Title

Canonical and non-canonical functions of STAT in germline stem cell maintenance

Journal

Developmental Dynamics, 252(6)

ISSN

1058-8388

Authors

Xing, Yalan

Larson, Kimberly

Li, Jinghong

et al.

Publication Date

2023-06-01

DOI

10.1002/dvdy.576

Canonical and non-canonical functions of STAT in germline stem cell maintenance

Yalan Xing[1,2,a] , Kimberly Larson[1,2,b], Jinghong Li[1], and Willis X. Li[1,2,*].

[1]Department of Medicine, University of California at San Diego, La Jolla, CA 92093

[2]Department of Biomedical Genetics, University of Rochester Medical Center, Rochester, NY 14642

[a]Current Address: Meta, Bellevue, WA 98005

[b]Current Address: Boehringer Ingelheim, Fremont, CA 94555

[*] Corresponding author: Willis X. Li (wxli@health.ucsd.edu)

Funding
This work was supported, in part, by grants from the National Institutes of Health (R01GM131044 to W.X.L).

Running title: STAT and heterochromatin in stem cell maintenance

Key words: Non-canonical STAT, heterochromatin, germline stem cell (GSC), *Drosophila*

Abstract

Background
Maintenance of the *Drosophila* male germline stem cells (GSCs) requires activation of the Janus Kinase/Signal Transducer and Activators of Transcription (JAK/STAT) pathway by niche signals. The precise role of JAK/STAT signaling in GSC maintenance, however, remains incompletely understood.

Results
Here, we show that, GSC maintenance requires both canonical and non-canonical JAK/STAT signaling, in which unphosphorylated STAT (uSTAT) maintains heterochromatin stability by binding to Heterochromatin Protein 1 (HP1). We found that GSC-specific overexpressing STAT, or even the transcriptionally inactive mutant STAT, increases GSC number and partially rescues the GSC-loss mutant phenotype due to reduced JAK activity. Furthermore, we found that both HP1 and STAT are transcriptional targets of the canonical JAK/STAT pathway in GSCs, and that GSCs exhibit higher heterochromatin content.

Conclusion
These results suggest that persistent JAK/STAT activation by niche signals leads to the accumulation of HP1 and uSTAT in GSCs, which promote heterochromatin formation important for maintaining GSC identity. Thus, the maintenance of *Drosophila* GSCs requires both canonical and noncanonical STAT functions within GSCs for heterochromatin regulation.

Introduction

The male reproductive system in *Drosophila* provides an excellent model for understanding the fundamental mechanisms underlying stem cell regulation and the interaction between stem cells and their niches [1; 2; 3; 4; 5]. *Drosophila* male germline stem cells reside at the apex of the testis, next to a group of post-mitotic somatic cells called hub cells, which comprise a key component of the male GSC niche. Hub cells produce the cytokine-like ligand Unpaired (Upd), which activates the JAK/STAT pathway in the GSCs and somatic cyst stem cells (CySC), instructing their self-renewal. The GSCs are connected to the hub cells via adherens junctions. A GSC divides asymmetrically to produce two daughter cells, one of which retains contact with the hub and maintains stem cell identity, while the other is displaced from the hub and begins to differentiate as a gonialblast. Gonialblasts go through four synchronous mitotic divisions as spermatogonia, resulting in 16 spermatocytes, which enter meiosis and give rise to mature sperms. Early studies have shown that without JAK/STAT signaling, GSCs differentiate but do not self-renew, while ectopic JAK/STAT signaling greatly expands the stem cell population [6; 7].

The JAK/STAT pathway is conserved from invertebrates to mammals and is involved in various developmental processes and human diseases, including almost all types of leukemia and lymphoma [8; 9], mouse embryonic stem cell self-renewal [10], and multiple somatic stem cells maintenance and immune response in *Drosophila* [5; 11; 12; 13; 14; 15]. In *Drosophila*, the JAK/STAT pathway was found to control male germ-line

stem cell (GSC) self-renewal [6; 7]. The JAK/STAT pathway ligand Upd is expressed in the GSC niche, and overexpression of Upd greatly increases GSC number [6; 7]. Flies mutant for hopscotch (*hop*; encoding JAK), lose all early-stage germ line cells in testes and are male sterile, and GSCs that have lost *stat92E* are unable to self-renewal [7].

Despite the early findings that the JAK/STAT signaling pathway is activated in GSCs and is required for GSC maintenance, the precise role of JAK/STAT signaling in GSCs remains controversial and unclear. Later studies have shown that JAK/STAT activation is required not in GSCs, but rather in the adjacent somatic CySCs, which in turn maintains GSCs, and that STAT activation in GSCs functions to merely ensure their adhesion to the hub [14; 16]. Importantly, these authors show that despite losing contact with the niche, STAT-depleted GSCs are retained as stem cells by adjacent CySCs [14; 16]. However, other studies have shown that CySCs are dispensable for GSC maintenance [17], and that JAK/STAT activation in GSCs is required for their maintenance [18; 19] possibly through regulating cytokinesis [20]. Moreover, it has been shown that STAT is required in GSCs for male sex identity through Phf7 [21], for adhesion to the niche through E-Cadherin [16], for GSC survival [22], and for F-actin regulation in GSCs [23]. Transcriptional targets of STAT crucial for stem cell maintenance have been sought and studied, but their precise molecular roles in GSC maintenance remain incompletely understood [4; 21; 24].

We have previously identified a non-canonical JAK/STAT pathway, in which a portion of unphosphorylated STAT (uSTAT) is localized in the nucleus in association with heterochromatin protein 1(HP1), and the heterochromatin-associated uSTAT is essential for maintaining HP1 localization and heterochromatin stability [25; 26]. Furthermore, we and other groups have previously shown that several heterochromatin components, HP1 and the H3K9 methyltransferases Su(var)3-9 and dSETDB1, or other heterochromatin regulators are required for *Drosophila* GSC maintenance [27; 28; 29; 30; 31; 32; 33; 34]. Whether non-canonical JAK/STAT signaling plays a role in regulating heterochromatin formation and maintaining GSCs has not been studied.

To understand whether non-canonical JAK/STAT signaling is required for GSC maintenance, we altered the levels of STAT and JAK/STAT signaling specifically in GSCs by genetic means and assessed the effects on GSCs. We found that GSC-specific changes in STAT levels significantly affect GSC number and that not only wild-type STAT, but the transcriptionally inactive STAT92E^{Y704F} can also rescue, to a certain extent, the GSC loss phenotype associated with a *hop* loss of function allele, *hop*^{25}. We have previously shown that unphosphorylatable mutant STAT can function in the non-canonical JAK/STAT pathway to promote heterochromatin formation [26; 35]. In addition, we found that canonical STAT signaling in GSCs induces STAT and HP1 expression. These results suggest that both canonical and non-canonical JAK/STAT signaling is required for GSC maintenance, possibly by promoting heterochromatin formation in GSCs.

Results

Altering JAK/STAT levels in the germline affects GSC numbers

To investigate the role of JAK/STAT signaling in GSCs for their maintenance, we overexpressed or knocked down Hop or STAT92E in the germline using the germline–specific *nanos-Gal4*, as has been previously done [29]. *Drosophila* male GSCs are Vasa-positive cells surrounding the hub and are marked by dotted fusomes [6; 7], which can be observed in most, but not all, of the GSCs (**Fig. 1A**). We have previously

estimated the number of GSCs using *esg*-GFP (**Fig. 1A**), an enhancer trap line in which GFP is inserted in the gene *escargot* (*esg*), which encodes a transcription factor important for germline stem cell function [36] and is expressed in many types of adult stem cells [37; 38; 39]. As previously shown, *esg*-GFP is expressed in GSCs and gonialblasts, but not in CySCs. In wild-type males carrying one copy of *esg-GFP*, 26.8±2.6 GFP^+ cells on average are found in the testis tip region [29]. Although *esg*-GFP is also expressed is hub cells, they are morphologically distinct from GSCs and can be easily excluded in counting. A previous report suggests that *esg*-GFP is expressed in CySCs [40]. However, we have previously shown that *esg*-GFP is not detectable in CySCs, and GSCs are clearly distinguishable (see Fig. S3 in [29]). The number of GFP+ germ cells in *esg-GFP* male testis correlates well with the known 6 to 12 GSCs plus their immediate progeny (gonialblasts) present in wild-type *Drosophila* testis. Thus, *esg-GFP* can be used as a convenient marker for visualization and quantification of GSCs without immunostaining, which is advantageous to the *esg-lacZ* enhancer trap line previously used to mark GSCs and gonialblasts [41]. We counted both *esg*-GFP+ cells and GSCs, Vasa+ cells with round fusome.

