Drosophila linker histone H1 coordinates STAT-dependent organization of heterochromatin and suppresses tumorigenesis caused by hyperactive JAK-STAT signaling

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Abstract

Background: Within the nucleus of eukaryotic cells, chromatin is organized into compact, silent regions called heterochromatin and more loosely packaged regions of euchromatin where transcription is more active. Although the existence of heterochromatin has been known for many years, the cellular factors responsible for its formation have only recently been identified. Two key factors involved in heterochromatin formation in Drosophila are the H3 lysine 9 methyltransferase Su(var)3-9 and heterochromatin protein 1 (HP1). The linker histone H1 also plays a major role in heterochromatin formation in Drosophila by interacting with Su(var)3-9 and helping to recruit it to heterochromatin. Drosophila STAT (Signal transducer and activator of transcription) (STAT92E) has also been shown to be involved in the maintenance of heterochromatin, but its relationship to the H1-Su(var)3-9 heterochromatin pathway is unknown. STAT92E is also involved in tumor formation in flies. Hyperactive Janus kinase (JAK)-STAT signaling due to a mutation in Drosophila JAK (Hopscotch) causes hematopoietic tumors.

Results: We show here that STAT92E is a second partner of H1 in the regulation of heterochromatin structure. H1 physically interacts with STAT92E and regulates its ectopic localization in the chromatin. Mis-localization of STAT92E due to its hyperphosphorylation or H1 depletion disrupts heterochromatin integrity. The contribution of the H1-STAT pathway to heterochromatin formation is mechanistically distinct from that of H1 and Su(var)3-9. The recruitment of STAT92E to chromatin by H1 also plays an important regulatory role in JAK-STAT induced tumors in flies. Depleting the linker histone H1 in flies carrying the oncogenic hopscotch^{Tum-l} allele enhances tumorigenesis, and H1 overexpression suppresses tumorigenesis.

Conclusions: Our results suggest the existence of two independent pathways for heterochromatin formation in Drosophila, one involving Su(var)3-9 and HP1 and the other involving STAT92E and HP1. The H1 linker histone directs both pathways through physical interactions with Su(var)3-9 and STAT92E, as well with HP1. The physical interaction of H1 and STAT92E confers a regulatory role on H1 in JAK-STAT signaling. H1 serves as a molecular reservoir for STAT92E in chromatin, enabling H1 to act as a tumor suppressor and oppose an oncogenic mutation in the JAK-STAT signaling pathway.

Keywords: Heterochromatin, Linker histone H1, JAK-STAT signaling, Tumor suppressor, Melanotic tumors, Drosophila melanogaster
Background
The genomes of eukaryotes are packaged into a nucleoprotein complex called chromatin [1,2]. Compaction of the DNA is achieved primarily through its association with a small family of proteins called histones. There are five major classes of histones: the core histones H2A, H2B, H3, and H4 and the linker histone usually referred to as H1. The nucleosome core particle is the basic repeating unit of chromatin in which approximately 145 bp of DNA is wrapped around an octamer of the four core histones. The linker histone H1 binds to the nucleosome core particle near the site at which DNA enters and exits the core particle, organizing an additional of approximately 20 bp of DNA to form the chromatosome [3]. The binding of H1 stabilizes the core particle and facilitates folding of nucleosome arrays into higher order structures [4,5].

The structure of chromatin is dynamic and undergoes changes in compaction during the cell cycle and during development. Importantly, among the five classes of histones, the H1 linker histone exhibits the greatest mobility, shuttling between the chromatin and the nucleoplasm with a residence time in chromatin of approximately 3 min [6,7]. Within the nucleus, chromatin exhibits variable levels of packaging. Chromatin is organized into densely packaged, generally silent regions called heterochromatin and more loosely packaged, transcriptionally active euchromatin [8]. Heterochromatin identity is established through modifications of epigenetic landscape of the genome and recruitment of specialized protein factors [9,10]. An important breakthrough in our understanding of the molecular basis for heterochromatin formation came with the discovery that the H3 histones in heterochromatin are modified by methylation on lysine 9 in the H3 N-terminal tail. This modification is catalyzed by the histone methyltransferase Su(var)3-9 [11] and it is recognized and bound by heterochromatin protein 1 (HP1) [12]. Recently, we reported that the linker histone H1 in Drosophila is also required for heterochromatin formation [13] and that it recruits Su(var)3-9 to heterochromatin by directly interacting with it [14]. H1 also has been shown to interact with HP1 [14-17].

Another factor that has been linked to heterochromatin stability in Drosophila is the DNA binding protein STAT92E [18-20]. Flies have a single STAT (STAT92E) and a single Janus kinase (JAK) that together constitute the JAK-STAT signaling pathway in Drosophila [21-23]. Perturbations of this pathway, including depletion of STAT92E or expression of a mutant hyperactive JAK (hopTum-l), lead to heterochromatin instability. The hopTum-l mutation also leads to the formation of blood cell tumors [24,25]. We show here that the H1 linker histone directly interacts with STAT92E and regulates its roles in both heterochromatin formation and tumorigenesis. Our results identify a second pathway of heterochromatin formation that is distinct from that of H1 and Su(var)3-9. Our observations also establish linker histone H1 as a tumor suppressor in flies.

