# Heterochromatin protein 1 (HP1) is intrinsically required for post-transcriptional regulation of *Drosophila* Germline Stem Cell (GSC) maintenance

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## Abstract

A very important open question in stem cells regulation is how the fine balance between GSCs self-renewal and differentiation is orchestrated at the molecular level. In the past several years much progress has been made in understanding the molecular mechanisms underlying intrinsic and extrinsic controls of GSC regulation but the complex gene regulatory networks that regulate stem cell behavior are only partially understood. HP1 is a dynamic epigenetic determinant mainly involved in heterochromatin formation, epigenetic gene silencing and telomere maintenance. Furthermore, recent studies have revealed the importance of HP1 in DNA repair, sister chromatid cohesion and, surprisingly, in positive regulation of gene expression. Here, we show that HP1 plays a crucial role in the control of GSC homeostasis in *Drosophila*. Our findings demonstrate that HP1 is required intrinsically to promote GSC self-renewal and progeny differentiation by directly stabilizing the transcripts of key genes involved in GSCs maintenance.

## Introduction

Stem cells are undifferentiated cells defined by their unique capacity to maintain self-renewing potential at every cell division, while producing differentiating daughter cells to ensure the correct development and maintain tissues homeostasis[1-3]. A better understanding of stem cells biology will not only reveal the crucial molecular mechanisms that control the formation and maintenance of tissues, but will also influence stem cell-based therapies in regenerative medicine[2,4,5] and cancer treatments[6].

In view of this, deepening the molecular mechanisms that control the fine balance between stem cell self-renewal and differentiation represents one of the fundamental goals of stem cell biology. This balance often depends on the coordinated regulation of complex transcriptional and post-transcriptional hierarchies.

The best way to investigate the molecular basis of stem cell regulation involves *in vivo* approaches, in the whole organism, since the removal of stem cells from the contexts of their "niches", in tissue cultures, could irreversibly change their properties[7]. In this context, the *Drosophila* ovarian germline stem cells (GSCs) represent an excellent and attractive model system to study the molecular basis of adult stem cell behavior and regulation[8-11].

The *Drosophila* ovary is composed of about 20 functional units called ovarioles[12]. The most anterior part of the ovarioles consist of a germarium, a structure containing two or three asymmetrically dividing germline stem cells each of which produce another self-renewing GSC that remains anchored to the stromal somatic cap cells and a cystoblast (CB) committed to differentiate to sustain the later stages of the oogenesis.

The CB undergoes four synchronous divisions with incomplete cytokinesis to produce a 16-cell germ line cyst[12,13] and steadily moves in a posterior direction through the germarium. Of these, one cell will differentiate into an oocyte, while the remaining cells will become polyploidy nurse cells[14]. The 16 cells cyst becomes surrounded by a monolayer of follicle cells and buds off from the

posterior germarium to form an egg chamber[15,16] which ultimately gives rise to a single mature oocyte ready for fertilization.

The activity of GSCs is controlled by extrinsic and intrinsic signaling pathways that finely regulate the balance between stem cell self-renewal and differentiation through the coordination of complex transcriptional and post-transcriptional hierarchies.

Decapentaplegic (Dpp) and Glass bottom boat (Gbb) are produced from the somatic niche and activate bone morphogenetic protein (BMP) signaling in the GSC to directly repress the Bam-dependent differentiation pathway and to maintain GSC identity[17-20]. Besides extrinsic mechanisms, stem cell intrinsic programs are crucial to control the binary germ line cell fate in *Drosophila*. Nanos and Pumilio are intrinsic factors essential to maintain stem cell identity[21-23]. They are key components of an evolutionarily conserved translational repressor complex[24-28] that bind to specific recognition sequences in the 3'untranslated regions (3'UTRs) of differenziating mRNAs to repress their translation[24,29,30].

Other intrinsic factors necessary for GSC maintenance include components of the microRNA (miRNA) silencing machinery, indicating a central role for miRNA-dependent gene silencing in GSC identity[31-34]. Additionally, many genes involved in piRNA pathway appear to be crucial for proper GSC lineage development in *Drosophila*[22,35-38].

A fast-growing body of experimental data provide strong evidences that also epigenetic mechanisms involving chromatin architecture and histone modification are equally important for the regulation of GSC maintenance and differentiation in *Drosophila*[39-43]. For example, the chromatin remodeling factor Iswi and the putative transcription factor Stonewall are intrinsically required for GSC maintenance[39-43]. The H3K4 demethylase Lsd1 controls non-autonomously the germ cell differentiation presumably through repressing *dpp* expression[41]. Moreover, other interesting studies show that the histone H2B ubiquitin protease Scrawny (Scny)[40] and the histone H3K9 trimethylase Eggless (Egg) are required for maintaining self-renewal of GSC[42].

Although different experimental evidence confirms the relevance of epigenetic regulatory programs in the GSC regulation, a complete picture of such mechanisms is still far to be resolved.

Heterochromatin protein 1 (HP1) is an evolutionarily conserved multifunctional epigenetic adaptor that is involved in heterochromatin formation and epigenetic gene silencing in different species including humans[44-46]. In addition to its role in heterochromatin structural organization, emerging evidence in *Drosophila* and mammals has highlighted the importance of HP1 in telomere capping, telomere length homeostasis[47,48] and, more surprisingly, in positive regulation of gene expression[49-54].

A recent study showed that HP1 and Su(var)3-9 are both necessary for GSC maintenance and that HP1 is sufficient for GSC self-renewal in *Drosophila* testis[55]. It has also been demonstrated that planarian HP1, induced upon injury, is able to promote regenerative proliferation of adult stem cells[56]. In mice, loss of HP1 gamma significantly reduces the number of primordial germ cells (PGCs) by regulating their cell cycle progression[57]. Moreover, HP1 gamma is essential for male germ cell survival and spermatogenesis[58]. Recently, a large-scale RNAi screen in *Drosophila* female germline stem cells identified HP1 as potentially involved in oogenesis[59] even though the precise molecular mechanisms by which it exerts its function still remain elusive and need to be defined.

Here, we report our experiments showing an important function for *Drosophila* HP1 in female gametogenesis. In this study, we establish that HP1 is necessary for *Drosophila* oogenesis and is required cell autonomously to control the fine balance between stem cell self-renewal and differentiation. Finally, we show that HP1 exerts its functions, positively regulating the stability of key mRNAs involved in the control of female germ line stem cells development.

## Results and Discussion

### Functional inactivation of HP1 by *in vivo* RNA interference (RNAi) causes severe germ line defects that result in agametic ovarioles and female sterility

HP1 is a protein constitutively expressed in almost all larval and adult tissues with highest enrichment in adult ovaries (flybase.org). Immunostaining experiments performed by a specific anti-HP1 antibody on wild type ovaries, showed that HP1 localizes in the nucleus of both somatic and germline cells, from the anterior tip of the germarium (GSCs and CBs) until late stages of oogenesis (Fig. 1a). Specifically, HP1 immunosignals were mainly detected in dense pericentric heterochromatic foci in all germarium and developing egg chamber cells; HP1 also accumulated in the germinal vesicle and on the karyosome of the oocyte (Fig. 1a). HP1 was particularly enriched within and next to heterochromatic regions also in larval and pupal gonads (Supplementary Fig. S1).