We found that overexpressing Hop or STAT92E in the germ line resulted in a significant increase in the number of GSCs, whereas knocking down *Stat92E* in the germ line caused a dramatic decrease of GSC number (**Fig. 1B-F**). In *Stat92E* knockdown, branched fusomes (the marker of cyst cells) were observed at the GSC location, a defect consistent with the loss of GSCs (**Fig. 1D,** arrow). Moreover, loss of *Stat92E* may lead to premature differentiation of GSCs, as indicated by premature expression of *bag of marbles* (*bam*), a GSC differentiation marker [42; 43] (**Fig. 1G; Table 1**). As shown with a *bam-GFP* reporter [14], *bam* is normally expressed in a band three cell diameters away from the hub [42], which consists of differentiated cysts with 4-16 spermatogonia, but not in GSCs or gonioblasts. We have previously shown that loss of heterochromatin components also leads to premature *bam* expression [29]. These results indicate that STAT level in GSCs might be critical for maintaining proper GSC population. This is consistent with previous studies [6; 7; 20], but in contrast to other studies showing that STAT activation in GSCs is not essential for their identity or maintenance [14; 16].

Unphosphorylated STAT or non-canonical JAK/STAT signaling is involved in GSC maintenance

It has been previously demonstrated that activation of canonical JAK/STAT pathway is required for GSC maintenance [6; 7; 20], presumably due to its transcriptional activation of target genes in GSCs [5; 21; 44]. On the other hand, in a non-canonical mode of JAK/STAT signaling, the transcriptionally inactive uSTAT is required for directly maintaining heterochromatin stability [25; 26]. Moreover, we and others have shown that heterochromatin components including HP1 and Su(var)3-9 are required for GSC maintenance [27; 28; 29; 30; 31; 32; 33]. We thus decided to investigate whether non-canonical JAK/STAT signaling is required for GSC maintenance by regulating heterochromatin.

One way to distinguish canonical from non-canonical JAK/STAT function is to examine the effects of unphosphorylatable mutant STAT (uSTAT). In the canonical pathway, expressing uSTAT has the same effect as loss of STAT, because uSTAT is not able to activate STAT transcriptional targets, and may even act in a dominant negative fashion, interfering with transcription activities of pSTAT [45]. In the non-

canonical mode of JAK/STAT signaling, however, mSTAT positively promotes heterochromatin formation, having the opposite effect to loss of STAT. Thus, the effects of uSTAT can be used to determine whether canonical or non-canonical STAT function is involved.

To study the effect of uSTAT on GSCs, we expressed STAT92E^{Y704F}, which cannot be phosphorylated by JAK and is transcriptionally inactive [46]. After expressing STAT92E^{Y704F} using the germline-specific *nos-Gal4* [47], we found that both esg-GFP+ and GSCs in the adult testes were significantly increased, although these testes exhibited a pointed shape (**Fig. 1E, F**). This observation suggests that non-canonical JAK/STAT signaling, which promotes heterochromatin formation, might be required for maintaining GSCs.

Overexpression of unphosphorylatable STAT partially restores GSC to JAK loss-of-function mutants

To further investigate the role of non-canonical JAK/STAT signaling in GSC formation and/or maintenance, we expressed the unphosphorylatable mutant STAT92E^{Y704F} in *hop^{25}* hemizygous males and tested to what extent uSTAT can rescue or support GSC development/maintenance. It has been shown that *hop^{25}* hemizygous male survivors/escapers are completely sterile, with no GSCs [6; 7] or *esg-GFP^{+}* cells or any small cells with condensed nuclei that are characteristic of GSCs [29], which normally exhibit intense fluorescence after DAPI staining [7; 41]. However, we have previously found that testes of *hop^{25}* hemizygous male escapers contain large Vasa-positive germ cells, albeit with lower Vasa levels (**Fig. 2A**) [29], in contrast to a previous report that *hop^{25}* hemizygous testes had no germ cells [7]. This discrepancy might be due to an accumulation of unknown genetic modifications in the *hop^{25}* stock over the years. We have previously shown that over-expressing HP1 increases the number of GSCs and rescues the GSC loss phenotype and infertility associated with *hop^{25}* hemizygous males [29].

We used the *nanos-Gal4* driver to express wild-type Hop, STAT92E, and STAT92E^{Y704F}, respectively, in *hop^{25}* hemizygous testes, and observed different degrees of rescue of GSC formation. We found small Vasa-positive cells that contained dotted fusome and were brightly stained with DAPI in the testes, resembling GSCs (**Fig. 2A-D; Table 2**). Furthermore, the large Vasa-positive germ cells in *hop^{25}* hemizygous testes expressing Hop, STAT92E, and STAT92E^{Y704F}, respectively, contained branched fusomes, indicative of spermatogonia that have differentiated from GSCs (**Fig. 2A-D**). Consistent with this interpretation, numerous thick bundles of differentiated spermatids were also observed (**Fig. 2A-D**, left panels). These results indicate that expressing the transcriptionally inactive STAT92E^{Y704F} mutant can partially rescue the GSC loss phenotype due to lack of JAK/STAT signaling, suggesting that the requirement of JAK/STAT in GSC formation is mediated, at least in part, by the non-canonical mode of JAK/STAT, in which uSTAT promote heterochromatin formation.

Unphosphorylated STAT promotes heterochromatin formation in male germline

As has been previously shown, uSTAT or unphosphorylatable mutant STAT (e.g., STAT92E^{Y704F} or STAT5A^{Y694F}) can directly associate with HP1 to stabilize heterochromatin [26; 35]. The finding that over-expressing STAT92E^{Y704F} in germline can

increase GSC numbers raises the possibility that the non-canonical mode of JAK/STAT signaling might operate in *Drosophila* male GSC maintenance by regulating heterochromatin. To determine whether noncanonical JAK/STAT signaling indeed promotes heterochromatin formation in *Drosophila* male testes, we expressed $STAT92E^{Y704F}$ from an inducible transgene in marked random cells. By quantification, the mean HP1 immunofluorescence intensity in random GFP+ cells was 68.1±9.2 (n=10), while their next neighbors had a mean of 41.7±4.5 (n=10), which is significantly lower ($p=4.08x10^{-6}$, Student's t-Test). Thus, compared with neighboring cells, cells expressing $STAT92E^{Y704F}$ in either GSCs or spermatogonia resulted in higher levels of HP1 foci (**Fig. 2E**), indicative of higher levels of heterochromatin. Thus, as previously shown for non-canonical JAK/STAT signaling [26], uSTAT promotes heterochromatin formation in *Drosophila* male reproductive tissues as well, including GSCs.

Drosophila male germline stem cells contain high levels of heterochromatin

Cells located in the apex region of the *Drosophila* male testis (including GSCs and the somatic hub cells and CySCs) exhibit characteristic features. First, as has been previously reported [7; 41], GSCs and their immediate daughter cells, gonialblasts (GBs), as well as Hub cells and CySCs contain highly condensed DNA, which is brightly stained with the DNA dye DAPI (**Fig. 3B, D**). Highly condensed DNA is characteristic of heterochromatin, which was originally defined as regions of the nucleus densely stained with DNA dye [48; 49; 50]. Second, consistent with the presence of highly condensed DNA, the nuclei of these cells are much more compact and smaller in size compared with the differentiating spermatogonia (**Fig. 3C**). Third, GSCs exhibit distinct gene expression profiles. For instance, certain common housekeeping genes, such as the ubiquitin promoter, are repressed, whereas a few stem cell or germline-specific genes, such as *Vasa* and *escargot* (*esg*), are expressed at higher levels [24; 36; 51] (**Fig. 3C, D**). Thus, GSCs (and GBs) are small Vasa-positive cells that are brightly stained with DAPI. These features are consistent with the idea that GSCs have unique epigenetic properties and high levels of heterochromatin, which might play a role in the repression of differentiation genes and in the maintenance of the GSC cell fate.

To confirm that GSCs indeed contain high levels of heterochromatin, we stained testes for the heterochromatin marker histone H3 methylated at Lys9 (H3K9me3). At high magnification, GSCs exhibited many DAPI-dense foci in the nuclei (**Fig. 3D**), and these foci coincided with higher levels of H3K9me3 (**Fig. 3E**), consistent with them being heterochromatin. In contrast, the differentiated gonioblasts and spermatogonia (located farther away from the hub), exhibited a reduced number of DAPI dense foci and lower levels of H3K9me3 signal (**Fig. 3D, E**).