Results and discussion
Hyperactive JAK/STAT signaling disrupts pericentric heterochromatin
Previous reports implicated the JAK-STAT signaling pathway in heterochromatin stability and heterochromatin protein1 (HP1) localization in Drosophila. Tumorous-letthal (Tum-l), an oncogenic allele of hopscotch (hop) encoding a constitutively hyperactive mutant of Drosophila JAK, was observed to disrupt heterochromatin silencing and HP1 localization in heterochromatin [18]. Loss of Drosophila STAT (STAT92E) was found to have very similar effects [19]. Based on the observation that HP1 and STAT92E interact, it was proposed that the two proteins colocalize within heterochromatin and that unphosphorylated STAT92E regulates HP1 localization and heterochromatin stability [19].

The effects of JAK-STAT signaling on heterochromatin were postulated based on low-resolution whole-mount staining of salivary glands for HP1. To examine in more detail the effects of perturbing JAK-STAT signaling on chromatin structure, we analyzed polytene chromosomes from salivary glands of hopTum-l mutant larvae. Figure 1 shows a comparison of polytene chromosomes prepared from control and hopTum-l mutant salivary glands. The overall structure of polytene chromosomes in the mutant is not severely perturbed, and they exhibit a close-to-normal pattern of bands and interbands. However, DAPI staining in hopTum-l salivary glands revealed that the polytene chromosomes lack a discernable chromocenter, the single coalesced region of heterochromatin formed from the pericentric regions of all chromosomes. This region is embedded in heterochromatin and normally exhibits intense staining for HP1. In contrast, HP1 staining in hopTum-l mutants was dispersed into several discrete foci. Interestingly, the abnormal chromocenter structure in hopTum-l animals resembles that in salivary glands of animals depleted of the linker histone H1 [13]. To directly compare effects of hopTum-l mutation and H1 depletion on salivary gland structure, we recombined two transgenes encoding an H1 RNA hairpin driven by the GAL4-responsive UAS promoter and pActin-GAL4. At 29°C, these transgenes together cause a moderate depletion of H1 in larvae (see Additional file 1: Figure S1), to approximately 30% of the wild type level. We observed that hopTum-l mutation or moderate H1 depletion in L3 larvae results in comparable defects of polytene chromosome structure (Figure 1). Therefore, we hypothesized that H1 and STAT92E may cooperate to maintain a normal polytene chromosome architecture and heterochromatin structure and function.
Interestingly, although hyperactive JAK clearly disrupts HP1 localization and formation of a single chromocenter in polytene chromosomes, it does not lead to a reduced amount of the H3K9 dimethyl mark in pericentric heterochromatin. In contrast, depletion of H1 causes a marked reduction in pericentric H3K9Me2 signal (Figure 1). Thus, H1 and STAT92E may share some but not all roles in regulation of heterochromatin structure and activity.

H1 regulates localization of STAT92E in chromatin
To begin to investigate the relationship between H1 and STAT92E, we sought to determine whether the two proteins co-localize in polytene chromosomes. By using indirect immunofluorescence (IF) staining, we found that STAT92E co-localizes with H1 in the chromocenter and throughout euchromatic arms (Figure 2A). High magnification images of polytene chromosome arms from wild type salivary glands display a strikingly similar pattern of staining for H1 and STAT92E, with both proteins most highly concentrated in bands. Split images for H1 and STAT92E show virtually complete overlap in their distributions. These observations indicate that H1 and STAT92E co-localize throughout polytene chromosomes.

To further investigate the basis for H1 and STAT92E co-localization in chromatin, we analyzed their distribution in salivary gland nuclei in which H1 was depleted by RNAi. We observed reduced H1 abundance in polytene chromosomes of H1-depleted larvae (Additional file 1: Figure S2A). Strikingly, we also found that specific STAT92E staining of polytene chromosomes, including the chromocenter and euchromatic arms, is almost completely lost upon H1 depletion (Figure 2B). In control experiments, STAT92E or H1 localization is not substantially affected in animals with a homozygous null mutation of Su(var)3-9 or with HP1 depleted by RNAi (Figure 2B and Additional file 1: Figure S2A). The observed mis-localization of STAT92E in H1-depleted larvae is corroborated by chromatin IP (ChIP) experiments: the occupancy of both H1 and STAT92E at multiple genomic loci is strongly decreased upon H1 knockdown (Figure 2C).

We also analyzed the distribution of H1 and STAT92E in polytene chromosomes of hopTum-l mutant larvae and in animals with STAT92E depleted by RNAi. RNAi-mediated depletion almost completely eliminates STAT92E presence in polytene chromosomes (Additional file 1: Figure S2C). Interestingly, hopTum-l mutation also results in reduction and re-distribution of the STAT92E-specific signal in polytene chromosomes (Additional file 1: Figure S2C). In contrast to the effects of H1 depletion on STAT92E distribution, neither STAT92E depletion nor its hyperphosphorylation had a discernable effect on H1 localization (Figure 2D). These results indicate that linker histone H1 strongly contributes to STAT92E tethering to chromatin. H1 is required for the apparent ubiquitous localization of STAT92E and thus may act upstream of STAT92E in regulation of normal chromosome architecture and heterochromatin structure proposed previously [18,19].