Since homozygous HP1 mutants die at third instar larvae, to investigate *in vivo* the function of HP1 in adult female germline, we took advantage of the Gal4-UAS binary system[60]. We performed tissue-specific HP1 knockdown by independently crossing two different transgenic lines carrying HP1 short small hairpin RNAs (shRNA)[61] under the control of Gal4-responsive UAS promoter, with nanos-Gal4-NGT (hereafter referred as nos-Gal4) that provides a robust and uniform Gal4 expression in the germarium[62]. We found that the functional inactivation of HP1 in the F1 female progeny resulted in complete sterility thus suggesting an essential role for HP1 in female gametogenesis.

In order to further investigate the molecular basis underlying this female sterility, ovaries from *nos-Gal4>HP1*$^{RNAi}$ females ranging from 1- to 15-day-old, were dissected and immunostained with a specific antibody against Vasa, a DEAD-box RNA helicase which is a well-characterized marker of germ cells lineage in insects and vertebrates[63,64]. We found that knocking down HP1 upon nos-Gal4

driver expression (Supplementary Fig. S2), resulted in ovaries that were completely agametic (Fig. 1b, c) as compared to control ovaries (Fig. 1d); 86% of HP1 depleted germaria from 0- to 1-day-old females were completely devoid of germ cells (Fig. 1b) whereas 14% contained only a few germ cells at the tip of the ovariole (less than 10 per germarium) and one or two abnormal egg chambers (Fig. 1c) (n = 250 ovaries). From 5- to 15-day-old females, all the HP1 depleted ovaries exhibited a typical germline less morphology confirmed by the total absence of Vasa-positive cells (data not shown).

These findings strongly suggest for HP1 a specific and crucial role in germ line stem cell maintenance and differentiation; we could not, however, completely exclude a general role for HP1 in cell viability.

To discriminate between these possibilities, we knocked down HP1 with a maternal tubulin (Mat) Gal4 that induces transgenic expression of short hairpin RNAs against HP1 outside the germarium, starting in stage 2[65] (Supplementary Fig. S3a, b). We found that HP1 knockdown females were fertile and showed no obvious oogenesis defects (Supplementary Fig. S3c) thus suggesting for HP1 an essential and cell autonomous function in early oogenesis and not a general requirement for cell survival.

**HP1 is required during multiple processes in early oogenesis**

Germ cell-specific knockdown of HP1 causes almost complete loss of germ cells before adulthood. In order to determine the phenocritical period for HP1 requirement during normal oogenesis, we cytologically examined larval and pupal HP1 depleted ovaries following the germ cells fate, starting from early stages of germ-cell development to adulthood (Fig. 2 and Supplementary Fig. S4)

Vasa staining analysis showed that larval ovaries from *nos-Gal4>HP1*$^{RNAi}$ females displayed a normal cellular organization as compared to control; consistent with this the total number of PGCs resulted unaffected (*nos-G4/+*, 107.5 ± 8.5; *nos-G4>HP1*$^{RNAi}$, 106.6 ±7.0) (Fig. 2a).

On the contrary, HP1 depleted pupal ovaries were almost completely devoid of differentiated egg chambers when compared to the control pupal gonads (Fig. 2b).

Taken together, these findings suggest that HP1 is required during the earliest stages of oogenesis at the larval/pupal transition when GSCs are established[66].

In order to gain a more complete understanding of the altered phenotypes observed in pupal ovaries and to better investigate how HP1 regulates the behavior of germ cells, we performed an accurate cytological analysis on *nos-Gal4>HP1*$^{RNAi}$ pupal ovaries. We performed double-immunostaining experiments with antibodies against Vasa and α-Spectrin; α-Spectrin is a cytoskeletal protein that specifically labels spectrosomes and fusomes and can be used to trace the germline differentiation. Spectrosomes are spherical and mark GSCs and cystoblasts, whereas fusomes are branched and mark 2, 4, 8, and 16-cell cysts[67].

The results of this cytological analysis (152 ovarioles scored) demonstrated that HP1 depleted pupal ovaries exhibited several remarkable and complex phenotypes including: empty germaria (27%, n=41 ovarioles), germaria with germ cells containing spectrosomes only (33%, n=51 ovarioles), germaria with germ cells containing both spectrosomes and fusomes carrying a single developing cyst connected by an incompletely branched fusome (26%, n=39 ovarioles) and germaria with few germ cells containing only fusomes (14%, n=21 ovarioles) (Fig. 3a, b).

These complex phenotypic defects suggest for HP1 a functional role in regulating the germline stem cell (GSC) maintenance.

We asked whether the low number of germ cells in HP1 depleted ovarioles could be related to defects in the division rate of ovarian stem cells and their progeny. These defects might contribute to germ line cells loss over time. In order to verify the capacity of germ cells to undergo mitotic divisions, we immunostained wild type and HP1 knockdown ovaries with a specific antibody to phosphorylated H3S10 (phospho-H3, PH3) to detect germline cells undergoing mitosis at a given time (Fig. 3c). In HP1 depleted ovaries we observed an almost complete loss of PH3 positive nuclei

(5%, n=56 ovarioles) respect to the control ovaries (35%, n=46 ovarioles) (Fig. 3d); this result establishes that the functional inactivation of HP1 severely impairs the correct germ cells division. We also assessed apoptosis by using anti-cleaved Caspase-3 antibody that is a proven marker for cells that are dying. The results clearly indicated that the few remaining germline cells detected in HP1 depleted ovaries are strongly stained with cleaved Caspase-3 suggesting that the germ cells that fail to properly divide die prematurely (Fig. 3e).

**HP1 promotes germ cell differentiation by post-transcriptionally regulating *bam* expression and function**

Germline division defects are often associated to an altered differentiation program. Previous studies demonstrated that Bag of Marbles (Bam) protein is necessary and sufficient for promoting GSC and cystoblasts differentiation, since *bam* mutations completely block germ cell differentiation (causing GSC hyperplasia), whereas *bam* ectopic expression in GSCs results in their complete and precocious differentiation[18,68,69].

To determine whether the phenotypic defects observed in HP1 depleted pupal ovaries could be related to *bam* repression, we firstly evaluated, by quantitative real-time PCR (qRT-PCR), the expression of *bam* gene in HP1 knockdown pupal ovaries. We found that ovaries lacking HP1 exhibited a significant reduction of *bam* transcript levels (close to about 80%) as compared to control ovaries (Fig. 4a).

Consistent with the down regulation of *bam* mRNAs, we also observed a drastic diminution of Bam protein by immunostaining with a specific monoclonal antibody against Bam (Fig. 4b). In wild type ovaries, Bam protein was detected, as expected, in cystoblasts and early developing cysts (2-, 4-, and 8-cell cysts) whereas in HP1 mutant ovaries Bam protein was almost undetectable (Fig. 4b). Altogether, these data strongly suggest that HP1 blocks Bam driving germ cell differentiation.