Furthermore, we examined the testes with transmission electron microscopy (TEM), which has been used to define heterochromatin as electron-dense regions in the nucleus. Indeed, we found that GSCs exhibited more prominent electron-dense regions, i.e., heterochromatin, when compared with the differentiated spermatogonia (SG) (**Fig. 3F-H**), such that the former had a significantly higher heterochromatin to nucleus ratio (**Fig. 3I**). These observations support the idea that GSCs have higher heterochromatin content than the differentiated SGs.

HP1 and STAT92E are among transcriptional targets of canonical JAK/STAT signaling in the male germline

We have previously shown that, in somatic tissues, JAK overactivation leads to reduced heterochromatin levels due to phosphorylating STAT and consequently reducing uSTAT levels [26; 52]. In GSCs, however, we have found that JAK activation increases GSC number (**Fig. 1**), consistent with other group's previous findings [7; 41]. These observations seem contradictory to the idea that heterochromatin is required for GSC maintenance. One possible explanation for this paradox is that JAK activation in GSCs, in contrast to somatic tissues, leads to increased heterochromatin. This could be accomplished if JAK activation in GSCs induces production of HP1 and STAT, both of which promote heterochromatin formation. STAT activation indeed induces its own expression by autoregulation [12; 25; 53; 54]. Previous studies have shown that STAT is indeed highly enriched in GSCs [34; 39; 55].

To test the possibility that canonical JAK/STAT signaling in GSC induces HP1 and STAT92E expression, we examined transcript levels of *HP1* and *Stat92E* in testes with different levels of JAK/STAT signaling. Indeed, we detected significantly higher transcription levels of both HP1 and Stat92E in testes overexpressing JAK, which leads to activation of JAK/STAT signaling (**Fig. 4A**). Conversely, when STAT92E was knockdown in the germline by over-expressing a *Stat92E RNAi* transgene using the *nanos-Gal4* driver, HP1 mRNA level was significantly reduced (**Fig. 4A**). Interestingly, although $STAT92E^{Y704F}$ was able to partially rescue GSCs (see **Fig. 1E**), it was unable to induce expression of two of the canonical STAT targets, Ptp and HP1 (**Fig. 4B**), suggesting that $STAT92E^{Y704F}$ lacks transcriptional activity. The results were confirmed with traditional qPCR (**Fig. 4C**). Together, these results indicate that both HP1 and Stat92E are transcriptional targets of canonical JAK/STAT signaling in *Drosophila* male germline.

To confirm that expressing $STAT92E^{Y704F}$ led to an increase in heterochromatin, especially on differentiation genes, we carried out chromatin immunoprecipitation (ChIP) on testis tips expressing the indicated transgenes and using anti-H3K9me3 vs control antibodies. We found that expressing HP1 or $STAT92E^{Y704F}$ greatly increased H3K9me3 abundance on *bam* promoter, whereas their knockdown by RNAi decreased H3K9me3 levels in *bam* promoter (**Fig. 4D**). These results are consistent with a potential role of heterochromatin in repressing the expression of the differentiation gene *bam* in GSCs.

Discussion

We have investigated the role of noncanonical functions of STAT in *Drosophila* male GSC maintenance using genetic and immunobiological means. Our results suggest that both canonical and noncanonical JAK/STAT pathways may play important roles in maintaining GSC numbers of adult male flies. We speculate that regulated heterochromatin dynamics might be an important factor contributing to GSC maintenance by epigenetic mechanisms.

Although studies have shown that JAK/STAT activation is required only in the adjacent somatic CySCs and not in GSCs for maintaining their identity [14; 16], other studies have shown that CySCs are dispensable for GSC maintenance [17], and that JAK/STAT activation in GSCs is required for their maintenance by regulating various

aspects of GSC biology, including asymmetric cell division [19], cytokinesis [20], male sex identity through Phf7 [21], survival [22], and F-actin regulation [23]. We have shown here that STAT functions in regulating heterochromatin in GSCs for their maintenance. We suggest that STAT in GSCs regulates multiple processes including epigenetic status, sex identity, asymmetric division, and survival, which all contribute to maintaining the identity of GSCs.

Previous studies have suggested that epigenetic regulation, including heterochromatin dynamics, is important for stem cell maintenance or self-renewal. In mammalian systems, constitutive DNA methylation has been shown to be essential for hematopoietic stem cell (HSC) self-renewal [56]; chromatin modification by Polycomb group proteins (PcGs) is required for maintaining embryonic and adult stem cells via gene repression and delayed differentiation [34; 57]. Consistent with the idea that heterochromatin dynamics is important for stem cell maintenance, studies have shown that when mammalian embryonic stem cells are induced to differentiate in vitro, the pattern of H3K9me3 and HP1 localization go through dynamic changes at each step of differentiation [58]. In planarians, an HP1 protein is induced upon injury and functions specifically to promote the regenerative proliferation of adult stem cells [59]. In *Drosophila*, we and other groups have previously shown that heterochromatin components such as HP1 and Su(var)3-9 are important for maintaining GSCs [27; 28; 29; 30; 31; 60; 61]. However, the potential role for heterochromatin formation in suppressing developmental genes, especially in the context of stem cell maintenance, has not been investigated.

Stem cells are capable of long-term maintenance of an undifferentiated state yet poised for differentiation, and this is accomplished through unique epigenetic modifications and gene expression profiles [2; 3; 62]. Epigenetic repression of developmental or differentiation genes is a salient feature of stem cells [63; 64]. H3K9me3-dependent heterochromatin thus could serve as a barrier to cell fate changes, preventing differentiation of stem cells. Indeed, the stem cell marker Esg and the differentiation marker of Bam seem to be mutually exclusive in expression in the testis tip. Loss of STAT by RNAi results in a decrease in the number of Esg-GFP+ cells, with a concomitant increase of or ectopic Bam-GFP+ cells near or next to the hub (**Fig. 1G**, arrowheads), suggesting premature differentiation of GSCs.

Multiple reasons might contribute to the GSC loss due to decreased heterochromatin levels. First, other than the well-known function associated with gene silencing, key heterochromatin components are involved in general cell survival functions, such as the maintenance of genomic integrity [65; 66; 67; 68]. Thus, the reduction of GSC numbers when HP1 is eliminated may be attributable to cell death caused by genome instability. A second possible explanation for the GSC population decrease could be, GSCs with low heterochromatin levels encounter difficulties going through normal cell-cycle progression, thus are unable to proliferate properly.

It has been shown that activation of canonical JAK/STAT signaling is essential for maintaining GSCs [6; 7; 14; 16]. However, we have also shown that JAK activation in somatic cells reduces heterochromatin due to the noncanonical mode of STAT function [26; 52]. This finding appears paradoxical to the observation that GSCs contain higher levels of heterochromatin, which we suggest is important for their maintenance. We suggest the following to explain the differences between somatic cells and GSCs regarding JAK/STAT activation and heterochromatin changes. In somatic tissues, JAK/STAT activation (e.g., in response to immune challenges) is often transient, which causes a spike in pSTAT levels and concomitantly a drop in uSTAT levels, leading to

heterochromatin disruption, as proposed in the non-canonical STAT model. However, when the ligand Upd is present for a long duration, as in the GSC niche, cells eventually accumulate and sustain higher levels of uSTAT due to canonical JAK/STAT signaling, in which STAT itself is a transcription target. This may explain that GSCs can have higher heterochromatin levels even with all other parameters of JAK/STAT signaling unchanged.

Alternatively, there might be different expression levels of JAK/STAT signaling regulatory components in GSCs vs somatic cells, resulting in different responses to ligand stimulation. For instance, it has been shown that the negative regulator Suppressor of Cytokine Signaling 36E (Socs36E) is required only in CySCs but not in GSCs, whereas only GSCs (but not CySCs) accumulate high levels STAT [55]. Differential expression of STAT inhibitors/target genes could contribute the observed accumulation of uSTAT in GSCs. Further research will clarify the differences between somatic and stem cells regarding JAK/STAT activation and heterochromatin content.

In addition to STAT and other regulators of this pathway, we have found that HP1 is also a transcriptional target of canonical JAK/STAT signaling in GSCs. Our results are consistent with the hypothesis that GSCs require both canonical and noncanonical JAK/STAT signaling, with both uSTAT and HP1 being among transcriptional targets of the canonical pathway. This may explain why $STAT92E^{Y704F}$ is less able than $STAT92E^{WT}$ in rescuing hop^{25} mutants, in which HP1 might be below a threshold level due to reduced canonical JAK/STAT signaling. The reduced, but not completely absent, JAK activity in hop^{25} mutants might be compensated for by $STAT^{WT}$. HP1 is central for heterochromatin formation, which might be an important epigenetic mechanism in regulating GSCs, consistent with a higher heterochromatin content and DNA compaction of GSCs. We have previously shown that increasing HP1 can rescue the GSC loss phenotype of hop^{25} mutants [29], implying the importance of heterochromatin in GSCs. The precise mechanisms of heterochromatin regulation in GSCs are currently under investigation.

