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Heterochromatin Protein 1 (HP1) inhibits stem cell proliferation induced by ectopic activation of the Jak/STAT pathway in the *Drosophila* testis

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Abstract

Stem cells can divide asymmetrically with respect to cell fate, producing a copy of themselves (self-renewal), while giving rise to progeny that will differentiate along a specific lineage. Mechanisms that bias the balance towards self-renewal or extend the proliferative capacity of the differentiating progeny can result in tissue overgrowth and, eventually, the formation of tumors. Recent work has explored the role of heterochromatin and heterochromatin-associated proteins in the regulation of stem cell behavior under homeostatic conditions, but less is known about their possible roles in potentiating or suppressing stem cell overproliferation. Here we used ectopic activation of the Jak/STAT pathway in germline and somatic stem cells of the *D. melanogaster* testis as an *in vivo* model to probe the function of Heterochromatin Protein 1 (HP1) in stem cell overproliferation. Forced expression of HP1 in either early germ or somatic cells suppressed the overgrowth of testes in response to ectopic Jak/STAT activation. Interestingly, HP1 expression led to distinct phenotypes, depending on whether it was overexpressed in somatic or germ cells, possibly reflecting different cell-autonomous and non-autonomous effects in each cell type. Our results provide a new framework for further *in vivo* studies aimed at understanding the interactions

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Author Contributions

M.L.C., M.B. and D.L.J. designed the experiments; M.L.C., C.C.P. and M.B. performed the experiments and M.L.C., C.C.P., M.B. and D.L.J. analyzed the data. M.L.C. and D.L.J. wrote the paper.

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Statement of Competing Interest

The authors declare no competing interests in the present work.

between heterochromatin and uncontrolled stem cell proliferation, as well as the complex cross-regulatory interactions between the somatic and germline lineages in the *Drosophila* testis.

Keywords

stem cell; microenvironment; HP1; Jak/STAT; *Drosophila*

Introduction

Much of the research on stem cells has focused on cell-autonomous mechanisms regulating the decision between self-renewal and differentiation. However, previous work has also demonstrated that stem cells must reside within a permissive microenvironment (or niche) to remain functional, which has led to the development of robust *in vivo* models required to study the interaction between stem cells and the niche ¹⁻⁴.

One well-studied animal model for stem cell biology is the fruit fly (*Drosophila melanogaster*) testis ^{1,5-8}. The *Drosophila* testis is a coiled tube, with germline stem cells (GSCs) and cyst stem cells (CySCs) residing at the apical tip (Fig. 1a). During spermatogenesis, GSCs undergo asymmetric self-renewing divisions, giving rise to a new stem cell and a gonialblast. Each gonialblast undergoes four rounds of mitoses with incomplete cytokinesis to form a germline cyst of 16 interconnected spermatogonia, which will later undergo meiosis to form a bundle of 64 sperm. Germline cysts are ensheathed by a pair of somatic cyst cells that originate from asymmetric divisions of CySCs and ensure the proper differentiation of spermatogonia into mature sperm ⁷.

GSCs and CySCs are in contact with a cluster of somatic cells called the “hub” ⁹, which functions as a niche to support both stem cell populations. Hub cells secrete the cytokine Unpaired (Upd), which activates the Janus Kinase and Signal Transducer and Activator of Transcription (Jak/STAT) pathway in adjacent GSCs and CySCs to regulate their behavior ^{10,11}. Activation of STAT in CySCs is required autonomously for their maintenance and proliferation ¹², as well as restraining the activation of the MAPK pathway, which ensures that CySCs do not outcompete GSCs for attachment to the hub ¹³. GSCs, on the other hand, are maintained by signals emanating from the CySCs, and the activation of the STAT pathway in GSCs is required to ensure their attachment to hub cells, keeping them in close proximity to the proliferative and self-renewal signals emanating from adjacent CySCs ^{14,15}. In accordance with this model, ectopic expression of Upd throughout the apical tip of the testis results in a dramatic accumulation of early germ and somatic cells, including GSCs and CySCs, which eventually leads to the formation of large, “tumor-like” masses in the testis (Fig. 1b)^{10,11,14}. A recent report by Lenhart *et al.* also demonstrated that STAT activation in GSCs is critical for the completion of cytokinesis between GSCs and gonialblasts, highlighting a role for the STAT pathway not only in stem cell maintenance but also in ensuring the proper execution of early differentiation stages in differentiating progeny ¹⁶.

Heterochromatin is traditionally associated with global transcriptional repression in pericentric and telomeric regions of chromosomes. Two key heterochromatin regulatory

proteins are the histone H3 lysine 9 methylase (coded by the *Su(var)3-9* gene in *D. melanogaster*) and heterochromatin protein 1a (HP1a, commonly referred to as simply HP1, coded by the *Su(var)25* gene). HP1 binds heterochromatin through its interaction with histone H3 dimethylated on lysine 9 (H3K9me2) and induces further formation of heterochromatin by recruiting H3K9 methyl-transferase to DNA. Numerous other functions have more recently been described for HP1 proteins, ranging from transcriptional regulation of euchromatic loci to telomere capping^{17–20}. Interestingly, previous work unveiled a specific role for histone H3K9 tri-methylation, a defining feature of heterochromatin formation, in repressing the expression of cell lineage-specific genes throughout development, via the formation of so-called facultative heterochromatin²¹. Comparatively, less is known about the potential relevance of heterochromatin regulation in adult stem cell biology.

