The trithorax group proteins Kismet and ASH1 promote H3K36 dimethylation to counteract Polycomb group repression in Drosophila

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SUMMARY
Members of the Polycomb group of repressors and trithorax group of activators maintain heritable states of transcription by modifying nucleosomal histones or remodeling chromatin. Although tremendous progress has been made toward defining the biochemical activities of Polycomb and trithorax group proteins, much remains to be learned about how they interact with each other and the general transcription machinery to maintain on or off states of gene expression. The trithorax group protein Kismet (KIS) is related to the SWI/SNF and CHD families of chromatin remodeling factors. KIS promotes transcription elongation, facilitates the binding of the trithorax group histone methyltransferases ASH1 and TRX to active genes, and counteracts repressive methylation of histone H3 on lysine 27 (H3K27) by Polycomb group proteins. Here, we sought to clarify the mechanism of action of KIS and how it interacts with ASH1 to antagonize H3K27 methylation in Drosophila. We present evidence that KIS promotes transcription elongation and counteracts Polycomb group repression via distinct mechanisms. A chemical inhibitor of transcription elongation, DR8, had no effect on ASH1 recruitment or H3K27 methylation. Conversely, loss of ASH1 function had no effect on transcription elongation. Mutations in kis cause a global reduction in the di- and tri-methylation of histone H3 on lysine 36 (H3K36) – modifications that antagonize H3K27 methylation in vitro. Furthermore, loss of ASH1 significantly decreases H3K36 dimethylation, providing further evidence that ASH1 is an H3K36 dimethylase in vivo. These and other findings suggest that KIS antagonizes Polycomb group repression by facilitating ASH1-dependent H3K36 dimethylation.

KEY WORDS: Polycomb group, Trithorax group, Chromatin, Transcription, Histone methylation

INTRODUCTION
The Polycomb group of repressors and trithorax group of activators play key roles in the control of cell fate by maintaining heritable states of transcription during development. Polycomb and trithorax group genes were first identified as regulators of Hox gene expression in Drosophila and were subsequently shown to play conserved roles in mammalian development, the maintenance of stem cell pluripotency, and cancer (Fisher and Fisher, 2011; Grimaud et al., 2006; Mills, 2010; Richly et al., 2011). These findings have stimulated great interest in the mechanisms by which Polycomb and trithorax group proteins regulate transcription.

Many Polycomb and trithorax group proteins regulate transcription by altering chromatin structure (Lanzuolo and Orlando, 2012; Schuettengruber et al., 2011). The majority of Polycomb group genes encode subunits of two protein complexes: PRC1 and PRC2 (Levine et al., 2004). The Enhancer of zeste [E(Z)] subunit of PRC2 methylates lysine 27 of histone H3 (H3K27) (Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002); this histone modification is crucial for Polycomb group repression and is thought to promote the association of PRC1 with chromatin leading to chromatin compaction (Lanzuolo and Orlando, 2012; Margueron and Reinberg, 2011). The trithorax group genes trithorax (trx) and absent, small or homeotic discs I (ash1) encode histone modifying enzymes that counteract Polycomb group repression by methylating lysine 4 (or other residues) of histone H3 (Schuettengruber et al., 2011). The trithorax group genes brahma (brm), moira (mor) and osa encode subunits of a Drosophila complex related to the SWI/SNF ATP-dependent chromatin-remodeling complex (Mohrmann and Verrijzer, 2005). Thus, both the covalent modification and remodeling of nucleosomes are crucial for the maintenance of heritable states of gene expression during development.

Although great progress has been made toward defining the biochemical activities of trithorax group proteins, much remains to be learned about how they interact with each other and the general transcription machinery to antagonize Polycomb repression. Some trithorax group proteins (e.g. BRM, MOR and OSA) play relatively general roles in transcription by RNA polymerase II (Pol II) (Armstrong et al., 2002; Reisman et al., 2009), whereas others (e.g. ASH1 and TRX) function primarily to antagonize Polycomb repression (Klymenko and Müller, 2004; Papp and Müller, 2006; Tie et al., 2009). Interestingly, the Drosophila trithorax group protein Kismet (KIS) displays properties of both classes of trithorax group proteins (Srinivasan et al., 2005; Srinivasan et al., 2008).

kis was identified in a genetic screen for factors that antagonize Polycomb group repression (Kennison and Tamkun, 1988) and was subsequently shown to maintain the expression of the Hox genes Sex combs reduced (Scr) and Abdominal B (Abd-B) (Daubresse et al., 1999). KIS is a member of the CHD subfamily of chromatin remodeling factors (Daubresse et al., 1999; Therrien et al., 2000) and its human homolog, CHD7, has ATP-dependent chromatin-remodeling activity in vitro (Bouazoune and Kingston, 2012). The loss of kis function leads to a dramatic decrease in the levels of ASH1 and TRX associated with salivary gland polytene chromosomes and a concomitant increase in the level of repressive

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H3K27 methylation (Srinivasan et al., 2008). In addition to antagonizing Polycomb repression, KIS plays a relatively global role in transcription elongation. KIS is associated with the vast majority of transcriptionally active genes and loss of kis function dramatically reduces the level of the elongating form of RNA Pol II (Pol II<sub>el</sub>) and of the elongation factors SPT6 and CHD1 associated with polytene chromosomes (Srinivasan et al., 2005). These findings suggest that the ability of KIS to antagonize Polycomb repression and promote transcription elongation might be intimately related.