Materials and Methods

Fly stocks and Genetics. The following fly stocks were from the Bloomington *Drosophila* Stock Center (Bloomington, IN). *hop*^{25}, *Su(var)205*^{05}, *Su(var)205*^{04}, *Su(var)3-9*^{2}, *nanos-Gal4*, and *hsp70-flp; Act5C>y*$^{+}$*>Gal4 UAS-GFP/CyO, and FRT82B, ubq-GFP* . RNAi lines including *UAS-HP1 RNAi,* and *UAS-Su(var)3-9 RNAi* were from Vienna *Drosophila* RNAi Center (VDRC; Vienna,Austria). Fly stocks of *esg-GFP* (YB0232; L. Cooley), *bam-GFP* (S. DiNardo), *UAS-Stat92E* (M. Zeidler; S. Hou), *UAS-Stat92E*^{Y704F} (M. Zeidler), and *UAS-Hop* (N. Perrimon) were generous gifts. All crosses were carried out at 25^{o}C on standard cornmeal/agar medium unless otherwise specified.

To clonally express a transgene randomly using the "Flp-out" method [69], *hsp70-flp; Actin>y*$^{+}$*>Gal4 UAS-GFP* flies were crossed to flies carrying *UAS-transgene*, and the F1 progeny were heat-shocked for 1 h at 37^{o}C and examined at indicated times after clone induction.

Quantification of Esg-GFP+ cells and GSCs. *Nos-Gal4; esg-GFP* virgin females were crossed to males carrying indicated UAS-transgenes. F1 male testes were dissected, immunostained (with anti-Vasa and anti-FasIII, anti-α-spectrin), and were imaged as a serial Z-sections using a Leica confocal microscope. Esg-GFP+ cells (including GSCs, GBs, and hub cells) in different optical sections were counted. Hub cells were recognizable by morphology and were excluded. GSCs were recognized as Vasa+ cells with round fusome,

Immunofluorescence and Western blotting. The following primary antibodies were used. Mouse monoclonal anti-HP1 (C1A9; 1:50), anti-FasIII (7G10; 1:200; for hub cells), anti-α-spectrin (3A9; 1:10; for fusome) were from Developmental Hybridoma Bank (University of Iowa). Rabbit antibodies against histone H3 (1:1000; Cat.No. 05-952), H3K4me3 (1:500; Cat.No.07-030), and H3K9me3 (1:250; Cat.No.07-422) were from Upstate Biotechnology/Fisher. These antibodies and rabbit anti-Vasa (1:1000; generous gifts from Ruth Lehmann) were used as primary antibodies and fluorescent (Molecular Probes) or HRP conjugated secondary antibodies were used in whole-mount immunostaining or Western blotting, respectively. Tissues were fixed in 4% paraformaldehyde and 0.3% Triton-X. Stained tissues were photographed with a Leica confocal microscope or a Zeiss epifluorescence microscope. Images were cropped and minimally processed with Adobe Photoshop.

Quantitative Real-Time PCR. Testes were dissected from individual 2-day old males of appropriate genotypes carrying esg-GFP under a stereo microscope and stored in ice-cold Schneider medium supplemented with 10% FBS. Dissected testes were severed into tip and body regions using dissecting forceps and a scalpel. The tip region was approx. 0.2 mm in length, twice the width of the testis tubule. 200 testis tips were used for total RNA isolation. Total RNA was isolated from sorted cells using trizol (Invitrogen) according to the manufacturer's instructions. The first strand complementary DNA (cDNA) was generated from 1 pair of testis-equivalent of purified total RNA using Superscript III reverse transcriptase (Invitrogen) and oligo(dT)12-18. The cDNA was used as template for qPCR analysis using SYBR green based detection on a BioRad iCycler. Reactions were carried out in triplicate, and melting curves were examined to ensure single products. Results were quantified using the "delta-delta Ct" method to normalize to rp49 transcript levels and to control genotypes. Data shown are averages and standard deviations from at least three independent experiments. The following primer pairs were used.

rp49: TCCTACCAGCTTCAAGATGAC, CACGTTGTGCACCAGGAACT
Su(var)205: GCAAGCAAGCGAAAGTCCGAAGAA,
AGGCGCTCTTCGTAGAAGTGGATT
Ptp61F: TAACCGATCTCGAAACGCAGCAGA,
ATTGAGCGAAAGTATACCGCCGGA
Stat92E: TGCCCGATCGGTCCTTTACATTCT,
TTGAGTAGAACTCACCGAAGGCGA

Chromatin immunoprecipitation (ChIP). Testes were dissected in batches of 25 from 3 day-old male flies in ice-cold S2 culture medium (Invitrogen) containing protease inhibitors. Testes were fixed in PBS containing 1% formaldehyde at 25°C, washed three times using ice cold PBS containing protease inhibitors and pelleted after each wash by centrifugation for 5 minutes at 4°C. Samples were homogenized in ChIP Lysis buffer using a pellet pestle in Eppendorf tubes. Soluble chromatin was prepared using the protocol of the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology). Sample corresponding to 100 pairs of testes was used for anti-H3K9me3 ChIP, while sample corresponding to 50 pairs of testes each was used for anti-H3K4me3 and anti-H3 ChIP respectively. Samples were pre-cleared using Protein A-conjugated magnetic beads (Dynal) for thirty minutes at room temperature, followed by incubation with antibody coated Protein A-conjugated magnetic beads (Dynal) for 2 hours at room temperature. Immune complexes were recovered by magnetic selection, washed and eluted using the protocol of the Chromatin Immunoprecipitation Assay Kit. Target DNA abundance in ChIP eluates was assayed by quantitative PCR. The primers used for amplifying the *bam* promoter sequence are as the following.
Forward: CAATGAACCAAAGGACCGCAGCAA
Reverse: ATGGGACTCGCGATGAACCACAAA

Transmission Electron Microscopy (TEM). TEM was carried out by the University of Rochester EM Core Facilities with standard procedures. Briefly, testis samples were fixed with 2.5 % glutaraldehyde in PBS for 1 h at room temperature. A secondary fixation was carried out using osmium tetroxide (OsO_4), and the sample was dehydrated and embedded in resin. The sample was cut into 50 nm sections with a microtone and was stained with uranium and scanned with a transmission electron microscope.

Acknowledgments

We thank Drs. S. DiNardo, Y. Yamashita, L. Cooley, R. Lehmann, T. Xie, D. Bohmann, M. Zeidler, S. Hou, and N. Perrimon for various *Drosophila* strains and reagents; the Developmental Hybridoma Bank (Iowa), the Vienna *Drosophila* RNAi Center, and the Bloomington *Drosophila* Stock Center for antibodies and fly stocks.

Competing interests
The authors declare no competing interests.

Table 1. STAT knock down in germ line causes premature *bam* expression

Transgene expressed	Testes with premature *bam* expression	Number of testes scored	p-value
UAS-GFP RNAi	0	15	–
UAS-STAT RNAi	9	13	0.0052

Testes from *nos-Gal4;bam-GFP* with the indicated UAS-GFP RNAi (control) or UAS-STAT RNAi were examined for the presence of premature bam-GFP expression in GSCs or gonialblasts (as shown in **Fig. 1G**). P-values are from Fisher's exact test comparing each genotype with UAS-GFP (control).

Table 2. Rescue of GSC in *hop^{25}* male testis

Transgene expressed	Testes with GSCs	Number of testes scored	p-value
UAS-GFP	0	26	–
UAS-hop	15	16	2.75e-05
UAS-STAT	22	27	1.08e-5
UAS-STAT^{Y704F}	10	23	0.0016

Testes from *hop^{25}/Y; nos-Gal4* with the indicated UAS transgenes were scored for the presence of GSCs (as shown in **Fig. 2**). P-values are from Fisher's exact test comparing each genotype with UAS-GFP (control).

Figure legends

Fig. 1. Effects of altering levels of JAK/STAT Signaling on *Drosophila* GSC number

(A-E) Testes from 3-day old males of indicated genotypes were stained anti-Vasa, mAb3A9 and anti-FasIII, as indicated, and imaged with a confocal microscope. Representative confocal images of testis apex are shown for esg-GFP+ cells (green), Vasa+ germline cells (magenta), fusome and hub cells (both red), and merged images. UAS-transgenes, as indicated to the right, were expressed using the *nos-Gal4* driver. Scale bar = 20μm. The control images **(A)** are the same as previously reported in Figure 5A in Xing and Li (2015)[29] and are reused with permission, because the same control was used in these experiments.