The *D. melanogaster* testis was recently used to investigate *in vivo* the role of HP1 and *Su(var)3-9* in the regulation of GSC behavior under homeostatic conditions²². Xing and Li found that loss of *Su(var)3-9* or HP1 function caused premature differentiation of GSCs, suggesting that their activity is required for GSC maintenance. Conversely, overexpression of HP1 in early germ cells appeared to delay differentiation, causing a modest expansion of GSCs in testes from young males and a further accumulation of early germ cells in testes of older males (40-days old). Interestingly, Xing and Li also showed that HP1 overexpression could suppress the loss of GSCs caused by a loss of function mutation in Hopscotch (Hop), the fly homologue of the Jak kinase, placing HP1 function parallel to or downstream of the Jak/STAT pathway in GSC regulation.

Jeon *et al.* also investigated the role of HP1 and *Su(var)3-9* function on the homeostatic regulation of adult stem cells but in a different organ (intestine) and in the context of animal aging. Their work showed that there exists a decline in heterochromatin stability as flies age, and that the forced downregulation of HP1 and *Su(var)3-9* function in young enterocytes causes an increased rate of intestinal stem cell proliferation and abnormal differentiation of their progeny²³, both of which phenocopy physiological aging of the intestinal tissue²⁴.

Here, we sought to explore the role of HP1 in the context of germline and somatic stem cell overproliferation. Due to the requirement for HP1 in GSC self-renewal, we hypothesized that HP1 overexpression might enhance the stem cell overproliferation phenotype caused by ectopic expression of Upd. Surprisingly, however, we found that HP1 overexpression in either germline or somatic cells suppressed the overproliferation of stem cells. Interestingly, when HP1 was overexpressed in germ cells, the suppression of stem cell overproliferation correlated with germline differentiation and a non-autonomous alteration of surrounding somatic cells, which showed morphological and molecular characteristics that partially resemble those of hub cells. Thus, our work provides a framework for future *in vivo* studies on the role of heterochromatin regulation during uncontrolled stem cell overproliferation, as well as the complex cross-regulatory interactions between overproliferative adult stem cells and their niche.

Materials and Methods

Fly husbandry and stocks.

Flies were maintained on standard cornmeal-molasses media. The following is an abbreviated list of stocks used and the colleagues who generously shared them (refer to the Supplementary Material for a detailed list of genotypes): UAS-*upd*, UAS-*hop^{umL}* (N. Perrimon), UAS-*HP1^{eCFP}* (W. Li), *nos*-Gal4:VP16 (R. Lehmann), *tj*-Gal4 (DGRC#104055 – NP1624) and *hsHP1* (J. Eissenberg), *bam*-GFP (D.M. McKearin), *Sa*-GFP (X. Chen). Crosses were set up and maintained at the temperatures indicated in Supplementary Material.

Heat shock induction.

To induce the expression of the *hs*-HP1 transgene, crosses were maintained at 18°C (*nos>Upd*) or room temperature (*tj>Upd* and *tj>hop^{umL}*) and heat shocked twice a day at 37°C for 45min each, from golden pupae to the time of dissection.

Antibodies and immunostaining.

Unless indicated otherwise, all testes were dissected from males 5-7 days-after-eclosion (dae). Testes were dissected in ice-cold PBS and immediately fixed in 2% para-formaldehyde PLP buffer at room temperature for 45 min. After fixation, the tissue was rinsed twice followed by three 10min washes in PBS/0.5% Triton X-100 buffer (PBS-T), blocked in 3% BSA/PBS-T for 30min at room temperature and incubated overnight at 4°C with primary antibodies diluted in 3% BSA/PBS-T. The tissue was then rinsed twice and washed three times as before, incubated with secondary antibodies for 2 hours at room temperature and mounted on VectaShield with DAPI (Vector Laboratories). For STAT92E detection, testes were dissected and fixed as usual, rinsed twice in PBS-T buffer, washed twice for 10min in 0.03% DOC/PBS-T buffer, rinsed twice more and washed overnight at 4°C in regular PBS-T buffer before incubation with anti-STAT antiserum (pre-absorbed with fixed embryos). The following is a list of antibodies indicating their working concentrations and source: rabbit anti-VASA (1:3,000, P. Lasko), guinea pig anti-TJ (1:3,000, D. Godt), rabbit anti-Stat92E (1:1,000, D. Montell), rabbit anti-GFP (1:5000, Molecular Probes), mouse phospho-Histone H3 (1:200, Cell Signaling). Alexa fluorophore-conjugated secondary antibodies were always diluted 1:500 (Molecular Probes). We used the following monoclonal antibodies from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology (Iowa City, IA 52242). Clone number, contributor and working dilution are respectively indicated: anti-Fasciclin III (7G10, C. Goodman – 1:50), anti-HP1 (C1A9, L.L. Wallrath – 1:20), anti-Armadillo (N27A1, E. Wieschaus – 1:100), anti-Bam (*bam*, D. McKearin – 1:10) and anti-Spectrin (3A9, D Branton & R Dubreuil - 1:10).