Eukaryotic transcription involves a highly ordered series of events, including the binding of transcription factors to cis-regulatory elements, the assembly of the pre-initiation complex and recruitment of Pol II to promoters, initiation, promoter clearance, elongation and termination (Buratowski, 2009). The phosphorylated C-terminal domain (CTD) of elongating Pol II recruits a host of factors required for the transcription and processing of nascent transcripts to the body of active genes, including the histone methyltransferase SET2, the histone deacetylase RPD3, transcription elongation factor SPT6, and the histone H3 K27 demethylase UTX (Bartkowiak et al., 2011; Smith et al., 2008; Zhang et al., 2012). Transcription elongation also promotes the replacement of histone H3 with the histone variant H3.3, which harbors covalent modifications characteristic of active genes, including elevated H3K4me3 and low H3K27me3 (McKittrick et al., 2004; Mito et al., 2007). Furthermore, the transcription of non-coding regulatory elements, including Polycomb-response elements (PREs), can prevent heritable silencing by Polycomb group proteins in Drosophila (Rank et al., 2002; Schmitt et al., 2005). Transcription elongation could therefore antagonize Polycomb repression by promoting histone exchange or recruiting trithorax group proteins, UTX or other factors to active genes.

The above observations prompted us to examine whether KIS recruits ASH1 and antagonizes Polycomb repression by promoting transcription elongation. We also compared the effect of mutations in kis and ash1 on transcription and the levels of both activating and repressive histone modifications. The findings suggest that KIS promotes transcription elongation and counteracts Polycomb repression via distinct mechanisms. Furthermore, we present evidence that KIS antagonizes Polycomb repression by promoting the ASH1-dependent dimethylation of H3K36 in vivo.

MATERIALS AND METHODS

Drosophila strains

Flies were raised on cornmeal, agar, yeast and molasses medium, supplemented with methylparaben (Tegosept) and propionic acid. Unless otherwise indicated, the Drosophila strains used in this study are described in FlyBase (http://www.flybase.org). The hypomorphic kis<sup>41446</sup> allele resulted from the insertion of a P-element in the first intron of the kis gene; kis<sup>41446</sup> homozygotes survive until late larval or early pupal stages, but express undetectable levels of KIS-L in salivary gland nuclei (Srinivasan et al., 2005). The amorphic ash1<sup>22</sup> and antimorphic ash1<sup>117</sup> alleles encode polypeptides lacking the C-terminal 2098 and 1655 residues of the 2144 amino acid ASH1 protein, respectively (Tripoulas et al., 1996). Oregon R was used as the wild-type strain for all experiments.

Immunostaining of polytene chromosomes

Drosophila salivary glands from third instar larvae reared at 18°C were fixed for 5 minutes in 45% acetic acid/1.85% formaldehyde and stained with rabbit polyclonal antibodies against ASH1 (Tripoulas et al., 1996), BRM (Elfring et al., 1998) and KIS-L (Srinivasan et al., 2005), mouse monoclonal antibodies against Pol IIa, Pol II<sub>a</sub> and Pol II<sub>el</sub> (Covance), rat antibodies against KIS-L (Srinivasan et al., 2005) and guinea pig antibodies against SPT6 (Kaplan et al., 2000) as previously described (Corona et al., 2004). For the heat-shock experiments, salivary gland polytene chromosome squashes were prepared from wild-type third instar larvae shifted to 37°C for 40 minutes immediately prior to dissection. The wild-type KIS-L protein or β-galactosidase was expressed in the salivary glands of third instar larvae using the GAL4 system and ey-GAL4 driver as previously described (Fasuolo et al., 2012).

To stain polytene chromosomes with rabbit antibodies against H3K27me3 (Millipore) or H3K36me2 (Abcam) and mouse antibodies against H3K36me2 (Wako) or H3K36me3 (Kimura et al., 2008), salivary glands were fixed in 6 mM MgCl<sub>2</sub>, 1% citric acid and 1% Triton X-100 for 2 minutes. Primary antibodies were used at dilutions of 1:50-1:100, with the exception of the histone antibodies, which were used at dilutions of 1:500. Fluorophore-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and were used at 1:200. Chromosome preparations were mounted in Vectashield containing DAPI (Vector Laboratories).

Inhibition of transcription elongation in larval salivary glands

To inhibit transcription elongation, salivary glands from wild-type third instar larvae were dissected in PBS and incubated for 1 hour at room temperature in Schneider’s Insect Medium (Sigma-Aldrich) containing 65 μM β-D-ribofuranoside (DRB) (Sigma-Aldrich) dissolved in DMSO, or DMSO alone as a control. Incubation times of up to 3 hours with DRB concentrations ranging from 0 μM to 650 μM were also tested. Images were captured using a Zeiss Axioskop 2 plus microscope equipped with an Axioplan HRm camera and Axiosvision 4 software (Carl Zeiss). Images were processed and split and merged images were generated using Adobe Photoshop CS4 software as described previously (Corona et al., 2004). Pixel intensities along a polytene chromosome arm were measured using ImageJ (NIH) and peak plots generated using Microsoft Excel. Pearson’s correlation coefficients and pairwise scatter plots of overlapping pixel intensities were generated using Velocity software (PerkinElmer).