Note that compared with control **(A)**, expressing Hop **(B)**, STAT **(C)**, and STAT^{Y704F} **(E)** in the germline caused increased GSCs, as assessed by Vasa-positive esg-GFP expressing cells, and that expressing STAT RNAi caused a dramatic reduction in the number of esg-GFP expressing cells, reducing esg-GFP expression in GSCs, but not in hub cells (asterisk) **(D)**. Also note that the Vasa+ cells next to the hub exhibited branched fusome (arrow in **D**) and increased nuclear size.
(F) Quantification of esg-GFP+ cells (upper) and traditional GSCs (lower) of indicated genotypes using a confocal microscope. n: number of testes analyzed; p: significance compared to control by Mann–Whitney *U* test.

(G) Testes from 3-day old males of the indicated genotypes were examined for bam-GFP expression. Asterisk indicates hub position. Dashed lines indicate normal *bam-GFP* expression. Arrow heads point to ectopic (premature) *bam-GFP* expression in GSC and gonialblast. The control images (top) are the same as previously reported in Figure 3A in Xing and Li (2015)[29] and are reused with permission, because the same control was used in these experiments.

Fig. 2. Rescue of GSC loss in JAK mutants by expressing JAK/STAT transgenes

Testes from 3-day old male survivors of indicated genotypes were stained with DAPI, anti-Vasa, mAb3A9 and anti-FasIII, as indicated. Scale bar = 20μm.

(A) A *hop^{25}/Y; nos-Gal4* testis showing no sperm bundles, with all Vasa-positive germ cells exhibiting large nuclei (arrow) and lacking intense DAPI signals. These control images are the same as previously reported in Figure 4A in Xing and Li (2015)[29] and are reused with permission, because the same control was used in these experiments.

(B–C) Expressing *hop* **(B)** or *Stat92E* **(C)** in germline cells lead to nearly complete rescue of the morphology of *hop^{25}/Y* testes, restoring the hub (circled by dashed line), which is surrounded by Vasa-positive germ cells with small DAPI-dense nuclei that resemble GSCs (arrowhead). The arrow points to a spermatogonia with large nucleus.

(D) Expressing *Stat92E^{Y704F}* in germline cells partially rescued the morphology of *hop^{25}/Y* testes. Thick sperm bundles were seen (left). A fraction of Vasa-positive germ cells contain small DAPI-dense nuclei, resembling GSCs (arrow), were observed. Branched fusome (yellow arrow) that associates with differentiated spermatocytes were also seen.

(E) GSC (top), spermatogonia (middle), and ejaculatory duct (bottom; somatic tissue) expressing STAT92E^{Y704F} in random clones (GFP+; green) by "flip-out" *Actin-Gal4* were stained with anti-HP1 (red; marker for heterochromatin), or Vasa (magenta). Note that HP1 levels are higher in GFP+ cells (white dashed line; expressing STAT92E^{Y704F}) than those in flanking control cells. Also note that the GFP+ cells in the germline (top, Vasa+), but not in somatic tissues (bottom), have smaller nuclear sizes than flanking cells.

Fig. 3. Unique chromatin content and gene expression of *Drosophila* GSCs

(A) Schematic representation of stem cell differentiation in the *Drosophila* testis. Hub cells (Red) express the ligand Upd, which activates STAT in germline stem cells (GSCs; green) and somatic cyst stem cells (CySCs; blue) that are in contact with the hub. GSCs and CySCs differentiate to give rise to gonialblasts (GBs; light green) and cyst cells (CCs; light blue), which are somatic support cells. JAK/STAT activation promotes GSC and CySC self-renewal by unknown mechanisms.

(B) A wild-type *Drosophila* male testis stained with DAPI is shown in an epifluorescence (left) and a phase-contrast (right) image. The somatic hub cells are located at the testis tip (marked with an asterisk). GSCs, CySCs, and GBs are located in the apex, and all cells in the tip region contain highly condensed DNA that is brightly stained by DAPI.

(C) A wild-type *Drosophila* male testis expressing a ubiquitin-GFP transgene (left; GFP signals green) was stained with anti-Vasa (magenta in right panels). Note that GSCs express lower levels of ubiquitin and higher levels of Vasa, and that GSCs have a smaller nuclear diameter compared with differentiated spermatogonia. The two right panels are higher magnifications of the boxed cells labeled 1 and 2 to the left, representing a GSC and spermatogonia, respectively.

(D) Merged stack of confocal images of the apex region of a wild-type testis stained with DAPI (left; white) and anti H3K9me3 (middle; green). Dashed white circle indicates hub cells, and three of the adjacent GSCs are circled with dashed yellow lines. Note that there are prominent DAPI-dense foci in the GSCs that colocalized with high H3K9me3 signal. Also note that H3K9me3 signal decreases in intensity in cells more distant from the hub. Quantifications of H3K9me3 in GSCs vs GBs as mean pixel are shown (right). Error bars are standard deviations and p-values are for Student's t-test.

(E) A transmission electron microscopic image showing the apex of a wild-type testis. Hub cells are colored artificially with green. Yellow box 1 contains the hub and surrounding GSCs. A region of differentiated GBs and SGs is indicated by yellow box 2. Heterochromatin regions (including the nucleolus) are seen as electron dense foci in the nucleus (white arrows). High magnifications of the box 1 and 2 region are shown. Cell types are labeled. Ratio of the area of electron dense region to the nuclear area for GSC vs SG are shown. Quantifications of H3K9me3 in GSCs vs GBs as mean pixel are shown (right). Error bars are standard deviations and p-values are for Student's t-test.

Fig. 4. HP1 and Stat92E are canonical JAK/STAT pathway targets in testis tip
(**A, B**) Male flies expressing the indicated (UAS) transgenes driven by nos-Gal4 (GFP) were dissected and the testis tip regions were subjected to RT-qPCR for *Stat92E*, *HP1*, and *Ptp61F* mRNA levels. Results were normalized to rp49 mRNA. Error bars and stand deviations are shown. *,** indicates $p<0.05$, $p<0.01$ (Mann-Whitney test). Note that (**A**) JAK (Hop) expression increased, STAT RNAi expression decreased Stat92E and HP1 mRNA levels, and that (**B**) $Stat92E^{Y704F}$ expression did not significantly alter Ptp and HP1 mRNA levels. (**C**) semi-quantitative RT-PCR on agarose gel and stained with ethidium bromide (left), and quantification of gel band intensity (right). (**D**) ChIP using testis tips expressing the indicated transgenes and using anti-H3K9me3 vs control antibodies. H3K9me3 abundance on *bam* promoter is shown as relative fold-increase compared with input (no ChIP), measured by quantitative RT-PCR.

References

1 LIN, H. The stem-cell niche theory: lessons from flies. **Nat Rev Genet,** v. 3, n. 12, p. 931-40, Dec 2002. ISSN 1471-0056 (Print). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12459723 >.

2 FULLER, M. T.; SPRADLING, A. C. Male and female Drosophila germline stem cells: two versions of immortality. **Science,** v. 316, n. 5823, p. 402-4, Apr 20 2007. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17446390 >.

3 MORRISON, S. J.; SPRADLING, A. C. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. **Cell,** v. 132, n. 4, p. 598-611, Feb 22 2008. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18295578 >.

4 DE CUEVAS, M.; MATUNIS, E. L. The stem cell niche: lessons from the Drosophila testis. **Development,** v. 138, n. 14, p. 2861-9, Jul 2011. ISSN 1477-9129 (Electronic)
0950-1991 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/21693509 >.

5 BAUSEK, N. JAK-STAT signaling in stem cells and their niches in Drosophila. **JAKSTAT,** v. 2, n. 3, p. e25686, Jul 1 2013. ISSN 2162-3988 (Print)
2162-3988 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24069566 >.

6 KIGER, A. A. et al. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. **Science,** v. 294, n. 5551, p. 2542-5., 2001. Disponível em: < http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=11752574 >.

7 TULINA, N.; MATUNIS, E. Control of stem cell self-renewal in Drosophila spermatogenesis by JAK- STAT signaling. **Science,** v. 294, n. 5551, p. 2546-9., 2001. Disponível em: < http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=11752575 >.

8 BROMBERG, J. Stat proteins and oncogenesis. **J Clin Invest,** v. 109, n. 9, p. 1139-42, May 2002. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11994401 >.

9 YU, H.; JOVE, R. The STATs of cancer--new molecular targets come of age. **Nat Rev Cancer,** v. 4, n. 2, p. 97-105, Feb 2004. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14964307 >.