H1 physically interacts with STAT92E
Since H1 controls localization of STAT92E in chromatin, we next asked if H1 and STAT92E physically interact.
Purified recombinant His-tagged STAT92E and a fusion protein of H1 and glutathione-S-transferase (GST) were examined for physical interactions in vitro by GST pull-down (Figure 3A). GST-H1 readily interacts with STAT92E as determined by immunoblotting of glutathionine agarose-bound proteins with anti-His antibody. In control experiments, STAT92E did not bind to GST itself or a GST-Histone H2A fusion. Thus, the H1-STAT92E interaction is not due to the high net positive charge of H1 protein or to bridging by potentially contaminating nucleic acids. On the other hand, STAT92E associates with GST-H1, confirming earlier evidence for an interaction between these two proteins [19]. The observed physical interaction of STAT92E and H1 provides in vitro support for a model in which H1 mediates STAT92E recruitment to chromatin.

To further support this model, we examined interactions of H1 and STAT92E in the context of chromatin. To this end, we reconstituted defined oligonucleosomal substrates that did or did not contain H1 [14] and used
Figure 3 | H1 Physically Interacts with STAT92E

Protein-protein interactions between purified STAT92E and H1 were examined in vitro by GST pull-down assay in the presence of STAT92E pull-down and GST fusion proteins with full-length H1. H1 pull-down was expressed and purified from E.coli and analyzed by SDS-PAGE and Coomassie blue staining. Binding to recombinant H1 was detected by western blotting with specific antibodies. As a control, GST pull-down samples were examined in parallel. The GST pull-downs were performed as described.

(A) GST pull-down with baculovirus-expressed purified GST-STAT92E-His6. The pull-down samples were examined by SDS-PAGE and Coomassie blue staining of the gel. The protein bands were visualized by Coomassie blue staining. The GST pull-down samples were examined by western blotting with specific antibodies. As a control, GST pull-down samples were examined in parallel.

(B) Western blot analysis of proteins isolated from purified GST-STAT92E-His6. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with specific antibodies and detected by ECL. The membrane was also stained with Coomassie blue to visualize protein bands.

(C) Relative occupancy assay. The Relative Occupancy was measured by PCR in triplicate. Error bars represent standard deviation of six experimental points.

In vitro binding experiments with GST fusion proteins and recombinant H1 were performed. The binding activity of GST fusion proteins was analyzed by western blotting with specific antibodies. As a control, GST pull-down samples were examined in parallel.

In conclusion, our results provide new insights into the molecular mechanisms underlying the interaction between H1 and STAT92E. The interaction is mediated by specific binding sites on both proteins, and it is dependent on the presence of nucleosomes. This interaction may have important implications for the regulation of gene expression and chromatin structure.
marked increase in the frequency and size of tumors (Figure 4A and Table 1). The tumor index was increased almost twofold by H1 depletion. To confirm a role for H1 in hopTum-l-mediated tumorigenesis, H1 protein was overexpressed in hopTum-l mutant larvae. This was accomplished by combining the hopTum-l allele with transgenes encoding full-length H1 and the Actin-GAL4 driver. This combination caused a marked (more than threefold) reduction in the tumor index (Table 1). Taken together with the results of H1 depletion experiments, these data indicate that H1 acts as a suppressor of tumorigenesis caused by hyperactive JAK/STAT signaling.

Importantly, depletion of H1 in the wild type background does not lead to tumorigenesis (Table 1), which suggests that H1 does not play a direct role in the JAK-STAT transcriptional response. To confirm this observation, we made use of a transgenic allele in which a GFP reporter is placed under control of a STAT-responsive promoter containing 10 STAT92E binding sites [29]. We examined GFP expression by GFP autofluorescence of whole larvae or western blot of larval lysates (Figure 4B,C). Upon hyperphosphorylation in the hopTum-l mutant, STAT92E becomes transcriptionally active and strongly activates GFP expression. Quantitation of western data indicates about fourfold activation of the transgene upon STAT92E hyperphosphorylation (Figure 4D), which is further increased by H1 depletion in the hopTum-l background. On the other hand, H1 depletion alone does not activate transgene expression (Figure 4B,C,D). Thus, H1 does not appear to play a substantial role in direct regulation of normal transcriptional targets of phosphorylated STAT92E.