Quantitative immunofluorescence microscopy

To compare the levels of proteins associated with wild-type and mutant polytene chromosomes, images were captured using the same exposure time and processed and analyzed at the same time under identical conditions. Images shown are representative of multiple experiments. To quantify relative fluorescence intensities, five representative images from each condition were chosen from a single experiment and were treated identically in parallel as follows. First, the level of background fluorescence was measured across all images and the black point of each image was adjusted to the average background fluorescence intensity using levels in Adobe Photoshop. When necessary, non-chromosomal fluorescent objects in the field were removed in Adobe Photoshop to obtain an accurate measurement of only the chromosomal fluorescence signal. The average fluorescence intensity of each image was measured using Velocity. Average values were recorded in Excel, normalized to the control values and plotted in Excel using standard deviation for error bars. Statistical significance was determined using Student’s two-tailed t-test assuming equal variance.

Protein extraction and analysis

Whole salivary glands from wild-type third instar larvae were dissected into cold M buffer (10 mM HEPES-KOH pH 7.6, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 5% glycerol) and treated with 65 μM DRB or DMSO as a control for 2 hours on ice. Chromatin extracts were prepared from treated salivary glands as described (Corona et al., 2007) and analyzed by SDS-PAGE and western blotting as described (Srinivasan et al., 2005), using PVDF membrane and rabbit antibodies against KIS-L (Dautresesse et al., 1999) and ISWI (Tsukiyama et al., 1995). For western blotting of H3K27 methylation, whole salivary glands were dissected into cold M buffer containing 1 mg/ml Complete Protease Inhibitor Cocktail (Roche) and treated with 65 μM DRB or DMSO as a control for 2 hours on ice. Treated salivary glands were homogenized in M buffer containing protease inhibitors, boiled in SDS loading buffer for 5 minutes and analyzed by SDS-PAGE and western blotting as described (Srinivasan et al., 2005), using PVDF membrane and rabbit antibodies against H3K27me3 (Diagenode).
RESULTS

We previously showed that KIS is required for the binding of ASH1 to polytene chromosomes, promotes transcriptional elongation, and counteracts repressive H3K27 trimethylation by Polycomb group proteins. In this study, we used two general approaches to examine the dependency relationships between these activities. First, we examined whether ASH1 is required for transcription elongation in the larval salivary gland. Second, we examined whether elongating RNA Pol II recruits ASH1 to active genes and counteracts H3K27 methylation catalyzed by PRC2.

ASH1 is not required for transcription elongation

ASH1 plays a key role in maintaining the expression of Hox genes during Drosophila development. ASH1 binds chromatin dynamically (Steffen et al., 2013) and localizes downstream of promoters at many transcriptionally active Polycomb group target genes, including Ubx (Papp and Müller, 2006; Schwartz et al., 2010; Tripoulas et al., 1994). In imaginal disc cells, ASH1 activates Ubx transcription by counteracting Polycomb repression, suggesting that ASH1 might function as a Polycomb anti-repressor as opposed to a transcriptional activator (Klymenko and Müller, 2004). However, the role of ASH1 in the activation of other genes has not been characterized in detail.

In the larval salivary gland, ASH1 associates with the vast majority of active genes (Srinivasan et al., 2008). In addition, loss of ASH1 binding to the majority of its sites on polytene chromosomes is correlated with the loss of transcription elongation in kis mutants.

To investigate whether KIS promotes transcription elongation by recruiting ASH1, we stained salivary gland polytene chromosomes from wild-type and ash1 mutant larvae with antibodies against ASH1 (Fig. 1A,B,G). By contrast, the loss of ash1 function had no effect on the level of Pol IIser2 or SPT6 associated with salivary gland polytene chromosomes (Fig. 1C-G). Thus, ASH1 is not required for elongation by RNA Pol II in this tissue.

Fig. 1. ASH1 is not required for transcription elongation. (A-F) Polytene chromosomes from wild-type (A,C,E) and ash1 mutant (B,D,F) Drosophila larvae were stained with antibodies against ASH1 (A,B), RNA Pol Iloser2 (C,D) and SPT6 (E,F). (G) The relative fluorescence intensity is significantly reduced for ASH1 (P=0.0003), but is not significantly reduced for SPT6 (P=0.11) or Pol Iloser2 (P=0.52). Error bars in all figures indicate s.d.