Previously we have demonstrated that in *Drosophila* HP1 takes part in positive regulation of gene expression by stabilizing RNA transcripts and protecting them against premature and rapid

231 degradation[53]; in particular, we found that HP1 is able to directly bind the transcripts of more than
232 one hundred euchromatic genes in *Drosophila* and physically interacts with DDP1[70], HRB87F[71]
233 and PEP[72], which belong to different classes of heterogeneous nuclear ribonucleoproteins (hnRNPs)
234 that are known to be involved in RNA packaging, stability and processing. Moreover, in our
235 previous work we also demonstrated that HP1 is cotranscriptionally recruited on nascent transcripts
236 through its chromodomain[49,53].
237
238 In order to verify if HP1 was directly involved in post-transcriptional regulation of *bam* gene by
239 binding *in vivo* its mRNA, we performed HP1 CLIP (UV cross-linking and immunoprecipitation)
240 experiments[73,74] on whole adult ovaries dissected from 0- to 1-day-old-wild type females.
241 The results of RT-PCR from HP1 CLIP experiments clearly showed that *bam* transcripts were
242 significantly enriched in the CLIP sample when compared to the mock control sample (Fig. 4c) and
243 demonstrated that HP1 is able to specifically bind *bam* transcripts *in vivo*.
244 In order to further investigate whether HP1 is cotranscriptionally recruited on *bam* nascent
245 transcripts, we performed ChIP experiments on cross-linked chromatin purified from 0- to 1-day-
246 old wild-type ovaries. To evaluate the presence of *bam* sequences among the immunoprecipitated
247 DNA, a PCR analysis was performed with specific primer pairs covering both the promoter and the
248 coding regions of *bam* gene.
249 The results of ChIP assays demonstrated that HP1 is clearly associated to *bam* gene (Fig. 4d). To
250 completely exclude any direct role for HP1 on *bam* transcriptional control and to confirm that HP1
251 binding on *bam* gene was exclusively mediated by the presence of *bam* nascent transcripts, ChIP
252 experiments were repeated in presence of RNaseA/T1 mix that specifically degrades single stranded
253 RNA (ssRNA). The RNase-ChIP results demonstrated that chromatin RNase treatment prior to
254 immunoprecipitation completely remove HP1 from *bam* gene thus confirming that the recruitment
255 of HP1 on *bam* gene is clearly RNA-dependent (Fig. 4d). RNase treatment did not affect, as

256 expected, the HP1 occupancy over Het-A telomeric retrotransposon (Fig. 4d) since, at the
257 telomeres, HP1 is capable to directly bind HeT-A sequences through its hinge domain[48].
258 To determine the stability of *bam* transcripts, we analyzed, by qRT-PCR, RNA samples purified
259 from wild type and HP1 knockdown ovaries treated with Actinomycin D to inhibit transcription and
260 *de novo* RNA synthesis. Previous analysis showed that a 30 min treatment was sufficient to inhibit
261 transcription in the ovaries[75]. As shown in Figure 4e, in HP1 lacking ovaries we observed a strong
262 and rapid decay rate of *bam* transcript when compared to the control (Fig. 4e).
263 These observations strongly suggest that HP1 may regulate *bam* mRNAs in a post-transcriptional
264 manner.
265 To confirm our findings and to verify if HP1 can effectively control germ cells differentiation in a
266 *bam*-dependent manner, we overexpressed *bam* from a heat shock inducible transgene carrying the
267 full-length bam cDNA[68] in the HP1 knockdown germ cells. To assess the effectiveness of hs-bam
268 transgene expression we analyzed bam mRNA and protein in HP1 depleted ovaries with or
269 without heat-shock (Supplementary Fig. S5).
270 *Nos-Gal4 /UAS-HP1$^{RNAi}$; P[hs-bam]/+* and *nos-Gal4 /HP1$^{RNAi}$; +/+* females were heat-shocked at
271 pupal stage (96 hours) at 37 °C for 1 hour and, 24 hours after heat shock (HS) treatment, adult
272 ovaries were dissected and stained with anti-Vasa antibody (Fig. 5a, b). As showed in Figure 5b,
273 heat-shock induced *bam* can only partially rescue the phenotypic defects induced by HP1
274 knockdown since its forced expression under control of the heat shock promoter generates only few
275 normally developed egg chambers (see Fig. 5c for quantification of ovarioles containing developing
276 egg chambers in heat shocked HP1 depleted females carrying the *P[hs-bam]* transgene). This
277 finding suggests that oogenesis defects observed in HP1 depleted ovaries may be only partially
278 imputable to defective differentiation mechanisms.
279 It is well known that loss of *bam* blocks germ cell differentiation resulting in GSC hyperplasia[68], a
280 characteristic phenotype that we never observed in HP1 depleted ovaries by nos-Gal4.

Altogether, these findings strongly suggest that the complex phenotypic defects arising from HP1 knockdown in the female germline are only partially dependent on *bam* repression and are probably due to a duplex coordinated control operated by HP1 in both GSCs self-renewal and differentiation. In order to verify this hypothesis we inactivated HP1 only in Bam-expressing germline cells by using P{bam promoter-Gal4:VP16}[17] that drives the expression of shHP1 only in the dividing cystoblast and cystocytes but not in GSCs where the function of HP1 protein remains completely wild-type. In this case, we observed the classical ovarian tumor phenotype (Fig. 5d) albeit at very low frequency (less than 1%) due to the low effectiveness of bam-Gal4 driver in knocking down HP1 protein (Supplementary Fig. S6).

**HP1 controls GSCs self-renewal by post-transcriptional regulation of stemness genes**

Consistent with the conclusion stated above, we wondered if HP1 was able to post-transcriptionally regulate also key stemness genes. First, we analyze by qRT-PCR the expression profiles of some important genes that are intrinsically involved in GSCs self-renewal by repressing Bam differentiation pathways[76-78].

We found that some of them as *nos*, *cup*, *piwi* and *vasa* were significantly down regulated in HP1 knockdown pupal ovaries respect to the control (Fig. 6a). These results allowed us to hypothesize that also *nos*, *cup*, *piwi* and *vasa* genes might be post-transcriptionally regulated by HP1. So we dissected ovaries from 0- to 1-day-old wild type females to repeat both CLIP and ChIP experiments. CLIP-PCR analysis, clearly showed that *nos*, *cup* and *piwi* RNAs were significantly enriched in the IP sample respect to the mock control sample whereas *vasa* mRNA did not (Fig. 6b). These genes resulted strongly enriched also in ChIP IP sample but not in RNAse-ChIP IP sample (Fig. 6c) indicating that their RNAs are co-transcriptionally bound by HP1. To determine the mRNA decay of these genes, we repeated the Actinomycin D treatment that allowed us to conclude that HP1 is able to stabilize *nos*, *cup* and *piwi* mRNAs (Fig. 6d).