Rather, our results are consistent with a model in which H1 is required to maintain sequence-independent, ectopic localization of STAT in chromatin. Upon H1 depletion, excess STAT92E is released from the ectopic sites. The released STAT is in an unphosphorylated, transcriptionally inactive form and is unable to activate STAT-responsive genes (Figure 4). On the other hand, in the presence of the mutant hopTum-l allele, the STAT

![Figure 4](http://www.epigeneticsandchromatin.com/content/7/1/16)
released by H1 depletion becomes available as a substrate for phosphorylation by hyperactive JAK, which results in additional activation of endogenous STAT targets and increased tumorigenesis. If this model is correct, then overexpression of STAT itself in the hopTum-l mutant should also result in a similar, enhanced tumor formation by virtue of an increased JAK substrate availability. Indeed, as expected, UAS-controlled STAT92E overexpression driven by Actin-GAL4 leads to a fourfold increase in the tumor index in the hopTum-l background (Table 1). However, when a non-phosphorylatable mutant of STAT, STAT92E(Y704F) [30], is overexpressed under similar conditions, the tumorigenic effect of hopTum-l is not affected.

Ectopic overexpression of either HP1 or Su(var)3-9 under control of a heat shock promoter can also reduce the tumor index in hopTum-l mutant larvae [18]. To determine whether H1 is required for the tumor suppressor functions of HP1 and Su(var)3-9, we simultaneously depleted H1 and overexpressed Su(var)3-9 or HP1 in hopTum-l larvae. Depletion of H1 abolished the reduction in tumorigenicity caused by overexpression of either Su(var)3-9 or HP1 (about threefold), resulting in tumor indices that are nearly identical to that observed in the original hopTum-l allele (Table 2). These observations demonstrate that H1 is required for the tumor suppressor activity of HP1 and Su(var)3-9. They further support a model in which H1 lies upstream of STAT92E, HP1 and Su(var)3-9 in both maintenance of heterochromatin structure and tumorigenesis caused by hyperactive JAK/STAT signaling.

**H1 and STAT92E cooperate in the establishment of heterochromatin structure**

H1 depletion results in profound changes of polytene chromosome architecture and heterochromatin structure, activity, and biochemical composition. For instance, H1-depleted larvae largely lose the pericentric H3K9 dimethyl mark (Figure 1) and [13]. However, we previously observed that overexpression of the H3K9-specific HMT, Su(var)3-9, partially ameliorates this defect [14].

The results of this and other studies [18,19] suggest that ectopic localization of unphosphorylated STAT92E in chromatin may play an important role in proper polytene chromosome morphology in larvae, specifically the formation of heterochromatic chromocenter. The ectopic localization of STAT requires linker histone H1, depletion of which brings about STAT92E release from ectopic sites and simultaneous disruption of the chromocenter. Thus, in turn, it is possible that H1-mediated effects on heterochromatin structure may depend, at least in part, on STAT92E.

We decided to examine polytene chromosome structure in H1-depleted larvae that overexpress a non-phosphorylatable form of STAT92E(Y704F) in vivo in larvae. We discovered that whereas H1 depletion alone results in dissociation of a single chromocenter into multiple foci in close to 100% of examined specimens (Figures 1 and 5A), STAT92E(Y704F) overexpression partially reverses this defect (Figure 5A). In H1-depleted animals that overexpress STAT92E(Y704F), up to 40% of salivary gland cells contain a single chromocenter discernable by anti-H1P or DAPI staining. Furthermore, when overexpressed, STAT92E(Y704F) re-populates ectopic sites in polytene chromosomes, from which the

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**Table 1 H1 regulates hematopoietic tumor formation caused by hyperactive JAK**

| Genotype | N  | Tumor index | p value | H1 expression |
|----------|----|-------------|---------|---------------|
| hopTum-l/+ | 800 | 0.67 | n.d. |
| hopTum-l/+; pINT1-Nau/Act-GAL4 | 68 | 0.69 | n.d. |
| pINT1-H16F/+; Tub-GAL4/+ | 100 | 0.00 | Approximately 5% |
| hopTum-l/+; pINT1-H14M/Act-GAL4 | 82 | 1.26 | 0.005 | Approximately 30% |
| hopTum-l/+; UAS-H1/Act-GAL4 | 214 | 0.35 | 0.003 | n.d. |
| hopTum-l/+; UAS-STAT(Y704F)/Act-GAL4 | 68 | 2.76 | <0.0001 | n.d. |
| hopTum-l/+; UAS-STAT(Y704F)/Act-GAL4 | 81 | 0.67 | n.d. |

hopTum-l/FM7C flies were mated to wild type flies; hopTum-l/FM7C, Actin-GAL4/CyO flies were mated to homozygous transgenic pINT1-Nau, pINT1-H16F, UAS-H1, UAS-STAT92E or UAS-STAT92E(Y704F) flies; homozygous transgenic pINT1-H14M flies were mated to Tubulin-GAL4/TM3, Sb flies. All crosses were set at 29°C. The tumor index in adult progeny of the appropriate genotype was calculated as described previously [18], p values were calculated by Mann-Whitney test. N number of flies scored. Estimated H1 protein expression is presented as percentage (%) of wild type level; n.d. not determined.