Fig. 2. DRB inhibits phosphorylation of RNA Pol II on serine 2. (A-L) Polytene chromosomes were treated with DMSO as a control (A,B,E,F,I,J) or the drug DRB (65 μM) (C,D,G,H,K,L) and stained with antibodies against RNA Pol Ila (A,C), RNA Pol Iloser5 (E,G), RNA Pol Iloser2 (I,K) and KIS-L (B,D,F,H,J,L). (M) The average fluorescence intensity normalized to the DMSO control shows an 85% reduction in RNA Pol Iloser2 fluorescence (P=5×10−5) on the DRB-treated chromosomes, but no change in KIS-L or RNA Pol Ila and only a 60% reduction in RNA Pol Iloser5 (P=0.0005).
Elongating RNA Pol II does not recruit ASH1 to active genes

ASH1 is recruited to its target genes in an activation-dependent manner through mechanisms that are not well understood (Gregory et al., 2007; Papp and Müller, 2006; Schwartz et al., 2010). We therefore considered whether elongating RNA Pol II recruits ASH1 to sites downstream of active gene promoters. This possibility was attractive because the CTD of RNA Pol II recruits a wide variety of factors involved in transcription (Zhang et al., 2012).

To determine if ASH1 is recruited by elongating RNA Pol II, we examined whether its association with polytene chromosomes is affected by an inhibitor of transcription elongation, DRB (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside). DRB is a nucleoside analog that primarily binds to and inhibits CDK9, the serine 2 CTD kinase subunit of p-TEFb (Bensaude, 2011), and has more moderate effects on CDK7, the serine 5 CTD kinase (supplementary material Figs S1, S2) (Mancebo et al., 1997). Owing to its effects on CTD phosphorylation, DRB and other drugs that inhibit p-TEFb are potent inhibitors of transcription elongation both in vivo and in vitro (Chodosh et al., 1989; Egyházi et al., 1996; Giardina and Lis, 1993; Ni et al., 2008; Wada et al., 1998). Salivary glands of wild-type larvae were dissected and treated with DRB; polytene chromosomes were then fixed and stained with antibodies against RNA Pol II to monitor the effect of DRB treatment on transcription elongation (Fig. 2). Treatment with 65 μM DRB led to a significant (85%) decrease in the level of elongating Pol II (Pol IIo ser2) on polytene chromosomes relative to controls (Fig. 2I,K,M). By contrast, DRB treatment did not decrease the level of initiating Pol II (Pol IIa) and caused only a modest (60%) decrease in the level of promoter-proximal Pol II (Pol IIo ser5) associated with chromosomes relative to controls (Fig. 2A-H,M).

Importantly, DRB treatment had no effect on the association of KIS with polytene chromosomes, relative to controls (Fig. 2; supplementary material Figs S1, S2). As expected, the chromosomal association of the elongation factor SPT6 was significantly decreased upon DRB treatment (Fig. 3A-E). Thus, DRB treatment mimics the effect of kis mutations on transcription elongation and SPT6 recruitment without altering the expression or recruitment of the KIS protein.

Using the same DRB treatment conditions described above, we next investigated whether elongating Pol II recruits ASH1 to active genes. We failed to observe any change in the level or distribution of ASH1 on polytene chromosomes following DRB treatment, relative to controls (Fig. 3F-J). We also compared the distributions of ASH1, elongating Pol II and SPT6 on salivary gland polytene chromosomes. SPT6 binds to the CTD of elongating Pol II (Yoh et al., 2007) and displays almost perfect colocalization with Pol IIo ser2 on polytene chromosomes (Fig. 4A-C; supplementary material Fig. S3). Although the distributions of ASH1 and elongating Pol II on polytene chromosomes were similar, their banding patterns were not identical and their relative levels were highly variable from site to site (Fig. 4D-F; supplementary material Fig. S3).

**Fig. 3. ASH1 does not require elongating RNA Pol II to localize to chromatin. (A-D,F-I) Polytene chromosomes were treated with DMSO (A,B,F,G) or DRB (C,D,H,I) and stained with antibodies against RNA Pol II oser2 (green, B,D,G,I) and either SPT6 (red, A,C) or ASH1 (red, F,H). (E,J) The relative fluorescence intensity of SPT6 was reduced 70% (P=0.001) on the DRB-treated chromosomes (E). By contrast, the fluorescence intensity of ASH1 was not significantly reduced (P=0.77) on the DRB-treated chromosomes (J).**
As an alternative approach for investigating whether elongating Pol II recruits ASH1 to transcriptionally active genes, we examined the association of ASH1 with heat-shock puffs of salivary gland polytene chromosomes. Heat shock leads to the rapid transcriptional activation of the Hsp70 genes at 87A and 87C accompanied by the accumulation of high levels of elongating RNA Pol II at heat-shock puffs. The distributions of KIS, ASH1 and Pol IIOser2 on polytene chromosomes were examined after shifting third instar larvae from 18°C to 37°C for 40 minutes. As expected, heat shock triggered the accumulation of high levels of elongating RNA Pol II at 87A/C and other heat-shock puffs on salivary gland polytene chromosomes (Fig. 5A,B; supplementary material Fig. S4A). By contrast, we failed to observe the recruitment of either KIS or ASH1 to these sites (Fig. 5C,D; supplementary material Fig. S4B). We therefore conclude that elongating Pol II does not recruit KIS or ASH1 to active genes.

