into pUAST vectors, in which MOF::GFP and H4::GFP were flanked by hsp70 basal promoter and the SV40 polyA tail. The H4K16A gene was then generated by converting the 16th codon AAG (Lysin) into GCG (Alanine). The plasmids were then injected into STO-embryos following the standard procedure. To generate random RNAi and overexpression mosaics to study salivary gland cells and polytene chromosomes, 

**FLP-out and OK107-induced clonal analysis.** Clones of RNAi cells in the ovary were generated by FLP/FRT-mediated recombination. To generate GSC clones, y, hs-FLP; nos<STOP<\textsc{GAL4};VP16, UASP-GFP/Cyo or y, hs-FLP, nos<\textsc{STOP-P}<\textsc{GAL4};VP16, UASP-GFP/Cyo; Dial-aacZ/TM6B was crossed with the UAS-H1KD RNAi line (TH00868.N). Adult females of the appropriate genotypes were heat-shocked at 37°C for 45 min 2 days after eclosion. The females were then transferred to fresh food at 29°C, and the ovaries were dissected at days 2, 7, 14 and 21 after heat-shock treatment for antibody staining. 

Mosaic salivary glands with RNAi cells were generated using the OK107-GAL4 driver. OK107-GAL4 is mostly expressed in the mushroom bodies, but is also expressed in a random manner in a few salivary gland cells. Thus, OK107 was applied to generate random RNAi and overexpression mosaics to study salivary gland cells and polytene chromosomes. 

**Immunostaining of ovaries.** Immunostaining of ovaries followed previously described protocols. Ovaries were dissected in cold PBS, 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. The ovaries were first incubated in 0.5% goat serum diluted in PBT for 1 h and then with the appropriate primary antibodies diluted in PBS at 4°C overnight. The samples were then washed with PBT five times for 15 min each, incubated with the appropriate secondary antibodies at 25°C for 2 h, then washed with PBT five times for 15 min each. After the last wash, the stained ovaries were mounted in Fluoromount mounting media (Sigma, F4680). Images were obtained with an inverted Zeiss LSM780 fitted with a ultraviolet laser. The NIS Elements BR programme was used for measurements.

The following primary antibodies were used: mouse monoclonal anti-Hts antibody 1B1 (Developmental Studies Hybridoma Bank (DSHB), 1:100), mouse monoclonal anti-LaminC antibody LC28.26 (DSHB, 1:100), rabbit polyclonal anti-Vasa (Santa Cruz Biotechnology, sc30210, 1:200), rabbit polyclonal anti-H1 (anti-H1C, Fang-Lin Sun)28, rabbit polyclonal anti-Hts (Abcam, ab290, 1:1,000), rabbit polyclonal anti-H4K12ac (Millipore, 07-030, 1:100), rabbit polyclonal anti-H3K27me3 (Cell Signaling, 9733, 1:200), and rabbit polyclonal anti-H4K5ac (Millipore, 07-327, 1:100), rabbit polyclonal anti-H4K16ac (Millipore, 07-329, 1:200), rabbit polyclonal anti-H4K12ac (Abcam, ab290, 1:1,000), mouse monoclonal anti-GFP (Roche, 1814460, 1:50), anti-pMad (Cell Signaling, 9516, 1:50), anti-beta-Galactosidase (Cappel, 53976, 1:1,000), rabbit polyclonal anti-cleaved Caspase-3 (Cell Signaling, 9661, 1:200), rabbit polyclonal anti-dBigH1 (a gift from Dr Fernando Azorín, 1:40), rabbit polyclonal anti-H4K16ac (Millipore, 07-329, 1:200), rabbit polyclonal anti-H4K12ac (Abcam, ab46983, 1:400), rabbit polyclonal anti-H4K8ac (Cell Signaling, 2594, 1:200), rabbit polyclonal anti-H4K5ac (Millipore, 07-327, 1:100), rabbit polyclonal anti-H3K4me2 (Millipore, 07-030, 1:100), rabbit polyclonal anti-H3K7me3 (Cell Signaling, 9733, 1:200), and rabbit polyclonal anti-H4K9me2 (Millipore, 07-212, 1:200). Various secondary antibodies (Jackson ImmunoResearch Laboratories) conjugated with FITC or TRITC were used at 1:200.