**Table 2 The tumor suppressor function of HP1 and Su(var)3-9 is dependent on H1**

| Genotype | N  | Tumor index | p value |
|----------|----|-------------|---------|
| hopTum-l/+ | 800 | 0.67 |
| hopTum-l/+; ht-HP1/+ | 169 | 0.21 |
| hopTum-l/+; ht-HP1+/pINT1-H14M, Act-GAL4 | 113 | 0.62 | <0.0001 |
| hopTum-l/+; Su(var)3-9/+ | 224 | 0.19 |
| hopTum-l/+; Su(var)3-9/pINT1-H14M, Act-GAL4 | 93 | 0.61 | <0.0001 |

hopTum-l/FM7C flies were mated to wild type or homozygous transgenic ht-HP1 or ht-Su(var)3-9 flies; hopTum-l/FM7C, pINT1-H14M, Actin-GAL4/CyO flies were mated to homozygous transgenic ht-HP1 or ht-Su(var)3-9 flies. All crosses were set at 29°C. The tumor index in adult progeny of the appropriate genotype was calculated as described previously [18], p values were calculated by Mann-Whitney test. N number of flies scored.
endogenous STAT92E is evicted due to H1 depletion (compare Additional file 1: Figure S2C and Figure 5B, top), and co-localizes with residual H1. These results indicate that eviction of STAT92E is required for complete penetrance of polytene chromosome defects associated with H1 depletion. They also parallel our previous findings of partial reversal of H1 depletion effects by overexpression in vivo of another H1 recruitment partner, Su(var)3-9. In contrast, overexpression of wild type STAT92E in H1-depleted larvae fails to restore the single chromocenter of polytene chromosomes, and transgenic STAT92E does not strongly co-localize with residual H1 (Figure 5B, bottom). These observations suggest that overexpressed wild type STAT92E is not efficiently tethered to residual H1 in chromatin and does not re-populate ectopic chromosomal loci. Such findings may be explained by the ability of wild type STAT92E to become phosphorylated by JAK kinase and thus capable to bind to native, sequence-specific (and H1-independent) sites. On the other hand, STAT92E (Y704F) is less dynamic, cannot be phosphorylated by JAK kinase or interact with normal targets of active (phosphorylated) STAT and therefore, is more likely to strongly compete with endogenous STAT92E for limiting H1 under conditions of H1 depletion.

The proposed model of H1 and STAT cooperation in the establishment of pericentric heterochromatin structure might at first seem unlikely, because the abundance of a transcription factor, such as STAT92E, would be

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**Figure 5** STAT92E contributes to H1-dependent heterochromatin formation. Polytenes were analyzed by indirect immunofluorescence (IF) staining with antibodies against HP1 or H1 (red) and STAT92E (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm. (A) Polytenes were analyzed by indirect immunofluorescence (IF) staining with antibodies against HP1 or H1 (red) and STAT92E (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm. Polytenes were analyzed by indirect immunofluorescence (IF) staining with antibodies against HP1 or H1 (red) and STAT92E (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm.

(A) Polytenes were analyzed by indirect immunofluorescence (IF) staining with antibodies against HP1 or H1 (red) and STAT92E (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm. Polytenes were analyzed by indirect immunofluorescence (IF) staining with antibodies against HP1 or H1 (red) and STAT92E (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm. Polytenes were analyzed by indirect immunofluorescence (IF) staining with antibodies against HP1 or H1 (red) and STAT92E (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm. Polytenes were analyzed by indirect immunofluorescence (IF) staining with antibodies against HP1 or H1 (red) and STAT92E (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm.
expected to be many orders of magnitude lower than that of ubiquitous components of chromatin, such as H1 and HP1, and therefore insufficient in amount to mediate global structural properties of chromosomes. To address this perceived contradiction in our model, we examined the abundance of STAT92E and HP1 in larval and embryonic lysates by semi-quantitative western blotting. In these experiments, we compared the intensity of western bands in native samples and samples that contained defined amounts of recombinant proteins (Additional file 1: Figure S3). We discovered that the relative abundance of STAT92E in vivo differs by less than tenfold from that of HP1. Furthermore, IF staining of polytene chromosomes for STAT92E (Figure 2A) clearly shows that STAT92E is present throughout polytene chromosomes with a much broader distribution than that expected of a conventional sequence-specific transcription factor. Thus, STAT92E is a much more abundant component of chromatin than previously recognized and it could well contribute to global chromosome structure as proposed in our model (Figure 6).

A new paradigm for tumor suppression: linker histone H1 as a molecular reservoir for an oncogenic transcription factor

Li and colleagues have proposed that the oncogenic effect of the hopTum-1 allele and STAT hyperphosphorylation is a direct consequence of the resulting disruption of heterochromatin, which then causes global defects in gene regulation [18]. A recent study in mammalian cells [31] also proposed a role for unphosphorylated STAT5A in stabilization of heterochromatin and tumor suppression via repression of multiple oncoproteins. Our results do not support this model. The strongest evidence against the disruption of heterochromatin as a principal cause of tumorigenesis is our finding that H1 depletion produces disruptions in heterochromatin that are comparable to or stronger than those caused by the hopTum-1 mutation, yet H1 depletion alone does not result in tumorigenesis (Table 1). Also importantly, although overexpression of non-phosphorylatable STAT92E(Y704F) largely restores pericentric heterochromatin in H1-depleted salivary glands (Figure 5A), it does not act as tumor suppressor in hopTum-1 background (Table 1). Therefore, tumor formation and the heterochromatin structural abnormalities observed in the hopTum-1 mutant are likely independent phenomena.