**KIS does not directly recruit ASH1 to chromatin in vivo**

We next examined whether KIS directly recruits ASH1 to transcriptionally active genes. Previous biochemical studies failed to detect stable interactions between KIS and other trithorax group proteins in the Drosophila embryo (Papoulas et al., 1998; Srinivasan et al., 2005; Srinivasan et al., 2008), and ASH1 and KIS-L (one of the two major isoforms of KIS) do not co-immunoprecipitate from embryo protein extracts (data not shown). We therefore used an alternative approach to investigate whether KIS recruits ASH1 to chromatin in vivo. We previously demonstrated that the expression of a GAL4-inducible transgene encoding wild-type KIS-L in salivary gland nuclei leads to a significant (~4-fold) increase in the level of KIS-L associated with polytene chromosomes (Fasulo et al., 2012). The chromosomal distribution of KIS-L in ey-GAL4; UAS-KIS-L larvae appears normal, as evidenced by its extensive overlap with BRM (supplementary material Fig. SSD-F), which is also observed in wild-type (Srinivasan et al., 2005) and control (ey-GAL4; UAS-lacZ) larvae (supplementary material Fig. S5A-C). The expression of elevated levels of KIS-L did not lead to a concomitant increase in the levels of ASH1 or BRM associated with polytene chromosomes (Fig. 6; supplementary material Fig. S5).

**Fig. 4. SPT6 but not ASH1 colocalizes with elongating RNA Pol II on polytene chromosomes.**

(A-C) Merged image (A) of polytene chromosomes stained with antibodies against SPT6 (red) and RNA Pol IIOser2 (green). The chromosome arm in A (boxed) is magnified in B, showing the banding patterns of SPT6 and Pol IIOser2, alone and split. A comparison of the band size and intensity for SPT6 and Pol IIOser2 (C) confirms the highly coincident pattern. (D-F) Merged image (D) of polytene chromosomes stained with antibodies against ASH1 (red) and RNA Pol IIOser2 (green). The chromosome arm in D (boxed) is magnified in E, showing the banding pattern of ASH1 and Pol IIOser2, alone and split. The band intensity distribution of ASH1 and Pol IIOser2 (F) shows that although Pol IIOser2 is present at many sites of ASH1, the intensities of each are not well correlated.

**Fig. 5. ASH1 is not recruited to heat-shock puffs by elongating RNA Pol II.**

(A) Merged image of polytene chromosomes from heat shocked third instar larvae stained with antibodies against ASH1 (red) and Pol IIOser2 (green). (B) Magnification of the region encompassing the 87A/C heat-shock puffs (arrowheads) from A. (C,D) ASH1 staining alone (C) and higher magnification (D) show that elongating RNA Pol II does not recruit ASH1 to heat-shock puffs (arrowheads).
We therefore conclude that KIS is necessary but not sufficient for the recruitment of ASH1 to active genes in vivo.

**KIS does not antagonize Polycomb repression by promoting transcription elongation**

H3K27 methylation catalyzed by PRC2 is essential for transcriptional repression mediated by Polycomb group proteins (Cao et al., 2002; Cao and Zhang, 2004). Many models exist for how repressive H3K27 methylation is held in check by KIS and other trithorax group proteins. Active transcription is associated with histone exchange (Henikoff et al., 2004), H3K27me3 demethylation (Smith et al., 2008) and inhibition of silencing at PREs (Rank et al., 2002; Schmitt et al., 2005). These observations suggested that KIS might counteract H3K27me3 by promoting transcription elongation.

A recent genome-wide survey of chromatin states in *Drosophila* cultured cells revealed that Polycomb domains marked by H3K27me3 occupy separate domains to those associated with active transcription (Kharchenko et al., 2011). This study also revealed that, although a small number of “balanced” Polycomb domains do have active transcription, these are notably lacking H3K27 methylation. Furthermore, boundaries of H3K27 methylation in *Drosophila* are correlated with gene promoters bound by RNA Pol II (Schuettengruber and Cavalli, 2013). To determine whether transcription elongation counteracts H3K27me3, we first examined the localization of Pol IIoser2 relative to domains of H3K27me3. If transcription elongation antagonizes the methylation of H3K27 on polytene chromosomes we might expect regions of elongating Pol II to be devoid of bands of H3K27me3. We found that, although Pol IIoser2 and H3K27me3 are largely non-overlapping, there are some regions of partial overlap, suggesting that the presence of elongating Pol II might not be sufficient to block H3K27 methylation (Fig. 7A-C; supplementary material Fig. S6A). Furthermore, the treatment of salivary glands with DRB for up to 3 hours did not increase levels of H3K27me3 on salivary gland chromosomes (Fig. 7D-H; supplementary material Fig. S7). After 3 hours of DRB treatment *ex vivo*, degeneration of the salivary gland makes immunostaining infeasible. Although it is possible that a prolonged block of transcription elongation might ultimately affect H3K27me3 levels, our data suggest that loss of transcription elongation is not responsible for the loss of ASH1 or the increase in H3K27me3 observed in *kis* mutants.

**KIS is required for H3K36 tri- and di-methylation**

The above findings suggested that KIS antagonizes H3K27 methylation and promotes transcription elongation via independent mechanisms. This prompted us to investigate alternative mechanisms by which KIS might antagonize Polycomb repression. We had previously determined that the loss of *kis* function increases the level of H3K27me3 on polytene chromosomes without causing obvious changes in the level or distribution of PRC2 (Srinivasan et al., 2008). Thus, KIS does not antagonize H3K27 methylation by blocking the recruitment of Polycomb group proteins to chromatin. Recent studies have reported that methylation of H3K4 and H3K36 can inhibit the methylation of H3K27 by PRC2 *in vitro* (Schmitges et al., 2011; Yuan et al., 2011). We have previously shown that loss of KIS has no effect on di- or tri-methylation of H3K4 (Srinivasan et al., 2008); however, the role of KIS in H3K36 methylation has not been explored.