306 Altogether, these data strongly indicate that HP1 is intrinsically required for post-transcriptional
307 regulation of *Drosophila* GSC maintenance. Our findings suggest that HP1 exerts its function
308 through the formation of an HP1-containing hnRNP nuclear complex that protects and stabilizes
309 key mRNAs involved in the control of GSC homeostasis and behavior. Intriguingly there are
310 different experimental evidences demonstrating that also mutations in genes coding for the HP1-
311 interacting hnRNPs DDP1, HRB87F and PEP induce female-sterile phenotypes[79-81]. For example,
312 the hypomorphic insertional allele of DDP1 ($Dp1^{15.1}$) causes complete sterility in females but not in
313 males. Homozygous $Dp1^{15.1}$ females show abnormal ovaries with ovarioles undeveloped, egg
314 chambers often fused and containing an irregular number of cells[79]; finally, also PEP and HRB87F
315 are essential for normal gonadal development and female fecundity[80,81].
316 In conclusion, the above results demonstrate, for the first time, an essential role for HP1 in post-
317 transcriptional regulation of GSC maintenance and certainly add a new dimension to our
318 understanding of HP1 targeting and functions in epigenetic regulation of GSC behavior.
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# Methods

## *Drosophila* Strains

All flies were raised at 24 °C on standard cornmeal-sucrose-yeast-agar medium.
For a detailed list of all stocks used in this study, see Supplementary Methods.

## Immunofluorescent staining of larval, pupal and adult whole-mount ovaries

Pupal and adult ovaries were stained according to Grieder[82].
Larval ovaries were dissected, fixed, and immunostained as described previously by Pisano[83].
Further details can be found in Supplementary Methods.

## Western Blot analysis

Protein extracts fractionated by 10% SDS-PAGE and electroblotted onto Immobilion-P polyvinyldifluoride membranes (Bio-rad) were probed with antibodies against HP1 (1:500, 9A9 monoclonal mouse), α-Tubulin (mouse 1:2000, Sigma). Proteins of interest were detected with HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody (1:10000, Santa Cruz) and visualized with the ECL Western blotting substrate (GE Healthcare), according to the provided protocol. The chemiluminescence detection was performed on the ChemiDoc XRS+ System (Bio-rad) and analyzed using the included ImageLab software.

## Cross-linking immunoprecipitation (CLIP) assay

CLIP assay was performed as previously reported[84] with some modifications. Approximately 20 mg ovaries from 0- to 1-day-old wild-type females were UV crosslinked (3x2000 μJ/cm$^2$), homogenized on ice in 1 mL RCB buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 250 mM sucrose, 1 mM DTT, 1× EDTA-free Complete Protease Inhibitors, 1 mM PMSF) supplemented with 300 U RNAseOUT (Invitrogen) and placed on ice for 30 min. The

356 homogenate was sonicated on ice, at 80% power, five times in 20 s bursts with a 60 s rest in
357 between using the Hielscher Ultrasonic Processor UP100H (100W, 30kHz) and centrifuged
358 (16000xg for 5 min at 4 °C). Soluble extract was precleared with 20 µl Protein-G dynabeads
359 (Invitrogen) for 20 min at 4 °C. After removal of samples for immunoblotting and quantitation of
360 RNA input (1%), HP1 was immunoprecipitated with anti-HP1 9A9 antibody from 450 µl precleared
361 extract by incubation for 4 h with 50 µl Protein-G dynabeads. Immunoprecipitates were washed 4
362 times with RCB. To elute the immunoprecipitated RNAs, the pelleted beads were boiled in 100 µL
363 of UltraPure DEPC-treated Water for 5 min. 900 µL Qiazol Reagent was added to the supernatant
364 recovered for RNA preparation. The RNA purified was used as a template to synthesize cDNA
365 using oligo dT, random hexamers and SuperScript reverse transcriptase III (Invitrogen) according to
366 the manufacturer's protocol.
367
368 **Chromatin immunoprecipitation assay**
369 Chromatin immunoprecipitation was performed according to the method described by Menet[85] with
370 minor modifications. Approximately 20 mg ovaries from 0- to 1-day-old wild-type females were
371 homogenized in 1 mL of NEB buffer (10 mM HEPES-Na at pH 8.0, 10 mM NaCl, 0.1 mM EGTA-
372 Na at pH 8, 0.5 mM EDTA-Na at pH 8, 1 mM DTT, 0.5% NP-40, 0.5 mM Spermidine, 0.15 mM
373 Spermine, 1× EDTA- free Complete Protease Inhibitors) with a Polytron homogenizer (Kinematica
374 Swizerland) with a PT300 tip for 1 min (at 3000 rpm). The homogenate was transferred to a pre-
375 chilled glass dounce (Wheaton) and 15 full strokes were applied with a tight pestle. Free nuclei
376 were then centrifuged at 6000xg for 10 min at 4 °C. The nuclei-containing pellets were resuspended
377 in 1 mL of NEB and centrifuged at 20000xg for 20 min on sucrose gradient (0.65 mL of 1.6 M
378 sucrose in NEB, 0.35 mL of 0.8 M sucrose in NEB). The pellet was resuspended in 1 mL of NEB
379 and formaldehyde to a final concentration of 1%. Nuclei were cross-linked for 10 min at room
380 temperature and quenched by adding 1/10 vol of 1.375 M glycine. The nuclei were collected by
381 centrifugation at 6000xg for 5 min. Nuclei were washed twice in 1 mL of NEB and resuspended in

1 mL of Lysis Buffer (15 mM HEPES-Na at pH 7.6, 140 mM NaCl, 0.5 mM EGTA, 1 mM EDTA at pH 8, 1% Triton X-100, 0.5 mM DTT, 0.1% Na Deoxycholate, 0.1% SDS, 0.5% N-lauroylsarcosine and 1× EDTA-free Complete Protease Inhibitors). Nuclei were sonicated using a Hielscher Ultrasonic Processor UP100H (100W, 30kHz) six times for 20 s on and 1 min on ice. Sonicated nuclei were centrifuged at 13000xg for 4 min at 4 °C. The majority of sonicated chromatin was 500 to 1000 base pairs (bp) in length. For each immunoprecipitation, 15 µg of chromatin was incubated in the presence of 10 µg of HP1 9A9 monoclonal antibody (3 h at 4 °C in a rotating wheel). Then, 50 µl of dynabeads protein G (Invitrogen) was added and incubation was continued overnight at 4 °C. The supernatants were discarded and samples were washed twice in Lysis Buffer (each wash 15 min at 4 °C) and twice in TE Buffer (1 mM EDTA, 10 mM TrisHCl at pH 8). Chromatin was eluted from beads in two steps; first in 100 µl of Eluition Buffer 1 (10 mM EDTA, 1% SDS, 50mM TrisHCl at pH 8) at 65 °C for 15 min, followed by centrifugation and recovery of the supernatant. Beads material was re-extracted in 100 µl of TE + 0.67% SDS. The combined eluate (200 µl) was incubated overnight at 65 °C to reverse cross-links and treated by 50 µg/ml RNaseA for 15 min at 65 °C and by 500 µg/ml Proteinase K (Invitrogen) for 3 h at 65 °C. Samples were phenol–chloroform extracted and ethanol precipitated. DNA was resuspended in 25 µl of water. For maximising the molecular analyses with DNA immunoprecipitated, candidate genes were amplified in pairs through an optimized duplex-PCR protocol by using two different sets of primers having similar melting temperatures in a single reaction.

RNAse-Chromatin immunoprecipitation was performed essentially as described for ChIP but with an important modification: sheared chromatin was treated with RNAse mix (Roche) for 1 h at 37 °C before immunoprecipitation.