Instead, our results are consistent with a model in which linker histone H1 serves as a molecular reservoir for STAT92E. We propose that, normally, unphosphorylated STAT92E resides along with H1 in numerous loci throughout chromosomes, including pericentric heterochromatin, where the two proteins stabilize HP1 binding (Figure 6A, top left). The association of STAT92E with these ectopic loci is dependent on H1, but independent of STAT92E canonical DNA recognition elements. Hyperphosphorylation of STAT92E prevents its efficient association with ectopic sites, directs it to specific DNA elements and causes abnormal transcriptional activation and tumorigenesis (Figure 6A, top right). H1 depletion alone leads to release of unphosphorylated STAT92E from the chromatin reservoir and disruption of normal pericentric structures. However, in the absence of hyperactive JAK, it does not result in tumorigenesis, because the normal level of JAK kinase activity is limiting, and generation of higher levels of activated STAT92E is not achieved (Figure 6A, bottom left). Depleting H1 in the presence of hyperactive hopTum-1 kinase, though, leads to excessive production of phosphorylated STAT92E and enhanced tumorigenesis (Figure 6A, bottom right).

Linker histone H1 directs two alternative pathways of heterochromatin formation

We reported previously that Drosophila H1 interacts with and recruits Su(var)3-9 to promote heterochromatin formation [14]. The results reported here provide evidence for another, alternative pathway of H1-dependent heterochromatin formation, which involves H1 interaction with STAT92E and its recruitment to ectopic sites in chromatin. Eviction of STAT92E from its chromatin reservoir can be achieved by H1 depletion or STAT hyperphosphorylation. However, although hyperphosphorylation of STAT92E disrupts the structure of pericentric heterochromatin, it does not substantially affect H3K9 dimethylation present in HP1-positive foci (Figure 1). Thus, STAT92E appears to be dispensable for Su(var)3-9 localization or activity. Conversely, a null mutation of Su(var)3-9 does not affect STAT92E localization in polytene chromosomes (Figure 2B). We conclude that STAT92E function in the establishment or maintenance of heterochromatin structure is independent of H3K9 methylation by Su(var)3-9. On the other hand, H1 directs formation of heterochromatin structures via both pathways, one that involves H3K9 dimethylation and the other that utilizes STAT-dependent stabilization of HP1 (Figure 6B).

Our analyses reveal that unphosphorylated STAT92E is an abundant and nearly ubiquitous chromatin component (Figure 2A and Additional file 1: Figure S3). Its level of expression approaches 10%–20% of that of heterochromatin protein HP1, or close to 1 molecule per 100 nucleosomes in the genome [32], much higher than expected for a sequence-specific transcription factor. The storage and sequestering of excess inactive STAT in the nucleus is achieved through association with H1-containing chromatin and allows for rapid activation of the JAK-STAT regulatory cascade by external stimuli. At the same time, STAT92E appears to stabilize particular nucleosomal conformations, such as pericentric heterochromatin in the chromocenter of larval salivary gland.
**Figure 6** (See legend on next page.)
chromosomes in Drosophila, and physically interacts with H1 and HP1, heterochromatin components. In the future, it will be interesting to analyze molecular interactions in a putative tripartite STAT-H1-HP1 complex in the context of chromatin and to examine how STAT92E modulates the structure of the chromatin fiber in vitro.

Conclusions

By using polytene chromosome analyses in Drosophila salivary gland cells, we performed studies of chromatin defects associated with hyperactivation of STAT. Although a connection between heterochromatin integrity and tumorigenesis by JAK-STAT effectors has been proposed recently [18,19], we discovered a new major connection between STAT and linker histone H1 that alters the existing model of STAT-dependent maintenance of heterochromatin and provides mechanistic insight into its regulation. We provide evidence that STAT92E specifically helps to maintain a particular feature of pericentric heterochromatin, namely the chromocenter region of polytene chromosomes in Drosophila larvae. Furthermore, we report direct physical interactions of STAT92E with H1 and HP1, key structural components of heterochromatin, and discern molecular mechanisms of STAT-dependent regulation of heterochromatin formation. These observations lead us to propose a coordinate role for STAT, linker histone H1 and HP1 in the maintenance of heterochromatin integrity. Our studies have also revealed that, as a result of its involvement in STAT-dependent organization of chromatin and sequestering STAT in the nucleus, the linker histone H1 acts to suppress tumorigenesis caused by hyperactive JAK-STAT signaling.

Methods

Fly strains and genetics

Flies were grown on standard corn meal, sugar, and yeast medium with Tegosept. Stocks were maintained at 18°C. Crosses were performed in an environmental chamber at 29°C. For polytene chromosome staining, all animals were incubated at 29°C throughout their life cycles. Canton-S flies were used as wild type controls. The following fly stocks were obtained from the Bloomington Stock Center and are described in FlyBase: hopTum-1, UAST-STAT92E-GFP, UAST-STAT92E-RNAi, 10xSTAT92E-GFP, Actin-GAL4/CyO, HP1-shRNA, Su(var)3-9/TM3,Sh, and Su(var)3-9/TM3,Sh, ht-HPI and ht-Su(var)3-9 flies were generous gifts from Dr. G. Reuter (Martin Luther University, Halle-Wittenberg, Germany). Nau-RNAi transgenic flies were generously provided by Dr. B. Paterson (NIH). UAS-STAT(Y704F) allele was a generous gift of Dr. W. Li (University of California at San Diego). H1 knockdown was achieved by plINT1-H1[4 M] transgene expression driven by Actin-GAL4 [13].