To investigate whether KIS antagonizes H3K27me3 by promoting H3K36 methylation, we stained polytene chromosomes from wild-type and *kis* mutant larvae with antibodies against ASH1 (green, B,E) or Pol Iloser2 (blue, C,F). (G) The average fluorescence intensity normalized to the *lacZ* control shows a ~3-fold increase in KIS-L staining (P=2×10^{-5}) on polytene chromosomes from KIS-L-expressing larvae. By contrast, the fluorescence intensity of ASH1 is not significantly increased (P=0.57) and Pol Iloser2 staining is only slightly increased (P=0.01) on polytene chromosomes from KIS-L-expressing larvae.

**ASH1 promotes dimethylation of H3K36 in vivo**

The concomitant reduction in the levels of H3K36me2 and ASH1 on the polytene chromosomes of *kis* mutants suggested a potential role for ASH1 in H3K36 methylation *in vivo*. ASH1 has been reported to methylate a number of histone residues, including H3K4, H3K9 and H4K20 (Beisel et al., 2002; Byrd and Shearn, 2003); however, more recent *in vitro* studies indicate that ASH1 specifically dimethylates H3K36 (An et al., 2011; Tanaka et al., 2007; Yuan et al., 2011). Interestingly, H3K36 methylation also inhibits the methylation of H3K27 by PRC2 *in vitro* (Schmitges et al., 2011; Yuan et al., 2011). Taken together, these data suggest that
KIS might counteract Polycomb repression by promoting ASH1-dependent H3K36 dimethylation. We therefore investigated the relationship between ASH1 and H3K36 dimethylation in vivo.

We first examined the distribution of ASH1 relative to H3K36me2 on polytene chromosomes. We reasoned that if ASH1 catalyzes H3K36me2 then we should observe a significant overlap between the chromosomal distributions of ASH1 and H3K36me2. ASH1 localizes to virtually every site of H3K36me2, but their relative levels vary considerably from site to site (Fig. 9A,B). We next examined whether the loss of ASH1 function affects the chromosomal levels of H3K36me2. Strikingly, we observed a significant (68%) reduction in the level of H3K36me2 on the
polytene chromosomes of ash1<sup>17</sup>/ash1<sup>22</sup> larvae compared with wild type (Fig. 9D,G,I). In contrast to the effect of ASH1 on H3K36me2, we did not observe a reduction in the levels of H3K36me3 in ash1 mutant larvae relative to controls (Fig. 9E,H,I). These findings suggest that ASH1 specifically dimethylates H3K36<sup>+</sup> in vivo.

To investigate whether H3K36me2 inhibits the ability of PRC2 to trimethylate H3K27<sup>+</sup> in vivo, we compared the distribution of the two modifications on salivary gland polytene chromosomes. Consistent with a functional antagonism between H3K36 dimethylation and H3K27 trimethylation, we observed very little overlap between these marks (Fig. 10A,B; supplementary material Fig. S6B). Furthermore, we observed that H3K36me2 often flanks regions of H3K27me3 (Fig. 10A,B). This juxtaposed pattern suggests that H3K36me2 might function as a barrier to the spread of H3K27me3. Finally, to determine whether the reduction in chromosomal H3K36me3 levels observed in ash1 mutants occurs concomitantly with the previously reported increase in H3K27me3, we co-stained polytene chromosomes from ash1 mutant larvae with antibodies against H3K27me3 and H3K36me3. Indeed, the increased levels of H3K27me3 on the polytene chromosomes of ash1 mutant larvae were accompanied by a reduction in the levels of H3K36me3 relative to controls (Fig. 10C-G). These observations suggest that ASH1 antagonizes H3K27 methylation catalyzed by PRC2 by dimethylating H3K36.

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Fig. 9. ASH1 promotes H3K36 dimethylation in vivo. (A,B) Colocalization of ASH1 (green) and H3K36me2 (red) on the distal arm of a polytene chromosome (A), together with a comparison of the band intensities along the arm (B). (C-H) Polytene chromosomes from wild-type (C-E) and ash1 mutant (F-H) larvae were stained with antibodies against ASH1 (C,F), H3K36me2 (D,G) and H3K36me3 (E,H). (I) A 68% reduction in the average fluorescence intensity of H3K36me2 (P=7.5×10<sup>–7</sup>) and no significant change in H3K36me3 (P=0.088) were observed in ash1 mutants.