**Primers design and PCR amplification**

All PCR specific primers (18–25 mers with a minimum GC content of 50% and average Tm of 60 °C) (listed in Supplementary Table S1) were designed using the Invitrogen OligoPerfect™ designer

web tool and oligonucleotide sequences were screened using a BLAST search to confirm the specificity. PCR amplifications were performed with Platinum® Taq DNA Polymerase Kit (Invitrogen) according to the manufacturer's instructions.

The thermal profile for PCR amplification of CLIP samples was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and ending with a final extension at 72 °C for 7 min.

The thermal profile for duplex-PCR amplification of ChIP samples was as follows: initial denaturation at 94 °C for 5 min, followed by 28 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and ending with a final extension at 72 °C for 7 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

**Total RNA extraction and qRT-PCR**

RNA samples from ovaries were isolated by Qiazol reagent (Qiagen) according to the manufacturer's instructions. The concentration and purity of RNAs were determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific). 5 µg of total RNA was reverse transcribed using oligo dT and SuperScript Reverse Transcriptase III (Invitrogen) according to the manufacturer's protocol. The qPCR reactions were carried out with QuantiFast SYBR Green PCR Kit (Qiagen) according to manufacturer's protocol. Relative abundance of the different transcripts was determined using the $2^{-\Delta\Delta Ct}$ method[86] using *rp49* transcript as control. qRT-PCR experiments were performed in three independent biological replicates; all reactions were run in triplicates in 96-well plates over 40 cycles of 95 °C for 15 s and 60 °C for 60 s in a two-step thermal cycle preceded by an initiation step of 95 °C for 10 min. Melting-curve analysis was performed on each sample to control for nonspecific amplification and primer dimer formation. Primer sequences were listed in Supplementary Table S1. Statistical significance was determined by Mann-Whitney tests using GraphPad Prism Software. A $p$ value $\leq 0.05$ was considered statistically significant.

**Actinomycin D treatment**

To assay for mRNA stability, ovaries dissected from 1-day-old females raised at lower temperature (18 °C) were treated with 20 µg/ml Actinomycin D in Schneider's medium with constant rocking at room temperature for 30 min (T0, sufficient to inhibit transcription as described in Jao and Salic[87]; total RNA was extracted at T0 and then T30 min, T60 min, T120 min. mRNA levels for *bam*, *nos*, *piwi* and *cup* were analyzed by qRT-PCR.

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## Author contributions

A.M.C. and U.C. contributed to experimental design, performed, and analyzed all experiments; L.F. contributed to data analysis. L.P. designed, analyzed, supervised all experiments and wrote the manuscript. All authors reviewed the manuscript.

## Additional Information

**Competing Interests:** The authors declare no competing interests.

## Figure Legends

**Figure 1.** HP1 is required for correct ovarian development.

**(a)** Wild type ovariole stained for DNA (red) and HP1 (green). Arrows indicate HP1 concentrated at domains of constitutive heterochromatin. Karyosome is identified by a white dashed circle. Dashed box is magnified in the right panel. **(b, c)** HP1 depleted ovaries stained for Vasa (green) and DNA (red). All ovarioles show an altered ovarian morphology, consisting in germaria completely empty **(b)** or germaria with only few germ cells **(c)**. **(d)** Developing wild-type ovaries obtained from newly eclosed females stained for Vasa (green) and DNA (red).

**Figure 2.** HP1 is required during the earliest stages of oogenesis at the transitional period of pupal stage. **(a)** Developing ovaries obtained from female wandering third-instar larvae stained for Vasa (green) and DNA (red). **(b)** Developing ovaries obtained from 72-96 h old pupae stained for Vasa (green) and DNA (red).

**Figure 3.** Loss of HP1 Causes a Complex GSC Phenotype.

**(a)** Representative images of each phenotypic class obtained from pupal HP1-depleted germaria (*nos-G4>HP1*$^{RNAi}$) stained for Vasa (red) and α-Spectrin (green). **(b)** Quantification of the prevalence of each phenotypic class in HP1 depleted pupal ovaries; the total number of germaria scored is shown within each bar. E, empty germaria; S, germaria with spectrosomes only; S+F, germaria with germ cells containing both spectrosomes and fusomes; F, germaria with only fusome-containing germ cells. **(c)** Double-staining immunofluorescence on control (*nos-G4/+*) and HP1 depleted (*nos-G4>HP1*$^{RNAi}$) pupal ovaries for α-Spectrin (green) and PH3 (red). The white asterisk indicates dividing follicle stem cell (FSC). **(d)** Quantification of PH3-positive cystoblast in HP1 knockdown pupal ovaries. Statistical significance was determined by Fisher's exact test (***$p <$

712 0.001). **(e)** Immunofluorescence on control (*nos-G4/+*) and HP1 depleted (*nos-G4>HP1$^{RNAi}$*) pupal
713 ovaries for cleaved Caspase-3 (green) and DNA (red).
714
715 **Figure 4.** HP1 regulates *bam* mRNAs in a post-transcriptional manner.
716 **(a)** qRT-PCR analysis showing that HP1 depleted pupal ovaries express significantly less *bam*
717 transcript respect to the control. Fold-changes in RNA levels relative to the control were normalized
718 to *rp49* levels. Error bars indicate ±SEM from three biological replicates (***$p < 0.001$). **(b)**
719 Double immunofluorescence on control (*nos-G4/+*) and HP1 depleted (*nos-G4>HP1$^{RNAi}$*) pupal
720 ovaries for Vasa and Bam. **(c)** RT-PCR analysis of RNAs immunoprecipitated with α-HP1 (HP1
721 CLIP sample) in newly eclosed females ovaries. The PCR data shown here are representative of
722 three independent CLIP experiments. The full-length versions of the cropped gels are reported in
723 Supplementary Fig. S7a. **(d)** Chromatin immunoprecipitation (ChIP) analysis of HP1 occupancy at
724 the *bam* promoter region (bam Silencer Element) and coding sequence in newly eclosed female
725 ovaries. The RNase sensitivity of this association was tested by pre-treating the extract with a
726 combination of RNase A and RNase T1 (right panel). Het-A was used as a positive control to check
727 whether the ChIP experiments were working. PCR reactions were carried out on 1% input DNA.
728 The PCR data shown here are representative of three independent ChIP experiments. The full-
729 length versions of the cropped gels are reported in Supplementary Fig. S7b, c. **(e)** qRT-PCR
730 analysis of *bam* mRNA transcript at different times after blockage of transcription by Actinomycin
731 D treatment. The green line and the red line indicate the *bam* transcript amount respectively in the
732 control (*nos-G4/+*) and HP1-depleted (*nos-G4>HP1$^{RNAi}$*) ovaries from 1-day-old females. Total
733 RNA was isolated at the indicated times (0, 30 min, 60 min and 120 min). The values shown are
734 averages ±SEM of three biological replicates. The dashed lines represent the best fit regression of
735 all data point and the slopes are shown on the graph. For each genotype, all data point vs T0
736 was statistically evaluated by one-sample t-test (**$p < 0.01$).
737