Apoptosis detection with ApoTag®.

In situ apoptosis detection was carried out with the ApoTag® kit, following the manufacturer's instructions (Millipore, USA – cat# S7160). Briefly, testes were dissected in PBS, fixed in 2% PFA/PLP for 30min at RT, rinsed twice in PBS-T, incubated twice in

0.3%DOC/PBS-T for 10min at RT and rinsed twice again in PBS-T. Samples were then incubated twice in Equilibration Buffer (2min each), and then incubated for 1 hour in Reaction Mix at 37C (using a 2.3:1 Reaction Buffer to TdT ratio). The labeling reaction was stopped by rinsing, followed by a 10min incubation in Stop Buffer. After rinsing once and washing twice for 10min in PBS-T, samples were blocked and incubated with primary antibodies for immunostaining as usual.

Measurements of testis size, mitotic rate, and statistical analysis.

We manually outlined testes in DIC images using the polygonal selection tool in Image J²⁵, and then extracted the surface area of each selection (Plugin>Analysis>Measure and Label) as measured in pixels. The actual values obtained depend on default image resolution parameters identical across all images, and we therefore communicate them here as arbitrary units. Where indicated, we normalized the testis surface data by natural logarithmic transformation. For mitotic index calculations, the total number of nuclei (based on DAPI staining) and the total number of pHH3+ cells were manually counted from two high magnification (1000×) images/per testis, from areas with 2 or more pHH3+ cells/field of view. The rate of pHH3+ cells was averaged between images corresponding to the same testis, and the average rate was used in subsequent analyses. The data were then exported, analyzed and graphed using the statistical package Prism5 (GraphPad). Data were analyzed by the recommended statistical tests following checks for their normality and equal variance. The specific tests used and their corresponding significance (p-values) are indicated in figure legends.

Results

HP1 overexpression suppresses the overgrowth of testes caused by ectopic Upd expression.

In *Drosophila*, the bipartite Gal4-UAS system allows for tissue and cell-type specific expression of specific transgenes, often in a temperature or drug-inducible manner²⁶. Previous studies had shown that hyperactivation of the Jak-STAT pathway, via the controlled overexpression of Upd throughout the apical of the tip of the testis, leads to the accumulation of germline and somatic stem cells^{10,11,14}. We used the Gal4/UAS system to drive the expression of a UAS-*Upd* transgene in either early germline or somatic cells, using the *nanos* (*nos*)-Gal4 and *traffic jam* (*tj*)-Gal4 drivers, respectively, and reproduced the previously observed testis overgrowth phenotype (Fig. 1b).

Given a recent report showing that HP1 up-regulation caused a moderate accumulation of GSCs within testes over time²², we sought to determine whether global HP1 overexpression throughout the testis could enhance the stem cell overproliferation phenotype caused by ectopic Upd expression. Surprisingly, however, overexpression of HP1 from a heat-shock inducible *hs-HP1* transgene significantly suppressed the overgrowth phenotype caused by ectopic Upd expression in early germ or somatic cells (Fig. 1c and S1a, respectively).

Since induction of HP1 expression from a heat-shock promoter is expected to up-regulate HP1 expression in both germline and somatic cells, we wanted to determine whether using

the same early germline and somatic drivers to overexpress HP1 in either cell type (*nos*-Gal4 and *tj*-Gal4 - Fig. S1b and S1c), could also suppress the testis overgrowth phenotype induced by ectopic Upd expression. We found that, in both cases, the co-expression of UAS-*upd* and UAS-*HP1* led to significant reduction in testis growth (Fig. 1d). Given the previous finding by Xing and Li that germline overexpression of HP1 in an otherwise normal testis causes a slight but progressive expansion of GSCs²², we were particularly intrigued by the observation that germline HP1 overexpression could suppress the testis overgrowth induced by ectopic Upd expression. To further characterize this unexpected phenotype, we first sought to confirm that the growth-suppressive effect of co-expressing HP1 with Upd in early germ cells would not be attributable to decreased Upd expression caused by titration of Gal4 given due to the presence of two UAS constructs in the genetic background of the flies. Co-expression of an inert UAS-*mitoGFP* construct along with UAS-*Upd* did not cause a reduction in testis size, in contrast to the significant reduction in testis size caused by HP1 co-expression (Fig. S1d).

Phenotypes caused by HP1 overexpression in overproliferative germ cells.