Fig. 10. Decrease in H3K36me2 is correlated with increase in H3K27me3 in ash1 mutants. (A,B) The banding patterns of H3K27me3 (green) and H3K36me2 (red) from a polytene chromosome arm (A), together with the distribution of pixel intensities along an arm (B). (C-F) Polytene chromosomes from wild-type (C,D) and ash1 mutant (E,F) larvae stained with antibodies against H3K27me3 (C,E) and H3K36me2 (D,F). (G) Relative fluorescence intensities show a significant increase in H3K27me3 (P=4.8×10<sup>–6</sup>) and decrease in H3K36me2 (P=1.2×10<sup>–6</sup>).
DISCUSSION
KIS counteracts Polycomb repression independently of its role in facilitating transcription elongation
Since KIS promotes transcription elongation, promotes ASH1 binding and counteracts Polycomb repression, we suspected that these activities might be functionally interdependent. However, the loss of ASH1 function leads to an increase in repressive H3K27 trimethylation without affecting transcription elongation. Furthermore, the treatment of salivary glands with the elongation inhibitor DRB did not affect the level of ASH1 or H3K27me3 associated with polytenic chromosomes. We therefore conclude that KIS promotes transcription elongation and antagonizes Polycomb repression via distinct mechanisms.

Trithorax group proteins counteract Polycomb repression by methylating histone H3
Our findings suggest that the major mechanism by which KIS antagonizes Polycomb group repression is by promoting the association of the trithorax group histone methyltransferases ASH1 and TRX with chromatin. Recent biochemical studies have suggested several mechanisms by which ASH1 and TRX counteract Polycomb repression. A histone modification catalyzed by TRX in vitro – H3K4 trimethylation – disrupts interactions between PRC2 and its nucleosome substrate. H3K4me3 directly interferes with the binding of the PRC2 subunit NURF55 (CAF1) to nucleosomes and inhibits the catalytic activity of E(Z) allosterically through interactions with the SU(Z)12 subunit of PRC2 (Schmitges et al., 2011). The relevance of this modification to TRX function in vivo is not clear, however, as the bulk of H3K4 trimethylation in Drosophila is catalyzed by the histone histone methyltransferase SET1 (Ardehali et al., 2011; Hallson et al., 2012). Another mechanism by which TRX counteracts Polycomb repression was suggested by its physical association with the histone acetyltransferase CBP in the TAC1 complex (Petruk et al., 2001). The acetylation of H3K27 by CBP directly blocks the methylation of this residue by PRC2 (Tie et al., 2009). It is therefore tempting to speculate that the diminished binding of TAC1 to active genes contributes to the increased methylation of H3K27me3 observed in kis mutants.

KIS promotes H3K36 methylation
Other histone modifications, including both the di- and trimethylation of H3K36, also block the catalytic activity of PRC2 in vitro (Schmitges et al., 2011; Yuan et al., 2011). In Drosophila, H3K36 trimethylation is catalyzed by SET2, which associates with the elongating RNA Pol II via its phosphorylated CTD. In this way, H3K36me3 becomes concentrated at the 3’ ends of genes where it plays a role in preventing cryptic initiation (Bell et al., 2007; Stabell et al., 2007). Consistent with its role in transcription elongation, kis mutations decreased the level of H3K36me3 on polytenic chromosomes. Interestingly, H3K36me3 blocks the methylation of H3K27 at genes expressed in the C. elegans germline (Gaydos et al., 2012). Thus, H3K36 trimethylation might represent a conserved mechanism for antagonizing PRC2 function to maintain appropriate patterns and steady-state levels of transcription. Transcription-dependent H3K36 trimethylation is unlikely to be the sole mechanism by which KIS counteracts Polycomb repression, however, because blocking transcription elongation with DRB did not increase the level of H3K27me3 on polytenic chromosomes. Furthermore, ash1 mutants display elevated levels of H3K27 methylation without a reduction in H3K36 trimethylation or transcription elongation, suggesting that additional mechanisms exist to counteract repressive H3K27 methylation.

ASH1 dimethylates H3K36 in vivo
An antagonism between H3K36 dimethylation and H3K27 trimethylation was suggested by the recent discovery that H3K36me2 inhibits PRC2 function in vitro (Schmitges et al., 2011; Yuan et al., 2011). This finding, together with recent evidence that ASH1 dimethylates H3K36 in vitro (An et al., 2011; Tanaka et al., 2007; Yuan et al., 2011), prompted us to investigate whether ASH1 also dimethylates H3K36 in vivo. The chromosomal distributions of ASH1 and H3K36me2 overlap significantly, consistent with their localization at the 5’ end of active genes (Bell et al., 2007; Papp and Müller, 2006; Schwartz et al., 2010). Furthermore, the levels of H3K36me2 on the polytenic chromosomes of both ash1 and kis mutant larvae were significantly reduced, consistent with the role of KIS in promoting ASH1 binding. Taken together, these observations strongly suggest that KIS antagonizes Polycomb repression by promoting the ASH1-dependent dimethylation of H3K36.

The differences in the chromosomal distributions of ASH1 and H3K36me2 and the residual H3K36me2 observed in ash1 mutants are probably due to the presence of another H3K36 dimethylase – MES-4 – in Drosophila. In addition to dimethylating H3K36, MES-4 is required for SET2-dependent H3K36 trimethylation in vivo, as revealed by RNAi knockdown of MES-4 both in larvae and in cultured cells (Bell et al., 2007). By contrast, we failed to observe a significant reduction in H3K36me3 levels in ash1 mutant larvae. Our findings suggest that ASH1 and MES-4 play non-redundant roles in H3K36 methylation in vivo.