738 **Figure 5.** Heat-shock induced *bam* can only partially rescue the phenotypic defects induced by HP1
739 knockdown.
740 **(a,b)** Staining for Vasa (green) and DNA (red) on whole mounts adult ovaries from *nos-*
741 *G4>HP1*$^{RNAi}$ **(a)** and *nos-G4>HP1*$^{RNAi}$*; hs-bam* **(b)** females. **(b)** The dashed white box in the left
742 panel is magnified in the right panel. **(c)** Quantification of ovarioles containing developing egg
743 chambers in heat shocked HP1 depleted females carrying or not the *P[hs-bam]* transgene (454 and
744 784 ovarioles scored for *nos-G4>HP1*$^{RNAi}$ and *nos-G4>HP1*$^{RNAi}$*; hs-bam,* respectively). Statistical
745 significance was determined by Fisher's exact test (****$p < 0.0001$). **(d)** Staining for Spectrin
746 (green) and DAPI (red) on germaria obtained from *bam-G4>HP1*$^{RNAi}$ females.
747
748
749 **Figure 6.** HP1 is required for GSC self-renewal.
750 **(a)** Bar graph showing relative quantification of mRNA encoding GSCs key factors in HP1-
751 depleted or control pupal ovaries. Error bars indicate ±SEM from three biological replicates (**$p <$
752 0.01, *$p < 0.05$). **(b)** RT-PCR analysis of RNAs immunoprecipitated with α-HP1 (HP1 CLIP
753 sample) in ovarian extract from newly eclosed females. The PCR data shown here are
754 representative of three independent CLIP experiments. The full-length versions of the cropped gels
755 are reported in Supplementary Fig. S7a. **(c)** Chromatin immunoprecipitation (ChIP) analysis of HP1
756 occupancy at *cup*, *nos, piwi* and *vasa* genes in newly eclosed female ovaries. The RNase sensitivity
757 of this association was tested by pretreating the extract with a combination of RNase A and RNase
758 T1 (right panel). Het-A was used as a positive control. PCR reactions were carried out on 1% input
759 DNA. The PCR data shown here is representative of three independent ChIP experiments. The full-
760 length versions of the cropped gels are reported in Supplementary Fig. S7b, c. **(d)** Time course
761 degradation assay of *cup, nos*, and *piwi* RNAs measured by qRT-PCR in control (*nos-G4/+*) or HP1
762 depleted (*nos-G4>HP1*$^{RNAi}$) ovaries from 1-day-old females. Total RNA was isolated at the
763 indicated times (0, 30 min, 60 min and 120 min). The values shown are averages ±SEM of three

764 biological replicates. The dashed lines represent the best fit regression of all data point and the
765 slopes are shown on the graph. For each genotype, all data point vs T0 was statistically evaluated by
766 one-sample t-test (*$p < 0.05$).
767
768