We then sought to determine how HP1 expression in overproliferative germ cells led to suppression. We hypothesized that this might occur by enhanced apoptosis, limiting proliferation and/or forced differentiation. To compare levels of apoptosis in *nos*>Upd testes with or without co-expressed HP1, we used ApoTag (a modified TUNEL assay for detection of apoptotic DNA degradation in tissues). We rarely found pockets of apoptosis in *nos*>Upd testes (an example shown in Fig. S2a), and apoptosis was never observed in testes co-expressing HP1. Therefore, induction of apoptosis does not appear to be a prevalent mechanism for HP1-induced suppression of stem cell overproliferation.

We then explored the possibility that HP1 overexpression might decrease stem cell proliferation in response to ectopic Upd expression. When we immunostained testes with an antibody against the mitotic marker phospho-Histone H3 (pHH3), fewer total pHH3+ cells were observed upon HP1-expression, when compared to controls (Fig. S2b). We noted, however, that HP1-expressing testes often showed extended areas of weak DAPI staining, which is typically associated with more differentiated, non-proliferative cells. To determine if HP1 co-expression could affect the mitotic rate of cells that still maintain proliferative capacity, we compared the ratio of pHH3+ cells among brightly DAPI stained cells in testes overexpressing Upd alone or in combination with HP1. We observed that HP1 co-expression causes a slight but statistically significant decrease in the relative frequency of mitoses among cells that maintain replicative potential (Fig. 2a and S2c).

We also wanted to explore whether ectopic HP1 expression might inhibit excess stem cell proliferation by inducing differentiation. Consistent with this possibility, immunostaining for Vasa and Tj revealed clusters of Vasa+ cells with a size and morphology consistent with that of differentiating germline cysts (e.g. Fig. 2b – white arrow). These clusters of large Vasa+ cells presented a pattern of Spectrin staining consistent with that of normally differentiating spermatogonial cysts. Spectrin is a component of the fusome, a germline-specific organelle that connects spermatogonia within a cyst²⁷. Fusomes are spherical in GSCs and gonialblasts and are referred to as “dot fusomes” or spectroosomes (Fig. S2d – arrowheads).

As spermatogonia undergo mitotic amplification with incomplete cytokinesis, fusomes become progressively branched (Fig. S2d - arrows). Testes expressing Upd alone showed a characteristic pattern of dot fusomes, as shown previously and consistent with overproliferation of stem cells^{10,11}. In testes co-expressing Upd and HP1, small, single Vasa⁺ cells also contained dot fusomes; whereas clusters of larger Vasa⁺ germ cells contained branched fusomes (Fig. S2d). Finally, in testes co-expressing Upd and HP1, we found small and brightly DAPI-stained cells that also express bam-GFP and Sa-GFP (Fig. 2c), two markers of spermatogonial and spermatocyte differentiation, respectively^{28,29}. Taken together, these data are consistent with the idea that HP1 overexpression can induce the differentiation of excess germ cells due to overexpression of Upd. We also noted, however, that germline cysts occasionally presented a slightly abnormal composition, with either an uncharacteristically low number of large cells (e.g. Fig. 2b – open arrowhead) or an unusual large number of small cells (e.g. Fig. 2b – full arrowhead).

HP1 expression in overproliferative germ cells induces alterations in surrounding somatic cells.

In addition to inducing the differentiation of ectopic germ cells, overexpression of HP1 in early germ cells had a non-autonomous effect on surrounding somatic cells. Approximately 45% of testes co-expressing Upd and HP1 under *nos-Gal4* control had very large and tight clusters of somatic cells expressing the hub-specific marker Fasciclin III (n=35; Fig. 3a). Ectopic FasIII⁺-clusters could also be observed in control Upd-overexpressing testes, but they were much rarer (approximately 3%, n=29) and noticeably smaller (Fig. S3a). Given the morphology and FasIII expression, we speculated that these somatic cells might function as “ectopic” hubs. However, these cells do not express *upd*, as determined by the lack of expression of an *upd-lacZ* reporter (Fig. S3b), suggesting that despite other hallmarks, they have not undergone a full conversion to hub cells. Interestingly, ectopic FasIII⁺ clusters were never formed when HP1 was overexpressed with Upd in somatic cells using the *UAS*-Gal4 driver.

It is unclear whether and how the presence of FasIII⁺-clusters might affect the overproliferation of surrounding stem cells, since FasIII⁺-clusters were equally frequent among small and large HP1-overexpressing testes (Fig. S3c) and given that not all small *nos>Upd+HP1* testes had FasIII⁺-clusters within them. However, we noticed that the formation of FasIII⁺-clusters correlated with an intriguing phenotype: germ cells encapsulated by ectopic FasIII⁺-cells did not express detectable levels of Spectrin (Fig. 3b, bottom panels), despite a normal Spectrin staining pattern in germline cysts that are not surrounded by FasIII⁺-clusters within the same testis (Fig. 3b, top panels). This observation suggests that the formation of ectopic FasIII⁺ clusters triggered by HP1 overexpression in ectopic germ cells correlates with an abnormal differentiation of germ cell-containing cysts.