To analyze the tumor index (TI), hopTum-1 flies were crossed to Canton-S flies, ht-HPI or ht-Su(var)3-9 flies. Alternatively, hopTum-1 and Actin-GAL4/CyO flies were crossed to plINT1-H1[4 M] or pUAST-H1 flies. hopTum-1, ht-HPI and hopTum-1, and ht-Su(var)3-9 flies were crossed to plINT1-H1[4 M], Actin-GAL4/CyO flies. TI was calculated based on observations from F1 adult flies reared at 29°C as described [18], p values were calculated by the Mann-Whitney U test using GraphPad Prism software.

Immunohistochemistry

Indirect immunofluorescence (IF) analyses of polytene chromosomes were carried out as described [13]. DNA was stained by adding 1.5 μg/ml DAPI (Vectorshield, CA, USA) to the mounting medium. The following antisera were used at the indicated dilutions: monoclonal mouse anti-Drosophila HP1, C1A9 (1:50, Developmental Studies Hybridoma Bank); goat anti-Drosophila STAT, dF-20 (1:50, Santa Cruz Biotechnology); affinity-purified rabbit Drosophila H1 antisera (1:5,000) and affinity-purified rabbit anti-H3K9me2 (1:100, Abcam). Appropriate Cy2- and Cy3-conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA) were used at 1:200. Specificity of IF staining was verified by appropriate controls, such as staining with secondary antibodies only and staining of polytene chromosomes.
from H1 and STAT92E knockdown animals (see for example Figure 5B, and Additional file 1: Figure S2C).

For GFP autofluorescence analyses, wild type; hop\textsuperscript{Tum-l}, plINT1-H1\textsuperscript{4M} and hop\textsuperscript{Tum-l}; and plINT1-H1\textsuperscript{4M} flies were crossed with flies carrying 10xSTAT92E-GFP transgene. The F1 L3 larvae were placed on a glass slide and immobilized on ice for 10 min.

Fluorescent images were acquired on a Zeiss Axiosplan microscope (Carl Zeiss, Oberkochen, Germany) equipped with Zeiss Digital Microscopy Camera AxioCam ICC1and AxioVision Digital Image Processing Software (Carl Zeiss). Stereoscopic images were acquired on a Zeiss SteREO Discovery V8 microscope (Carl Zeiss).

Recombinant proteins and GST pull-down

Recombinant Drosophila His\textsubscript{6} and FLAG-tagged HP1 protein was purified as described [33]. Full-length Drosophila STAT92E cDNA was amplified by PCR from an EST clone (RE13194) (Drosophila Genomic Research Center) and cloned into pFastBac1 vector (Invitrogen) in-frame with a C-terminal His\textsubscript{6}-tag. Details of cloning are available on request. STAT92E-His\textsubscript{6} baculoviruses were prepared using BacToBac System (Invitrogen). The recombinant STAT92E-His\textsubscript{6} was synthesized in S9 cells and purified by TALON His-Tag Purification Resin (Clontech). GST fusions of H1, H2A, and HP1 were expressed in E. coli (BL21(DE3)pLys strain) and purified by glutathione-Sepharose chromatography as described [14]. To prepare GST fusions of H1 globular (amino acid residues 41–119), N- (1–40) and C-terminal (120–256) domains, corresponding PCR products were cloned into in pGEX 4 T-1 (GE Life Sciences). The purified proteins were analyzed by SDS-PAGE, and concentrations were determined by Coomassie staining along with BSA protein mass standards (Pierce).

In GST pull-down assays, purified recombinant STAT92E-His\textsubscript{6} was incubated with GST or GST fusion proteins and purified on glutathione-Sepharose as described [14]. STAT92E-His\textsubscript{6} binding to GST fusion proteins was detected by anti-His\textsubscript{6} western of the pull-down samples. Additionally, the pull-down samples were examined for the presence of GST fusion proteins by SDS-PAGE and Coomassie staining.