10 NIWA, H. et al. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. **Genes Dev,** v. 12, n. 13, p. 2048-60, Jul 1998. ISSN 0890-9369. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/9649508 >.

11 AGAISSE, H.; PERRIMON, N. The roles of JAK/STAT signaling in Drosophila immune responses. **Immunol Rev,** v. 198, p. 72-82, Apr 2004. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15199955 >.

12 ARBOUZOVA, N. I.; ZEIDLER, M. P. JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions. **Development,** v. 133, n. 14, p. 2605-16, Jul 2006. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16794031 >.

13 JIANG, H. et al. Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. **Cell,** v. 137, n. 7, p. 1343-55, Jun 26 2009. ISSN 1097-4172 (Electronic). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19563763 >.

14 LEATHERMAN, J. L.; DINARDO, S. Zfh-1 controls somatic stem cell self-renewal in the Drosophila testis and nonautonomously influences germline stem cell self-renewal. **Cell Stem Cell,** v. 3, n. 1, p. 44-54, Jul 3 2008. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18593558 >.

15 HERRERA, S. C.; BACH, E. A. JAK/STAT signaling in stem cells and regeneration: from. **Development,** v. 146, n. 2, 01 29 2019. ISSN 1477-9129. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/30696713 >.

16 LEATHERMAN, J. L.; DINARDO, S. Germline self-renewal requires cyst stem cells and stat regulates niche adhesion in Drosophila testes. **Nat Cell Biol,** v. 12, n. 8, p. 806-11, Aug 2010. ISSN 1476-4679 (Electronic) 1465-7392 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20622868 >.

17 LIM, J. G.; FULLER, M. T. Somatic cell lineage is required for differentiation and not maintenance of germline stem cells in Drosophila testes. **Proc Natl Acad Sci U S A,** v. 109, n. 45, p. 18477-81, Nov 2012. ISSN 1091-6490. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/23091022 >.

18 TARAYRAH, L. et al. Epigenetic regulator Lid maintains germline stem cells through regulating JAK-STAT signaling pathway activity. **Biol Open,** v. 4, n. 11, p. 1518-27, Oct 21 2015. ISSN 2046-6390. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/26490676 >.

19 CHEN, C. et al. Cytokine receptor-Eb1 interaction couples cell polarity and fate during asymmetric cell division. **Elife,** v. 7, 03 26 2018. ISSN 2050-084X. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/29578412 >.

20 LENHART, K. F. et al. Diminished Jak/STAT Signaling Causes Early-Onset Aging Defects in Stem Cell Cytokinesis. **Curr Biol,** v. 29, n. 2, p. 256-267.e3, 01 2019. ISSN 1879-0445. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/30612906 >.

21 BHASKAR, P. K. et al. Germline sex determination regulates sex-specific signaling between germline stem cells and their niche. **Cell Rep,** v. 39, n. 1, p. 110620, 04 05 2022. ISSN 2211-1247. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/35385723 >.

22 HASAN, S.; HÉTIÉ, P.; MATUNIS, E. L. Niche signaling promotes stem cell survival in the Drosophila testis via the JAK-STAT target DIAP1. **Dev Biol,** v. 404, n. 1, p. 27-39, Aug 01 2015. ISSN 1095-564X. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/25941003 >.

23 SHIELDS, A. R. et al. The actin-binding protein profilin is required for germline stem cell maintenance and germ cell enclosure by somatic cyst cells. **Development,** v. 141, n. 1, p. 73-82, Jan 2014. ISSN 1477-9129. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24346697 >.

24 TERRY, N. A. et al. Novel regulators revealed by profiling Drosophila testis stem cells within their niche. **Dev Biol,** v. 294, n. 1, p. 246-57, Jun 1 2006. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16616121 >.

25 LI, W. X. Canonical and non-canonical JAK-STAT signaling. **Trends Cell Biol,** v. 18, n. 11, p. 545-51, Nov 2008. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18848449 >.

26 SHI, S. et al. Drosophila STAT is required for directly maintaining HP1 localization and heterochromatin stability. **Nat Cell Biol,** v. 10, n. 4, p. 489-96, Apr 2008. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18344984 >.

27 RANGAN, P. et al. piRNA production requires heterochromatin formation in Drosophila. **Curr Biol,** v. 21, n. 16, p. 1373-9, Aug 23 2011. ISSN 1879-0445 (Electronic)
0960-9822 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/21820311 >.

28 WANG, X. et al. Histone H3K9 trimethylase Eggless controls germline stem cell maintenance and differentiation. **PLoS Genet,** v. 7, n. 12, p. e1002426, Dec 2011. ISSN 1553-7404 (Electronic)

1553-7390 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/22216012 >.

29 XING, Y.; LI, W. X. Heterochromatin components in germline stem cell maintenance. **Sci Rep,** v. 5, p. 17463, Dec 2 2015. ISSN 2045-2322.

30 FLORA, P. et al. Transient transcriptional silencing alters the cell cycle to promote germline stem cell differentiation in Drosophila. **Dev Biol,** v. 434, n. 1, p. 84-95, Feb 1 2018. ISSN 1095-564X (Electronic)
0012-1606 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/29198563 >.

31 JANKOVICS, F. et al. Drosophila small ovary gene is required for transposon silencing and heterochromatin organization, and ensures germline stem cell maintenance and differentiation. **Development,** v. 145, n. 23, Dec 4 2018. ISSN 1477-9129 (Electronic)
0950-1991 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/30389853 >.

32 CASALE, A. M. et al. Heterochromatin protein 1 (HP1) is intrinsically required for post-transcriptional regulation of Drosophila Germline Stem Cell (GSC) maintenance. **Sci Rep,** v. 9, n. 1, p. 4372, 03 2019. ISSN 2045-2322. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/30867469 >.

33 LOZA-COLL, M. A. et al. Heterochromatin Protein 1 (HP1) inhibits stem cell proliferation induced by ectopic activation of the Jak/STAT pathway in the Drosophila testis. **Exp Cell Res,** v. 377, n. 1-2, p. 1-9, 04 15 2019. ISSN 1090-2422. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/30817931 >.

34 DUTTA, P. et al. Drosophila SERTAD domain protein Taranis is required in somatic cells for maintenance of male germline stem cells. **Dev Dyn,** v. 250, n. 2, p. 237-248, Feb 2021. ISSN 1097-0177. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/32969117 >.

35 HU, X. et al. Unphosphorylated STAT5A stabilizes heterochromatin and suppresses tumor growth. **Proc Natl Acad Sci U S A,** v. 110, n. 25, p. 10213-8, Jun 18 2013. ISSN 1091-6490 (Electronic)
0027-8424 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/23733954 >.

36 STREIT, A. et al. mgm 1, the earliest sex-specific germline marker in Drosophila, reflects expression of the gene esg in male stem cells. **Int J Dev Biol,** v. 46, n. 1, p. 159-66, Jan 2002. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11902678 >.

37 MICCHELLI, C. A.; PERRIMON, N. Evidence that stem cells reside in the adult Drosophila midgut epithelium. **Nature,** v. 439, n. 7075, p. 475-9, Jan 26 2006. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16340959 >.

38 OHLSTEIN, B.; SPRADLING, A. The adult Drosophila posterior midgut is maintained by pluripotent stem cells. **Nature,** v. 439, n. 7075, p. 470-4, Jan 26 2006. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16340960 >.

39 VOOG, J.; D'ALTERIO, C.; JONES, D. L. Multipotent somatic stem cells contribute to the stem cell niche in the Drosophila testis. **Nature,** v. 454, n. 7208, p. 1132-6, Aug 28 2008. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18641633 >.

40 VOOG, J. et al. Escargot restricts niche cell to stem cell conversion in the Drosophila testis. **Cell Rep,** v. 7, n. 3, p. 722-34, May 8 2014. ISSN 2211-1247 (Electronic). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24794442 >.

41 TRAN, J.; BRENNER, T. J.; DINARDO, S. Somatic control over the germline stem cell lineage during Drosophila spermatogenesis. **Nature,** v. 407, n. 6805, p. 754-7, Oct 12 2000. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11048723 >.

42 GONCZY, P.; MATUNIS, E.; DINARDO, S. bag-of-marbles and benign gonial cell neoplasm act in the germline to restrict proliferation during Drosophila spermatogenesis. **Development,** v. 124, n. 21, p. 4361-71, Nov 1997. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9334284 >.

43 SCHULZ, C. et al. A misexpression screen reveals effects of bag-of-marbles and TGF beta class signaling on the Drosophila male germ-line stem cell lineage. **Genetics,** v. 167, n. 2, p. 707-23, Jun 2004. ISSN 0016-6731 (Print). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15238523 >.