HP1 and the STAT pathway in the context of stem cell overproliferation.

Previous studies have explored the functional relationship between HP1 and the STAT pathway in diverse systems. Xing and colleagues showed that HP1 overexpression can inhibit the growth of melanotic tumors induced by expression of *hop^{tumL}*, a gain of function allele of *hopscotch* (the fly homologue of Jak kinases)^{30,31}. In addition, work by Shi and

colleagues suggested that HP1 can titrate inactive STAT molecules in heterochromatin, thereby causing an overall reduction in STAT signaling within a cell³². On the other hand, Xing and Li recently showed that HP1 overexpression in germ cells could rescue the loss of GSCs in *hop* mutant testes, suggesting that HP1 and the STAT pathway may function synergistically to maintain male GSCs²². Given the complex and/or context-specific relationship between HP1 and the STAT pathway, we wanted to characterize this interaction in the context of testis stem cell overproliferation. To avoid the non-autonomous effects revealed by our previous experiments, we restricted the hyperactivation of the STAT pathway and HP1 overexpression to the same cell type by co-overexpressing HP1 and the constitutively active *hop*^{umL} in either germline or soma.

Restricting the hyperactivation of STAT to germline cells in *nos>hop*^{umL} testes was not sufficient to induce an overproliferation phenotype (Fig. S4a), as shown by previous reports^{6,12} and consistent with the model that the STAT pathway mediates proper attachment of GSCs to the hub rather than directly activating proliferation. However, the expression of *hop*^{umL} in early germ cells led to a noticeable alteration in the normal pattern of STAT expression. In wild type testes, STAT accumulates in the nuclei of all GSCs, and to a lesser extent in the nuclei of CySCs and gonialblasts³³, which results in a stereotypical ring of one or two tiers of STAT⁺ cells around the hub. Expressing *hop*^{umL} with the *nos*-Gal4 driver caused the ectopic accumulation of STAT in a wider range of early germ cells, a phenotype that was not noticeably affected by HP1 co-expression (Fig. 4a). HP1 has also been shown to co-localize with STAT at heterochromatic sites in the genome, which results in STAT accumulation in distinct nuclear foci detectable by immunostaining³². However, we could not observe any noticeable re-distribution of endogenous STAT in GSCs following overexpression of HP1 (Fig. 4b). These results would suggest that HP1 overexpression does not noticeably suppress Jak-STAT activation in early germ cells, in accordance with the findings by Xing and Li²², who showed that HP1 overexpression could rescue a loss of STAT function mutation in the testis.

We also sought to characterize the effect that overexpressing HP1 might have on STAT activation within somatic cells. To our surprise, HP1 overexpression caused a slight yet statistically significant enlargement of the testes, compared to control testes expressing *Hop*^{umL} alone (Fig. 4c). In contrast, the induction of HP1 expression via heat-shocking flies carrying a *hs*-HP1 transgene suppressed the overgrowth of *tj>Hop*^{umL} testes (Fig. 4d), presumably due to induction of HP1 expression in the germline in addition to somatic cells. Collectively, these observations strongly suggest that the functional interaction between HP1 and the STAT pathway may be highly cell type and context-specific.

Discussion

Here we show that overexpressing HP1 in either somatic or germ cells of the *D. melanogaster* testis can suppress the testis overgrowth induced by the ectopic expression of Upd (Fig. 1d). Overexpressing HP1 globally also suppressed the overproliferation of stem cells (Fig. 1c and S1a) although to a lesser extent, which may be due to a lower and/or intermittent induction of HP1 expression from the *hs-HP1* transgene.

In none of our experiments have we ever observed proliferative germline devoid of ectopic somatic cells, which may reflect the strict dependence of germ cells on the soma for maintenance and proliferation^{14,15}. Therefore, it seems plausible that HP1 overexpression in somatic cells may have cell-autonomously suppressed their Upd-induced proliferation and, indirectly, that of surrounding germ cells. Contrarily, whether overexpressing HP1 in early germline had a cell autonomous and/or non-autonomous effect is less clear. HP1 overexpression in germ cells led to a slight but significant decrease in the division rate among proliferative cells, led to the premature expression of germ cell differentiation markers and caused the formation of differentiating cysts. All of these could result from a cell-autonomous effect in germ cells; however, HP1 overexpression in germ cells also induced the formation of very large clusters of FasIII+ “hub-like” somatic cells (Fig. 3a). These “hub-like cells” do not express an *upd* reporter (Fig. S3b) and occasionally undergo mitosis (not shown), suggesting that these somatic cells adopted an altered state between cyst and hub cells.