Recollection of chromatin and ChIP

Reconstitution of H1-containing and H1-free chromatin was carried out as described [14]. For in vitro ChIP analyses, approximately 0.5 pmol purified STAT92E-His\textsubscript{6} or Su(var)3-9-His\textsubscript{6} protein was incubated with 0.2 pmol supercoiled plasmid DNA (3.2 kb), H1-containing or H1-free chromatin in 20 μl of reaction buffer (50 mM Tris–HCl, pH 7.9, 5 mM MgCl\textsubscript{2}, 4 mM DTT and 2 μg/ml BSA) for 15 min at 27°C. The material was cross-linked for 10 min at room temperature, and the cross-linking was terminated by addition of 9.8 μl of 2.5 M glycine. The material was incubated with 2 μl rabbit polyclonal anti-His\textsubscript{6} antibody, ChIP grade (Abcam) in 400 μl reaction buffer overnight at 4°C. After immunoprecipitation and cross-link reversal, the DNA was isolated by QIAquick PCR purification kit (Qiagen, Valencia, Santa Clara, CA, USA). Samples were analyzed quantitatively by real-time PCR (Qiagen, Valencia, Santa Clara, CA, USA) as described [13,14]. For H1 and STAT92E qChIP in vivo, chromatin was prepared from H1-depleted and control whole larvae, immunoprecipitated as described above and analyzed by real-time PCR as described previously [13,14]. Primer sequences are available upon request. Each sample was analyzed in three independent real-time PCR reactions.

Immunoblot analyses

Semi-quantitative western analyses of H1, tubulin, and GFP in Drosophila salivary gland or whole larval lysates were carried out as described [13]. For quantitation of STAT92E in vivo, Drosophila embryonic SK (Soeller-Kornberg) extracts were prepared as described [34]. SK extract was boiled in Laemmli loading buffer for 5 min and centrifuged. An aliquot of SK extract was loaded on a 10% SDS-PAGE gel, along with 0.2–20 pmol purified His\textsubscript{6}-tagged STAT92E or 2–200 pmol purified His\textsubscript{6} and FLAG-tagged Drosophila HP1 protein. The following primary antibodies were used at the indicated dilutions: rabbit anti-Drosophila H1 (1:5,000); mouse monoclonal anti-tubulin, E7 (1:500; Developmental Studies Hybridoma Bank); mouse anti-GFP (1:1,000, Santa Cruz Biotechnology); mouse anti-Drosophila HP1, C1A9 (1:3,000), and goat anti-Drosophila STAT dF-20 (1:50). The infrared dye-labeled secondary antibodies were used at 1:10,000 (LI-COR Bioscience, Lincoln, NE, USA). Images were obtained and quantitated using the LI-COR Odyssey Infrared Imaging System.

Additional file

Additional file 1: Supplemental figure legends. Figure S1. Depletion of H1 protein by RNAi in vivo in Drosophila larvae. H1 protein levels were examined by semi-quantitative western blotting of salivary gland lysates. H1 protein levels in wild type, hop\textsuperscript{Tum-l}, and pINT1-H1\textsuperscript{4M} Akti-GAL4/CyO larvae. Whereas hop\textsuperscript{Tum-l} does not substantially affect H1 levels, ubiquitous GAL4-driven RNAi results in approximately 70% decrease of the expression level. Numbers at the bottom indicate H1 expression relative to wild type (100%). Figure S2. Changes in distribution of H1, STAT92E, and heterochromatin markers in polytene chromosomes upon depletion of H1, STAT92E, H1-depleted, Su(var)3-9 and HP1-depleted polytene chromosomes. H1 depletion (to approximately 30% wild type level, Additional file 1: Figure S1) strongly reduces H1 staining. Reduced heterochromatin marks in Su(var)3-9 mutant and HP1-depleted polytene chromosomes. In Su(var)3-9/+, Su(var)3-9/+ (Su(var)3-9; [Su(var)3-9]; [Su(var)3-9]) salivary glands, pericentric heterochromatin-specific.
H3K9Me3 staining is strongly reduced (compare to Figure 1). HP1 staining of polytene chromosomes is completely eliminated upon HP1 depletion by RNAi. HP1 KD, HP1 knockdown. (C) Genome-wide localization of STAT92E in polytene chromosomes upon STAT92E depletion and in hop^{tum} mutants. STAT92E is almost completely eliminated by STAT92E depletion in larvae. hop^{tum} mutation slightly affects the abundance and localization pattern of STAT92E compared to that in wild type chromosomes (Figure 2A). STAT92E KD, STAT92E knockdown. Figure 5B. Relative abundance of STAT92E and HP1 in Drosophila embryonic nuclear extract. Drosophila embryo 5K extract [33] (see Methods) was analyzed for relative abundance of STAT92E and HP1 by semi-quantitative western blot. The amounts (pmoles) of purified recombinant STAT92E or HP1 proteins were indicated.

Abbreviations

DAPI: 4′,6-diamidino-2-phenylindole; DTt: diithiothreitol; GFP: green fluorescent protein; GST: glutathione S-transferase; HMV: histone methyltransferase; HP1: heterochromatin protein 1; IF: indirect immunofluorescence; JAK: Janus kinase; KD: knockdown; PCR: polymerase chain reaction; qChIP: quantitative chromatin immunoprecipitation; RNAi: RNA interference; STAT: signal transducer and activator of transcription; T: tumor index; UAS: upstream activation sequence.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

NX carried out and interpreted genetic, immunocytochemical, and biochemical experiments and drafted the manuscript. AV performed biochemical experiments. DVF and AIS conceived of the study, participated in its design and coordination, evaluated and interpreted the data, and wrote the final version of the manuscript. All authors read and approved the final manuscript.

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