44 MATUNIS, E. L.; STINE, R. R.; DE CUEVAS, M. Recent advances in Drosophila male germline stem cell biology. **Spermatogenesis,** v. 2, n. 3, p. 137-144, Jul 1 2012. ISSN 2156-5554 (Print)
2156-5554 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/23087833 >.

45 KAPTEIN, A.; PAILLARD, V.; SAUNDERS, M. Dominant negative stat3 mutant inhibits interleukin-6-induced Jak-STAT signal transduction. **J Biol Chem,** v. 271, n. 11, p. 5961-4, Mar 15 1996. ISSN 0021-9258 (Print)
0021-9258 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/8626374 >.

46 KARSTEN, P. et al. Mutational analysis reveals separable DNA binding and trans-activation of Drosophila STAT92E. **Cell Signal,** v. 18, n. 6, p. 819-29, Jun 2006. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16129580 >.

47 VAN DOREN, M.; WILLIAMSON, A. L.; LEHMANN, R. Regulation of zygotic gene expression in Drosophila primordial germ cells. **Curr Biol,** v. 8, n. 4, p. 243-6, Feb 12 1998. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9501989 >.

48 GREWAL, S. I.; JIA, S. Heterochromatin revisited. **Nat Rev Genet,** v. 8, n. 1, p. 35-46, Jan 2007. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17173056 >.

49 WANG, J.; JIA, S. T.; JIA, S. New Insights into the Regulation of Heterochromatin. **Trends Genet,** v. 32, n. 5, p. 284-94, May 2016. ISSN 0168-9525 (Print)
0168-9525 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/27005444 >.

50 IMAI, R. et al. Density imaging of heterochromatin in live cells using orientation-independent-DIC microscopy. **Mol Biol Cell,** v. 28, n. 23, p. 3349-3359, Nov 07 2017. ISSN 1939-4586. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/28835378 >.

51 HAY, B.; JAN, L. Y.; JAN, Y. N. A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. **Cell,** v. 55, n. 4, p. 577-87, Nov 18 1988. ISSN 0092-8674 (Print). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3052853 >.

52 SHI, S. et al. JAK signaling globally counteracts heterochromatic gene silencing. **Nat Genet,** v. 38, n. 9, p. 1071-6, Sep 2006. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16892059 >.

53 XI, R.; MCGREGOR, J. R.; HARRISON, D. A. A gradient of JAK pathway activity patterns the anterior-posterior axis of the follicular epithelium. **Dev Cell,** v. 4, n. 2, p. 167-77, Feb 2003. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12586061 >.

54 RAWLINGS, J. S.; ROSLER, K. M.; HARRISON, D. A. The JAK/STAT signaling pathway. **J Cell Sci,** v. 117, n. Pt 8, p. 1281-3, Mar 15 2004. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15020666 >.

55 ISSIGONIS, M. et al. JAK-STAT signal inhibition regulates competition in the Drosophila testis stem cell niche. **Science,** v. 326, n. 5949, p. 153-6, Oct 2 2009. ISSN 1095-9203 (Electronic) 0036-8075 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19797664 >.

56 BROSKE, A. M. et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. **Nat Genet,** v. 41, n. 11, p. 1207-15, Nov 2009. ISSN 1546-1718 (Electronic). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19801979 >.

57 PIETERSEN, A. M.; VAN LOHUIZEN, M. Stem cell regulation by polycomb repressors: postponing commitment. **Curr Opin Cell Biol,** v. 20, n. 2, p. 201-7, Apr 2008. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18291635 >.

58 DIALYNAS, G. K. et al. Plasticity of HP1 proteins in mammalian cells. **J Cell Sci,** v. 120, n. Pt 19, p. 3415-24, Oct 1 2007. ISSN 0021-9533 (Print). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17855382 >.

59 ZENG, A. et al. Heterochromatin protein 1 promotes self-renewal and triggers regenerative proliferation in adult stem cells. **J Cell Biol,** v. 201, n. 3, p. 409-25, Apr 29 2013. ISSN 1540-8140 (Electronic) 0021-9525 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23629965 >.

60 XI, R.; XIE, T. Stem cell self-renewal controlled by chromatin remodeling factors. **Science,** v. 310, n. 5753, p. 1487-9, Dec 2 2005. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16322456 >.

61 YOON, J. et al. dSETDB1 and SU(VAR)3-9 sequentially function during germline-stem cell differentiation in Drosophila melanogaster. **PLoS ONE,** v. 3, n. 5, p. e2234, 2008. ISSN 1932-6203 (Electronic). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18493619 >.

62 SPIVAKOV, M.; FISHER, A. G. Epigenetic signatures of stem-cell identity. **Nat Rev Genet,** v. 8, n. 4, p. 263-71, Apr 2007. ISSN 1471-0056 (Print). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17363975 >.

63 CHEN, D.; MCKEARIN, D. Gene circuitry controlling a stem cell niche. **Curr Biol,** v. 15, n. 2, p. 179-84, Jan 26 2005. ISSN 0960-9822 (Print). Disponível em: <

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15668176 >.

64 SZAKMARY, A. et al. Regulatory relationship among piwi, pumilio, and bag-of-marbles in Drosophila germline stem cell self-renewal and differentiation. **Curr Biol,** v. 15, n. 2, p. 171-8, Jan 26 2005. ISSN 0960-9822 (Print). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15668175 >.

65 PENG, J. C.; KARPEN, G. H. Heterochromatic genome stability requires regulators of histone H3 K9 methylation. **PLoS Genet,** v. 5, n. 3, p. e1000435, Mar 2009. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19325889 >.

66 KELLUM, R.; RAFF, J. W.; ALBERTS, B. M. Heterochromatin protein 1 distribution during development and during the cell cycle in Drosophila embryos. **J Cell Sci,** v. 108 (Pt 4), p. 1407-18, Apr 1995. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7615662 >.

67 PETERS, A. H. et al. Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. **Cell,** v. 107, n. 3, p. 323-37, Nov 2 2001. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11701123 >.

68 YAN, S. J. et al. Unphosphorylated STAT and heterochromatin protect genome stability. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology,** v. 25, n. 1, p. 232-41, Jan 2011. ISSN 1530-6860 (Electronic)
0892-6638 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20847228 >.

69 ITO, K. et al. The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. **Development,** v. 124, n. 4, p. 761-71, Feb 1997. Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9043058 >.

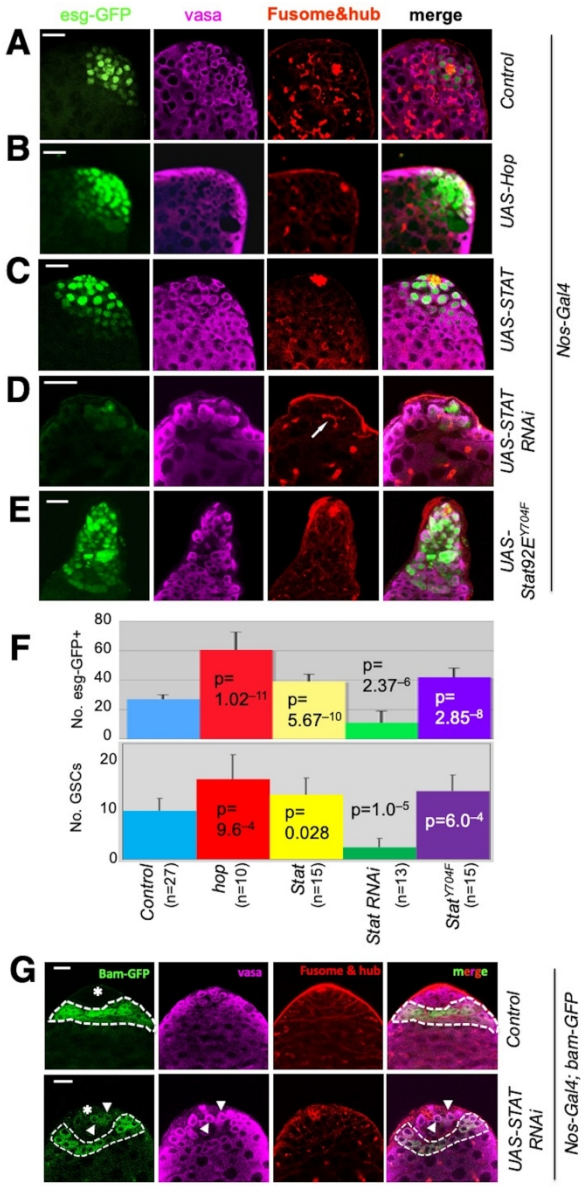

Figure 1

175x348mm (300 x 300 DPI)