An alternative and intriguing possibility is that HP1 overexpression in germline could have induced the feminization of surrounding somatic cells. Ma and collaborators showed that the loss of Chinmo function in CySCs and early somatic cells led them to express high levels of FasIII and other markers associated with somatic follicle cells in ovaries³⁴. Thus, overexpressing HP1 along with Upd in germ cells could have maintained surrounding somatic cells in an undifferentiated state due to activation of the Jak/STAT pathway, while promoting their feminization due to some non-autonomous mechanism that phenocopied the loss of Chinmo function in somatic cells. It should be pointed out that the FasIII+ clusters that we observed did not organize into the distinct columnar sheaths surrounding germ cells that are seen upon feminization of male somatic cells, as reported by Ma *et al.*³⁴. However, we cannot rule out that this difference in behaviors may be due to the hyperproliferative environment induced by the overexpression of Upd. In either case, we cannot rule out the intriguing possibility that, perhaps in addition to cell-autonomous effects, HP1 overexpression in germ cells may have affected the surrounding soma non-autonomously in a way that disrupts a delicate cross regulatory communication between both cell types, ultimately leading to a microenvironment that inhibits their overproliferation and/or forces their differentiation. In fact, when differentiating germline cysts were surrounded by clusters of FasIII+ cells, they were strikingly devoid of the characteristic Spectrin staining of branched fusomes (Fig. 3b), which could possibly reflect underlying abnormalities in these germ cells surrounded by dysfunctional soma. Interestingly, Tarayrah *et al.* previously demonstrated that dUTX (a Histone 3 Lysine 27 (H3K27) demethylase), plays both cell autonomous and non-autonomous roles in CySCs, controlling their level of Socs36E expression and Jak/STAT activation, as well as maintaining the molecular identity of hub cells, which supports the idea that chromatin regulation can elicit cross-regulatory interactions among cells of the testis niche³⁵. Furthermore Feng *et al.* showed that the loss of E(Pc) function in early cyst cells led to an accumulation of early germ cells that phenotypically resembled GSCs and gonialblasts but also expressed somatic markers, supporting the idea that chromatin regulation in somatic cells can non-autonomously affect the differentiation and lineage identity of the germline³⁶.

Another remarkable finding was that HP1 overexpression within somatic cells under *UAS*-Gal4 control suppressed the overgrowth induced by ectopic expression of Upd in somatic cells (Fig. 1d) but had the opposite albeit slight effect (i.e. growth promoting) when somatic cells expressed Hop^{tsL} (Fig. 4c). These differences could reflect the fact that both CySCs and GSCs are stimulated when Upd is expressed under *UAS*-Gal4, or that expression of Hop^{tsL} within somatic cells could lead to the activation of additional targets than those triggered by the STAT pathway. For instance, Singh *et al.* found that expression of Hop^{tsL}, but not that of a constitutively active mutant Stat92E, led to the nuclear translocation of Madm and repression of the EGFR ligand vein and integrin expression³⁷. In either case, the effect of overexpressing HP1 within somatic cells on the testis overproliferation phenotype appeared to be highly context dependent.

In summary, our findings reveal an unexpected role for HP1 in controlling cross-regulatory interactions between overproliferative stem cells and their microenvironment. We expect that the present work will provide a starting point to exploit the Upd-induced overproliferation of stem cells in the fly testis as a model to investigate genetic mechanisms regulating interactions between stem cells and their niche within a context of overproliferation and tumorigenesis.

Context-dependence of HP1 function.

Xing & Li showed that HP1 overexpression in an otherwise wildtype background caused a slight accumulation of early germ cells in young testes. By day 40 after eclosion, the testes were full of early germ cells, although they appeared to have a size and morphology seemingly comparable to that of aged-matched controls²². We propose that their observations are more consistent with a slow, linear accumulation of early germ cells that failed to differentiate, rather than the geometric accumulation of actively proliferating GSC-like cells. If so, our observations and the work by Xing & Li collectively support the possibility that HP1 overexpression may have highly context-dependent effects on germline stem cells: while HP1 may prevent GSC differentiation and allow their accumulation in an otherwise normal environment, it may induce germline differentiation in the context of stem cell overproliferation, possibly through a combination of cell autonomous and non-autonomous effects. Consistent with the idea that wild type GSCs and their GSC-like counterparts in an overproliferative testis may function under different regulatory environments, we observed that STAT accumulated noticeably in ectopic GSCs shortly after the onset of overproliferation induced by ectopic Upd expression but remained largely undetectable among ectopic germ cells at later stages of testis overgrowth (Fig. S4b). We rule out that decreased antibody penetration could explain the lack of STAT staining in larger testes because STAT accumulation could still be easily detected in a ring of 1-2 cell rows around the hub. This observation suggests that the mechanism leading to STAT accumulation in normal GSCs³³ is not present (or is effectively neutralized) at more advanced stages of overproliferation. Thus, in spite of the apparent similarities between normal and overproliferative GSCs, there appear to be significant underlying differences in their regulatory states.

Molecular mechanism(s) of HP1 Inhibition of stem cell overproliferation.