To quantify the immunostaining results of the mosaic studies, areas of interest (identified by markers including GFP, LaminC and 4,6-diamidino-2-phenylindole) were selected using the freeform tool in Imagej and measured to get the Mean Density readings. Then the light intensities of RNAi or overexpression GSC clones were directly compared with that of the neighbouring control GSCs (no GFP variability).
Figure 5 | An antagonism between H1 and MOF in GSC maintenance. (a,b) Control germarium (a) has two GSCs that can be identified by round spectrosomes (1B1; red) and attachment to the cap cells (asterisk). A loss of GSCs can be detected by 1B1 in mof germline overexpression flies (b). Note the prematurely differentiated germline cells neighbouring the cap cells identified by the branched fusome in (b). (c) Quantification of the results in a,b showing that mof overexpression in the germline causes GSC loss. n = 55 and n = 70 for control and experimental groups, respectively. (d) Column chart quantifying the results of the overexpression of a lysine to arginine H4 mutant (H4K16R) on GSC maintenance. The number of spectrosome-containing GSCs has significantly increased in H4K16R germline overexpression germaria, compared with that in nos-GAL4/+ controls. n = 21 and 24 for the two groups respectively. Data are shown as mean ± s.e.m. and evaluated with Student’s t-test. (e–g) H1 and mof double knockdown under the nos driver (g) results in normal numbers of GSCs similar to the control (e), which shows that mof-KD rescues the GSC loss phenotype caused by germline H1 KD (f). (h) Quantification of the results in e–g showing that mof-KD can rescue the GSC loss phenotype caused by H1 KD in the germline. n = 81, 114 and 103 for the three groups, respectively. (i) Quantification of 24-h egg production rates on the 4th, 5th and 6th day after eclosion (n = 15 for each group, mean ± s.d.). Note the H1 and mof double knockdown significantly increases egg production compared with H1 and GFP double knockdown flies. Germaria are from 3-day-old adult flies. Scale bars, 10 µm. DAPI, 4,6-diamidino-2-phenylindole.
(1% NP-40, 300 mM NaCl, 50 mM Tris at pH 7.8, Roche protease inhibitor). The protein concentration of the supernatant was measured by using Bio-Rad Protein Assay Reagent (500-0006). For SDS-polyacrylamide gel electrophoresis, 15 µl per lane were loaded. After electrophoresis, proteins were transferred from the gel onto polyvinylidene difluoride membrane (Bio-Rad), then hybridized with primary antibodies at the dilutions indicated: anti-H1C (1:2,000), anti-H3 (Abcam, ab1791, 1:5,000). The secondary antibodies were peroxidase-conjugated anti-rabbit IgG (H+L) (1:15,000). The ECL detection system (Thermo) was used to detect signals on the blots.

**Egg-laying assay.** To measure egg-laying rates, virgin females were collected within 6 h of eclosion, and two (for control vials) or seven (for experimental vials) females were mated to five w1118 males. Females were transferred to new vials containing fresh food with several grains of yeast every day for the duration of the experiment. Egg production by individual females was scored by counting the number of eggs laid in successive 24-h periods and dividing by the number of females in each vial. Approximately 15 vials were scored for each group.

**Polytene chromosome staining.** Immunostaining of third-instar larval polytene chromosomes was performed as previously described. Chromosomes were incubated with rabbit polyclonal anti-H1C (1:1000), rabbit polyclonal anti-acetylated histone H4K16 (anti-H4K16ac) or mouse monoclonal anti-GFP (MBL, M048-3, 1:500) at room temperature for 1 h. After washing with PBS, chromosomes were incubated with a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 and a goat anti-rabbit antibody conjugated to Texas red (1:100; Jackson ImmunoResearch Laboratories) at room temperature for 2 h. After one PBS wash, the slides were incubated with 4,6-diamidino-2-phenylindole to stain DNA (Sigma, 1:1000). The immunostained slides were mounted, and the images were taken using a Leica DMi 4000 B inverted microscope (Leica: ×40 model) and processed using Adobe Photoshop software.

**References**


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Author contributions

Additional information
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