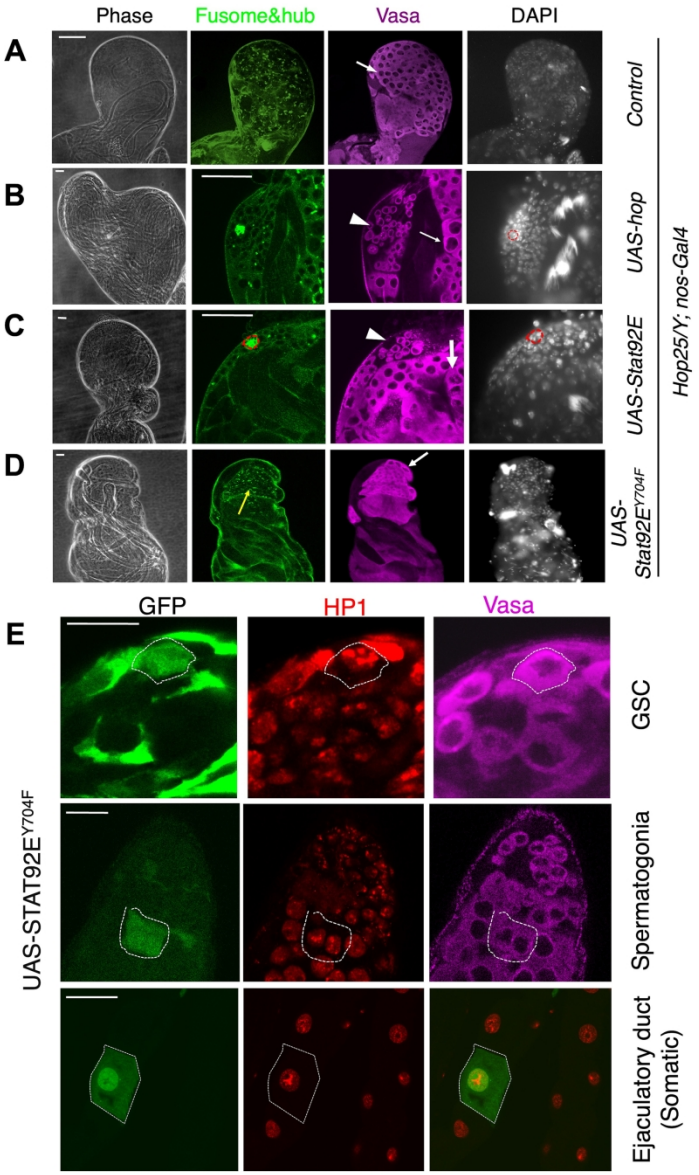

Figure 2

175x295mm (300 x 300 DPI)

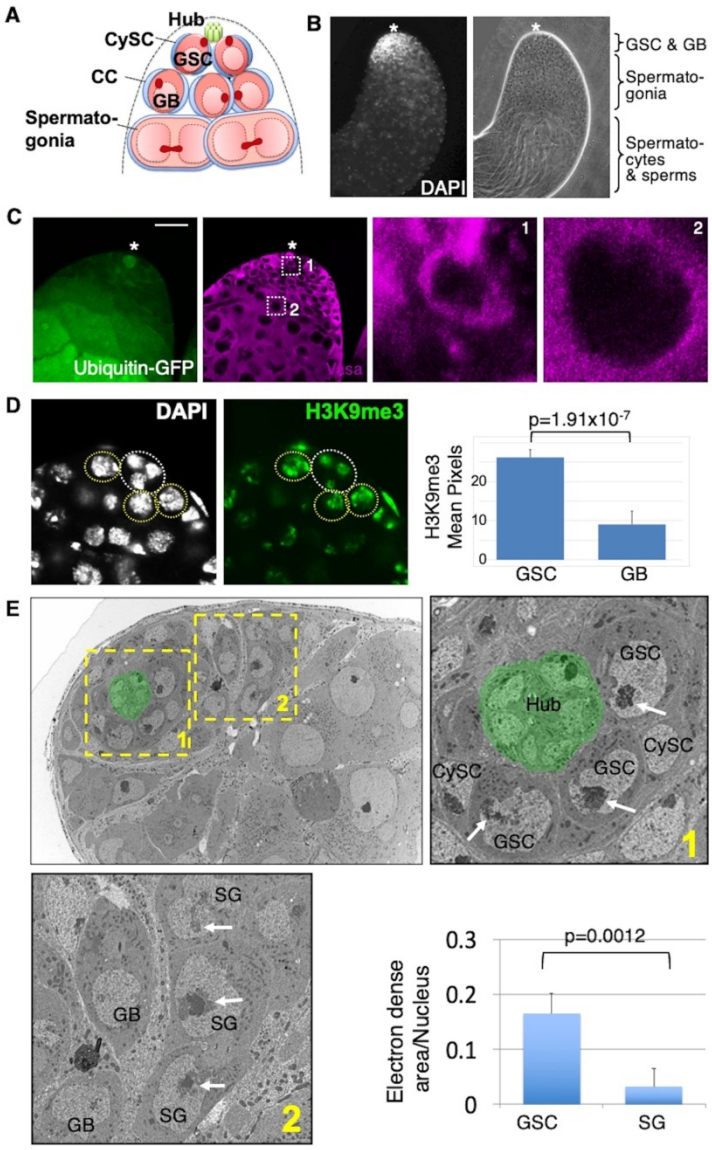

Figure 3

175x277mm (300 x 300 DPI)

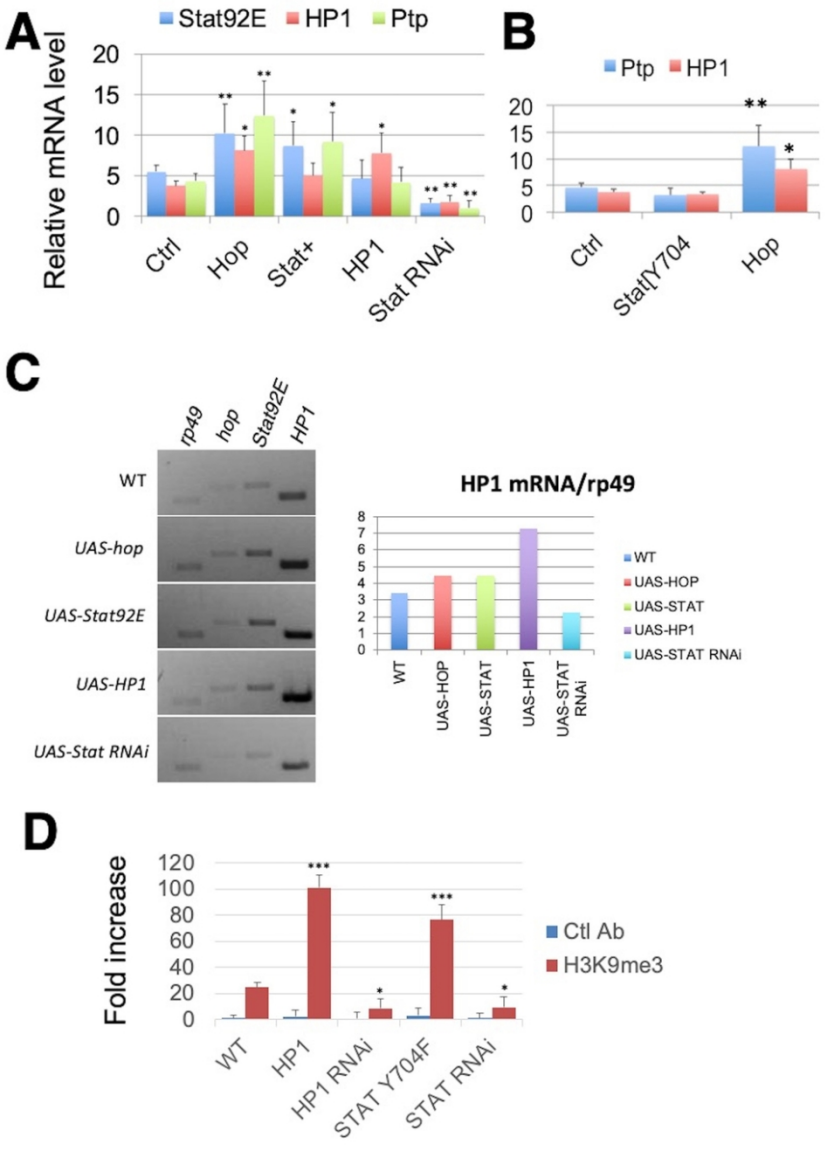

Figure 4

85x120mm (300 x 300 DPI)