To explore the mechanism by which HP1 inhibits the overproliferation of stem cells, we first focused on the STAT pathway itself, based on previous reports showing that HP1 can inhibit the formation of melanotic tumors in *hop^{umL}* mutants³⁰, as well as counteract STAT activation by sequestering unphosphorylated (inactive) STAT in the heterochromatin of normal cells³². However, we could not detect an effect of overexpressing HP1 on the STAT accumulation phenotype caused by *Hop^{umL}* in germline (Fig. 4a) nor could we detect any noticeable re-localization of STAT to heterochromatin foci following HP1 overexpression in otherwise normal GSCs (Fig. 4b). These observations are consistent with observations by Shi and collaborators, who used STAT-reporters to show that, in some cell types, HP1 and H3K9-methylase do not regulate physiological levels of Jak/STAT signaling³⁰. Therefore, while we cannot entirely rule out that overexpressed HP1 may have inhibited STAT activation in response to ectopic Upd within germline cells, our evidence is consistent with the possibility that HP1 may also have inhibited the overproliferation of stem cells through STAT-independent mechanisms. Clinical evidence has suggested that HP1 may function as a tumor suppressor in humans^{38,39}, and the identification of additional mechanisms by which HP1 may suppress the overproliferation of stem cells in flies may point to novel research leads in mammals with applicability to cancer research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used throughout the manuscript

HP1:	Heterochromatin Protein 1
Jak/STAT:	Janus Kinase/Signal Transducer and Activator of Transcription
Upd:	Unpaired
GSC:	Germline stem cell
CySC:	Cyst stem cell
Hop:	Hopscotch
nos:	nanos
tj:	traffic jam
H3K9/27:	Histone 3, Lysine residue 9/27

Socs36E:	Suppressor of Cytokine Signaling 36E
E(Pc):	Enhancer of Polycomb
Madm:	Myb1-adaptor molecule
dUtx:	Drosophila Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome
Chinmo:	Chronologically inappropriate morphogenesis
hs:	heat shock
dae:	days after eclosion
pHH3:	phosphor-Histone H3
bam:	bag of marbles
Sa:	spermatocyte arrest
FasIII:	Fasciclin III
PBS:	Phosphate buffer saline
BSA:	Bovine serum albumin
PLP:	Phosphate lysine periodate
DAPI:	4',6-diamidino-2-phenylindole
DOC:	Deoxy-cholate
GFP:	green fluorescent protein
PFA:	para-formaldehyde
DIC:	Differential Interference contrast
OE:	over-expression

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Manuscript Highlights

- HP1 overexpression suppresses the overgrowth of the *Drosophila* testis caused by ectopic Upd expression.
- HP1 overexpression induces the differentiation of overproliferative germ cells.
- HP1 expression in overproliferative germ cells induces alterations in surrounding somatic cells.

Figure 1. HP1 overexpression suppresses the overgrowth of testes caused by ectopic Upd expression.

(a) Schematic representation of the apical tip of a *D. melanogaster* testis. Germline Stem Cells (GSC – green) and Cyst Stem Cells (CySC – light gray) divide asymmetrically, renewing themselves and giving rise to a differentiating gonialblast and a cyst cell respectively. The hub (red) is a cluster of somatic cells critical for the maintenance of adjacent GSCs and CySCs. Encapsulated by two cyst cells, gonialblasts undergo four rounds of synchronized, amplifying mitoses with incomplete cytokinesis to form 2, 4, 8 and 16-spermatogonia cysts (cysts of up to 4-spermatogonia are shown in this diagram).

(b) DIC images of testes from 5-7 days old control males and males that overexpress Upd in early germ or somatic cells driven by the *nanos* (*nos*) and *traffic jam* (*tj*) Gal4 drivers respectively (see Materials and Methods for detailed genotypes). In the wild type testis, the asterisk denotes the approximate position of the hub. The bracket marks approximately the germline transit amplifying zone, where cysts of proliferating spermatogonia undergo 4 rounds of mitotic divisions to give rise to 16-spermatogonial cysts. The arrow points to mature sperm bundles that fill the rest of the testis. Notice that the *tj-Gal4>UAS-upd* testes are normally smaller than *nos-Gal4>UAS-upd* ones, which likely reflects differences in levels of Upd expressed by the two Gal4 lines. Scale bars = 50µm.

(c) Testes from control flies and flies overexpressing Upd in early germline, either alone (Upd OE) or in combination with HP1 (Upd OE+HP1), which was globally expressed from a heat shock-inducible promoter. The leftmost DIC images show the overall morphology of the testes at lower magnification (yellow insets indicate the area corresponding to the adjacent immunofluorescence images; scale bars = 100µm). DNA staining with DAPI is typically brighter in cycling cells at the apical tip of a wild type testis and throughout areas of stem cell overproliferation. Traffic jam (Tj - red) stains early cyst cells (and hub cells, though less brightly), whereas Vasa (green) stains early germ cells brightly and becomes progressively weaker as spermatogonia differentiate. All flies were subjected to the same heat shocks used to induce hs-HP1 expression (see Materials and Methods for details). Scale bars = 20µm.