Fig. 11. KIS promotes histone modifications that antagonize H3K27 methylation. KIS promotes H3K36 dimethylation in the vicinity of active genes by promoting the association of ASH1 with chromatin. KIS also promotes TRX binding, which acetylates H3K27 through its association with CBP in the TAC1 complex (Tie et al., 2009). KIS independently promotes transcription elongation, resulting in H3K36 trimethylation by SET2 at the 3’ end of genes. Each of these histone modifications antagonizes repressive H3K27 catalyzed by the E(Z) subunit of PRC2. GTFs, general transcription factors.
KIS regulates H3 methylation

It is becoming increasingly clear that multiple mechanisms – including the trimethylation of H3K4, the di- and tri-methylation of H3K36 and the acetylation of H3K27 – antagonize repressive H3K27 methylation catalyzed by Polycumb group proteins. Our findings suggest that KIS plays a central role in coordinating these activities (Fig. 11). By facilitating the binding of TRX and ASH1, KIS promotes H3K27 acetylation and H3K36 dimethylation in the vicinity of active promoters. By stimulating elongation, KIS promotes H3K36 trimethylation over the body of transcribed genes. Thus, KIS appears to counteract Polycumb group repression by promoting multiple histone modifications that inhibit H3K27 methylation by the E(Z) subunit of PRC2.

References


Fig. S1. DRB preferentially inhibits phosphorylation of the RNA Pol II CTD at serine 2. Salivary glands were treated with increasing concentrations of DRB to determine the optimal concentration to maximally inhibit elongating RNA Pol II (Pol II<sup>ser2</sup>) (red, upper panel) without affecting paused RNA Pol II (Pol II<sup>ser5</sup>) (red, lower panel). The chromosomes in each experiment were co-stained with antibodies against KIS-L (green), indicating that KIS-L localization is unaffected by loss of transcription elongation.
Fig. S2. Western blot analysis of RNA Pol II phosphorylation state and chromatin-associated proteins from salivary glands treated with DRB. (A) Protein extracts from salivary glands treated with 65 μM DRB or DMSO as a control were probed for the abundance of KIS-L, Pol II<sup>ser5</sup>, Pol II<sup>ser2</sup> and ISWI. (B) In a separate experiment, protein extracts from salivary glands treated with 65 μM DRB or DMSO as a control were probed for the abundance of histone H3 trimethylated on lysine 27 (H3K27).
Fig. S3. Polytene staining of SPT6, ASH1 and Pol IIoαα2. Individual images of the polytene chromosomes from wild-type larvae stained with antibodies against SPT6 (red, A) and Pol IIoαα2 (green, B) and against ASH1 (red, C) and Pol IIoαα2 (green, D) that were used to generate the merged images shown in Fig. 4A and Fig. 4D.
Fig. S4. KIS is not recruited to heat-shock puffs by elongating RNA Pol II. (A) Merged image of a region on polytene chromosomes from heat shocked third instar larvae stained with antibodies against KIS-L (red) and Pol IIαser2 (green). The 87A/C heat-shock puffs are indicated by arrowheads. (B) KIS-L staining alone shows that elongating RNA Pol II does not recruit KIS-L to heat-shock puffs (arrowheads).

Fig. S5. Ectopically expressed KIS binds to endogenous sites on polytene chromosomes. (A-F) Polytene chromosomes from larvae expressing transgenes encoding β-gal (A-C) or KIS-L (D-F) in the salivary gland under the control of a GAL4-inducible promoter were stained with antibodies against KIS-L (red, A,D) and BRM (green, B,E). The merged images (C,F) show that KIS-L binds to its native sites on polytene chromosomes, as evidenced by the yellow banding pattern in both the β-gal control and KIS-L expression. The intensity of the KIS-L signal was increased in the merged image of the β-gal control to visualize the colocalization. (G) The average fluorescence intensity normalized to the β-gal control shows an approximate 4-fold increase in KIS-L staining ($P=0.0001$) on polytene chromosomes from KIS-L-expressing larvae. By contrast, the fluorescence intensity of BRM is only slightly increased ($P=0.03$) on polytene chromosomes from KIS-L-expressing larvae relative to controls.
**Fig. S6. Colocalization analysis of chromatin marks and proteins.** Pairwise scatter plots of overlapping pixel intensities were generated from merged images of wild-type polytene chromosomes (shown above each plot) to examine the colocalization of: (A) Pol IIoser2 (green) and H3K27 (red); (B) H3K27me3 (green) and H3K36me2 (red); (C) SPT6 (green) and Pol IIoser2 (red); and (D) BRM (green) and KIS (red). The position and color of the dots indicate the level of colocalization at each pixel, with yellow dots along the diagonal axis indicating a perfect correlation. Pearson’s correlation coefficients (PCC) are shown for each image.
Fig. S7. Long-term DRB treatment does not affect H3K27 methylation. Treatment of salivary glands for 3 hours with a 10-fold higher concentration of DRB (650 μM) does not cause increased chromosomal levels of H3K27 trimethylation (A,B) despite the strong reduction in staining of Pol IIser2 (C,D).