**Figure 1**

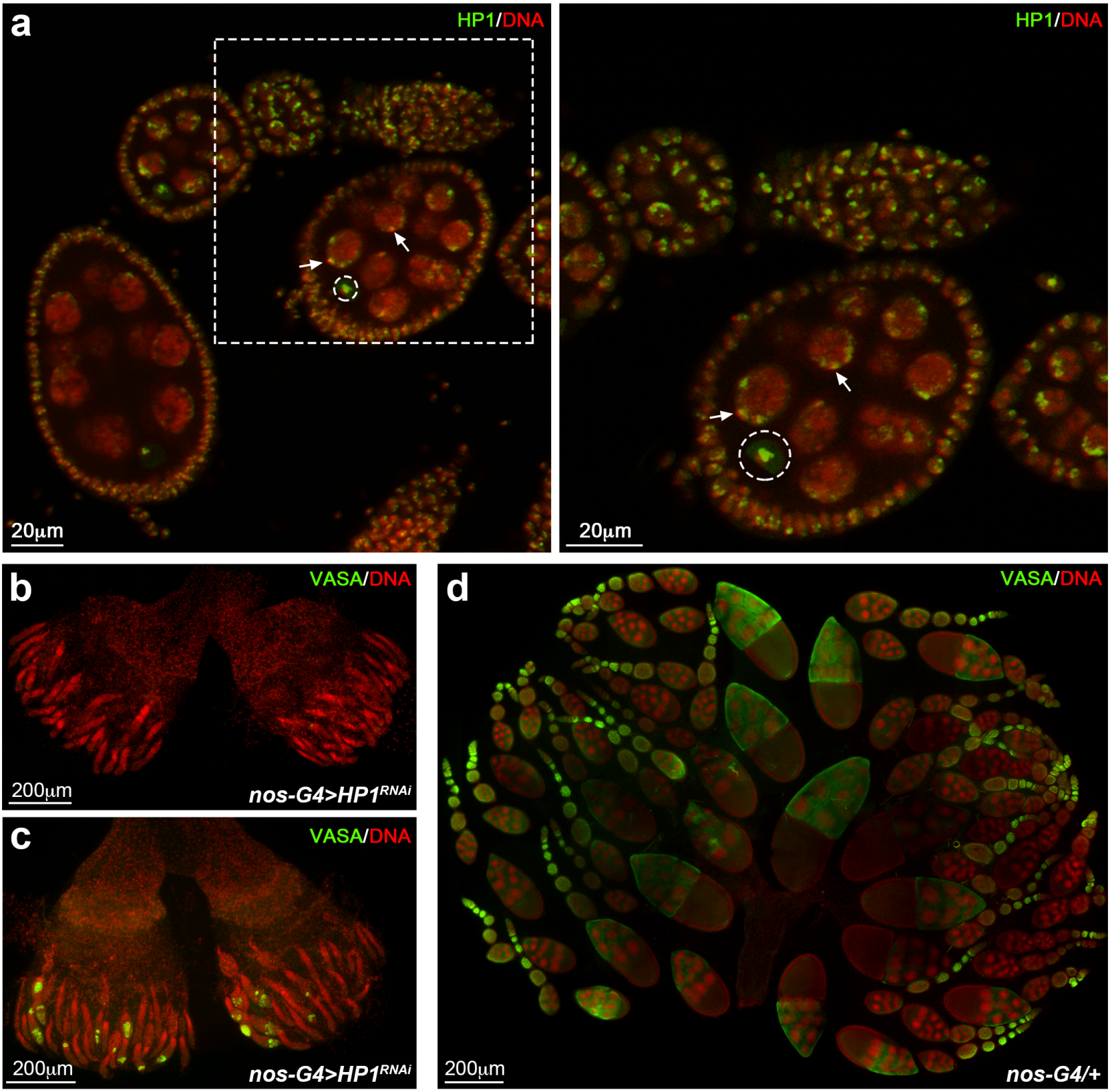

**Figure 2**

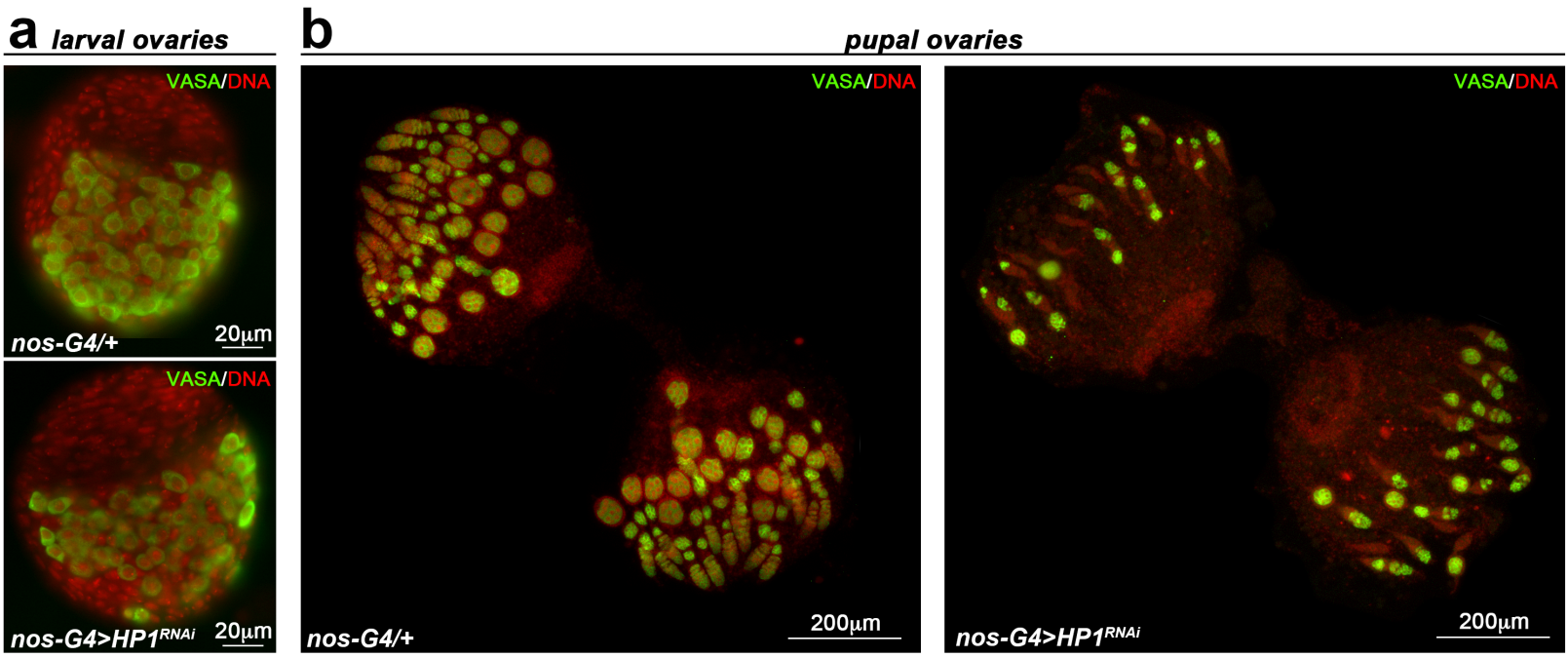

**Figure 3**