(d) Scatter plots of testis size in controls (gray, n=68), or flies overexpressing Upd alone or co-expressing Upd and HP1, as indicated, in early germline (blue, n=135; red, n=135) or early somatic cells (green, n=112; orange, n=140), respectively. A non-parametric ANOVA (Kruskal-Wallis) test indicates that all experimental groups had testes that are significantly larger than controls (****, $p < 10^{-4}$). Pairwise comparisons using a non-parametric Mann-Whitney test indicated that there is a significant reduction in testis size following co-expression of HP1 compared to Upd-only controls (**, $p = 0.0076$, *nos>Upd* vs. *nos>Upd* +HP1; **, $p = 0.0029$, *tj>Upd* vs. *tj>Upd*+HP1).

Figure 2. HP1 expression in overproliferative germ cells leads to decreased proliferation rate and differentiation of germ cells.

(a) HP1 expression in overproliferative germ cells causes a reduction in stem cell proliferation rate. Testes from 5-day old males overexpressing Upd alone (blue; n=22) or in combination with HP1 (red; n=32) driven by *nos*-Gal4 were stained with DAPI and the mitotic marker phospho-Histone H3 (pHH3). Data points represent the rate of pHH3⁺ per total number of small and brightly DAPI-stained cells (lines represent mean $\pm$ SD; and the

* denotes $p=0.0041$ based on a two-tailed Mann-Whitney test). Figure S2d shows examples of the high magnification images used to quantify the data represented in this plot.

(b) HP1 expression in overproliferative germ cells leads to the formation of germline cysts with signs of abnormal differentiation. Testes from 5-day old males overexpressing Upd alone or in combination with HP1 driven by *nos*-Gal4 were stained with DAPI, Tj, Vasa and the hub marker FasIII as indicated. The arrow in the lower panel points to an example of a seemingly normal spermatogonial cyst with a pair of adjacent Tj⁺ somatic cells. In contrast, some cysts had an uncharacteristically small number of large germ cells (open arrowhead) or supernumerary and uncharacteristically small germline cells (full arrowhead). Scale bars = 50µm.

(c) Brightly DAPI-stained germ cells overexpressing HP1 show premature expression of the germline differentiation markers bam-GFP and Sa-GFP. Testes from 5-day old males overexpressing Upd alone (top panels) or in combination with HP1 (bottom panels) were stained with DAPI and GFP, as indicated. Scale bars = 10µm.

Figure 3. Overexpression of HP1 in overproliferative germ cells causes non-autonomous alterations in somatic cells.

(a) HP1 expression in overproliferative germ cells under *nos*-Gal4 control often caused the formation of large clusters of somatic FasIII⁺-cells. Testes from males that overexpress Upd alone or in combination with HP1 were immunostained as indicated (scale bars = 50µm). Yellow insets in the DIC images mark the position of the corresponding immunofluorescence images (scale bars = 100µm).

(b) Two regions of the same testis overexpressing Upd and HP1 in early germ cells were immunostained with Vasa, FasIII and Spectrin as indicated. FasIII and Spectrin were detected using the same fluorescence channel but could be easily distinguished based on staining patterns and intensity. Germline cysts in areas devoid of ectopic FasIII⁺-clusters (top panels) showed a normal pattern of Spectrin staining (cfr. Fig. S2d). In contrast, germ cells encapsulated by ectopic FasIII⁺-clusters within the same testis (bottom panels) lacked any detectable Spectrin expression. Scale bars = 20µm.

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Figure 4. Probing the functional interaction between HP1 and the STAT pathway in germline and somatic cells.

(a) HP1 co-expression did not noticeably alter the pattern of STAT accumulation in several tiers of early germ cells around the hub caused by expressing UAS-*Hop^{tumL}* in early germ cells under *nos*-Gal4 control. Testes from males that express *Hop^{tumL}* alone or in combination with HP1 were immunostained for STAT and FasIII as indicated. Scale bars = 20µm.

- (b)** HP1 overexpression in early germ cells driven by *nos*-Gal4 did not cause a noticeable alteration in the localization of endogenous STAT within GSC nuclei. Testes from males of the indicated genotypes were dissected and stained for STAT and FasIII. Scale bar = 10µm.
- (c)** The testis overgrowth caused by expression of the constitutively active form of the STAT activator *hopscotch* (*hop^{tumL}*) in somatic cells under *tj*-Gal4 control (blue; n=81) was slightly but significantly enhanced by co-expression of HP1 (red; n=58). *. $p = 0.0129$ based on an unpaired, two-tailed Student's t-test.
- (d)** In contrast to the growth promoting effect of restricting HP1 expression to early somatic cells, global HP1 expression from a heat shock *hs*-HP1 transgene caused a significant reduction in testis overgrowth induced by *Hop^{tumL}* expression in somatic cells (*tj* > *hop^{tumL}*, blue, n=31; *tj* > *hop^{tumL}*+HP1, red, n=31).**. $p = 0.0058$, two-tailed Mann-Whitney test.