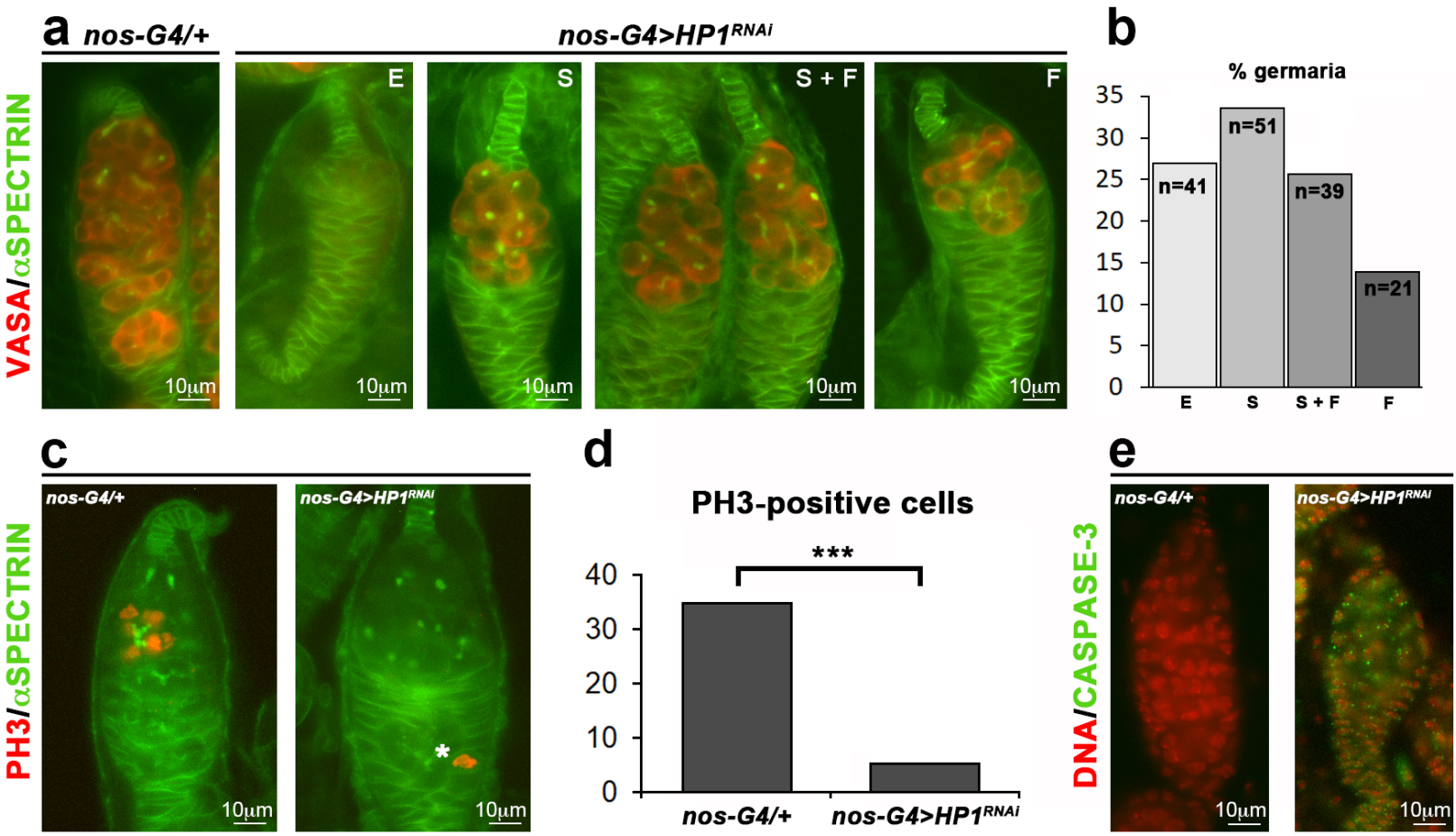

**Figure 4**

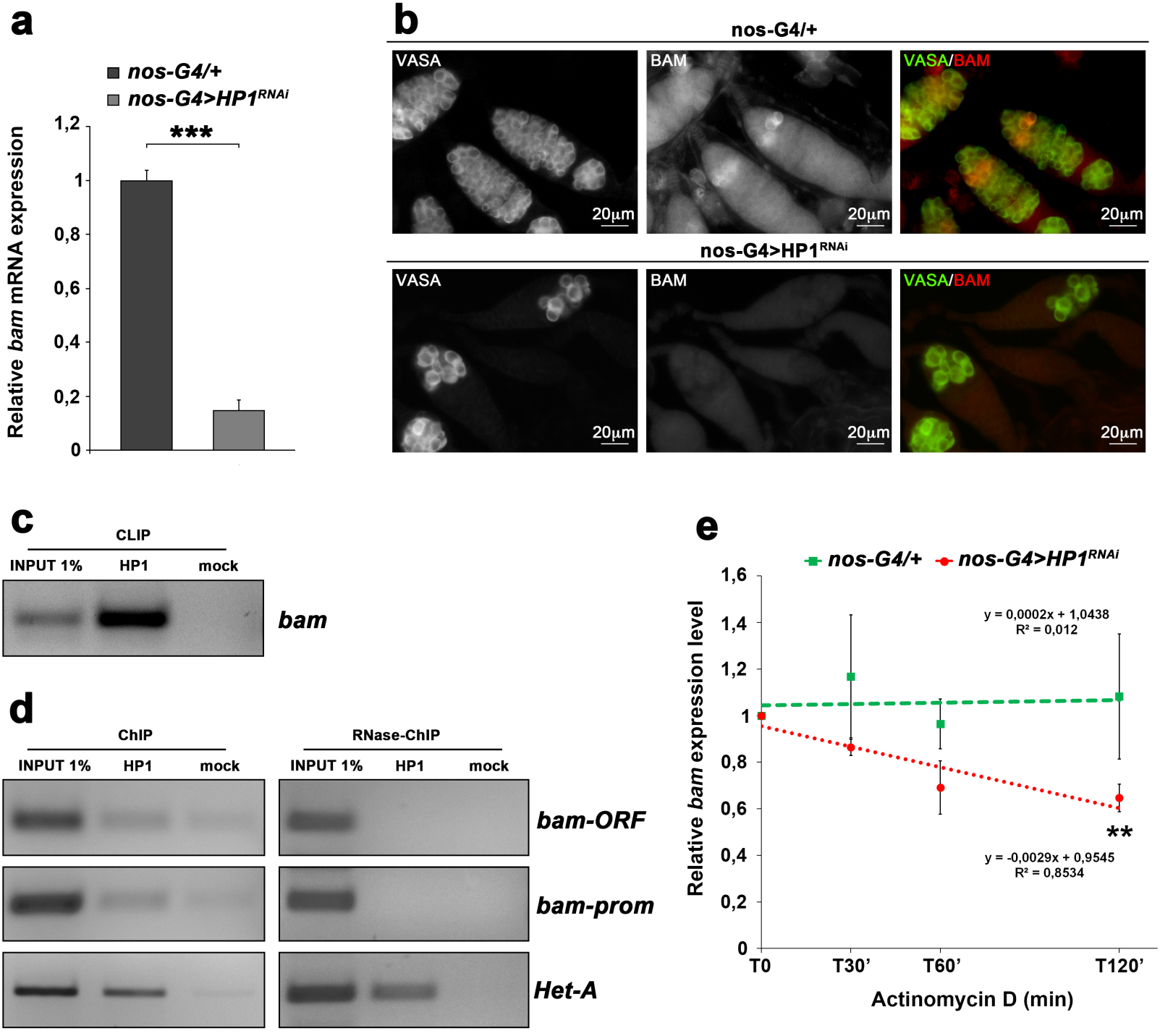

**Figure 5**

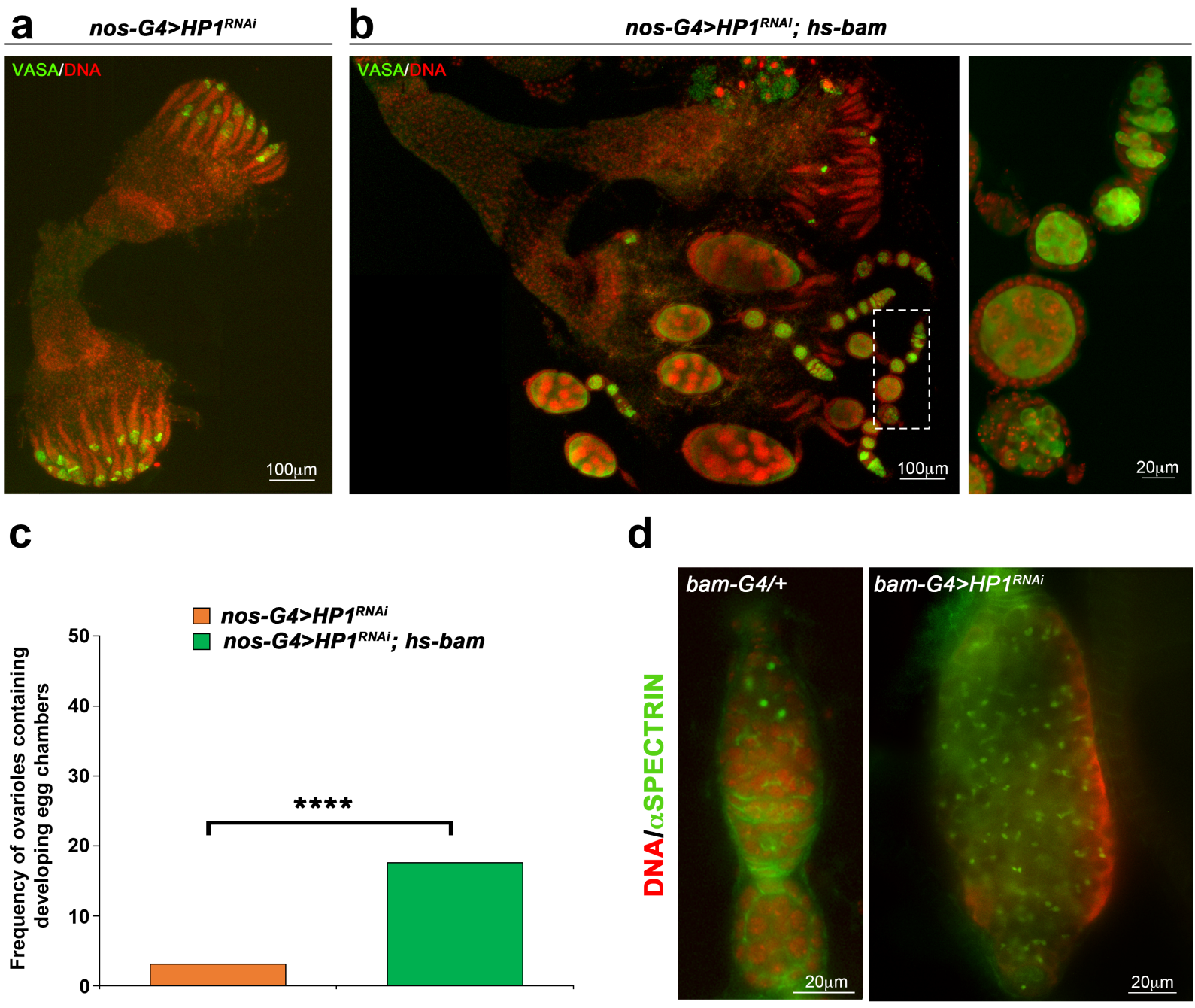

**Figure 6**