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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, DRB, 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 1* (*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 IIo^{ser2}) 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*^{k13416} allele resulted from the insertion of a P-element in the first intron of the *kis* gene; *kis*^{k13416} 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*^{22} and antimorphic *ash1*^{17} 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 IIo^{ser5} and Pol IIo^{ser2} (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 (Fasulo 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 $MgCl_2$, 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 5,6-dichlorobenzimidazole 1-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 Axiovision 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 Volocity 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 Volocity. 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 $MgCl_2$, 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 mouse antibodies against Pol IIo^{ser2} (H5) and Pol IIo^{ser5} (H14) (Covance) and rabbit antibodies against KIS (Daubresse 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 1 mM PMSF 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).

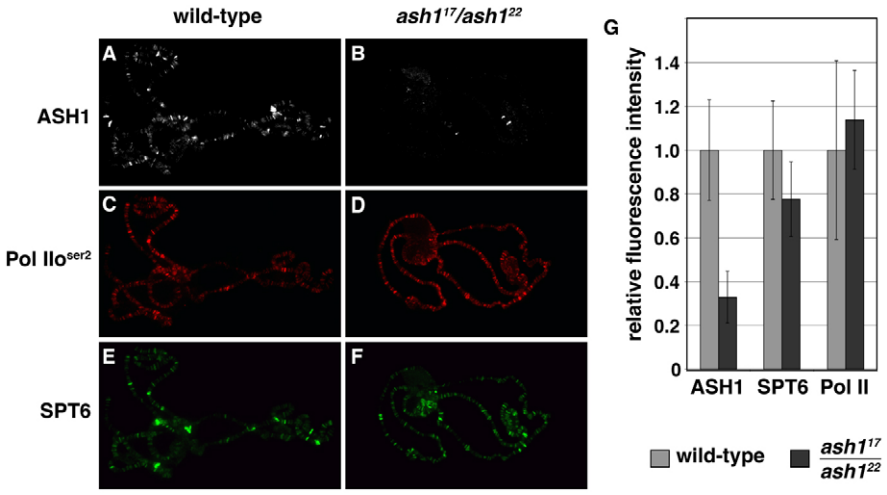

Fig. 1. ASH1 is not required for transcription elongation. (**A-F**) Polytene chromosomes from wild-type (A,C,E) and *ash1* mutant (B,D,F) *Drosophila* larvae were stained with antibodies against ASH1 (A,B), RNA Pol IIo^{ser2} (C,D) and SPT6 (E,F). (**G**) The relative fluorescence intensity is significantly reduced for ASH1 (P=0.0003), but is not significantly reduced for SPT6 (P=0.11) or Pol IIo^{ser2} (P=0.52). Error bars in all figures indicate s.d.

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, Pol IIo^{ser2} and the elongation factor SPT6. *ash1*^{22}/*ash1*^{17} transheterozygotes survive until the third larval instar and display significantly reduced levels of ASH1 on polytene chromosomes (Fig. 1A,B,G). By contrast, the loss of *ash1* function had no effect on the level of Pol IIo^{ser2} 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.

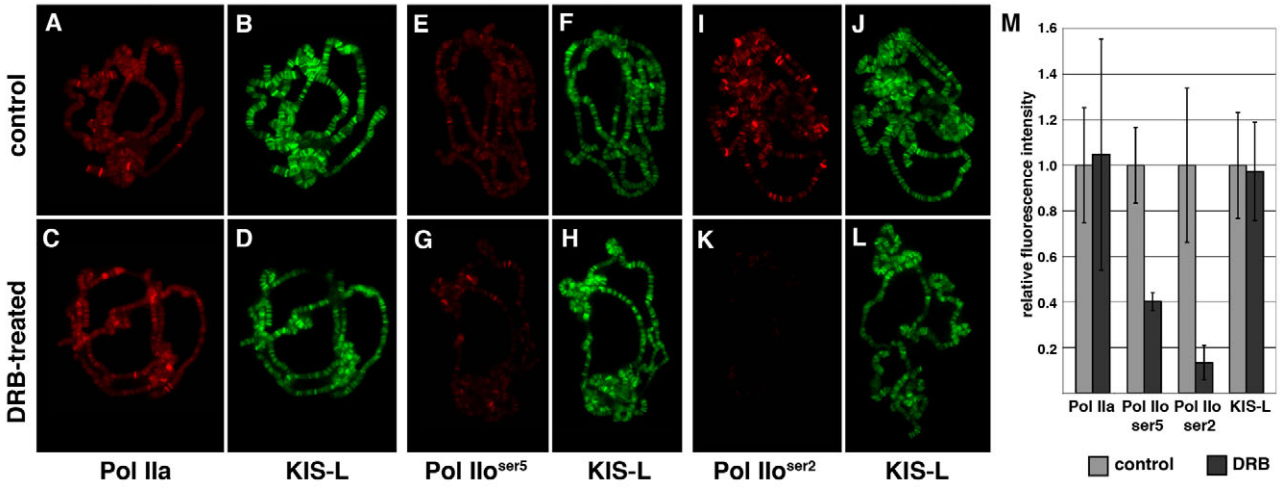

Fig. 2. DRB inhibits phosphorylation of RNA Pol II on serine 2. (**A-L**) Polytene chromosomes were treated with DMSO as a control (A,B,E,F,I,J) or the drug DRB (65 μM) (C,D,G,H,K,L) and stained with antibodies against RNA Pol IIa (A,C), RNA Pol IIo^{ser5} (E,G), RNA Pol IIo^{ser2} (I,K) and KIS-L (B,D,F,H,J,L). (**M**) The average fluorescence intensity normalized to the DMSO control shows an 85% reduction in RNA Pol IIo^{ser2} fluorescence (P=5×10^{-5}) on the DRB-treated chromosomes, but no change in KIS-L or RNA Pol IIa and only a 60% reduction in RNA Pol IIo^{ser5} (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).

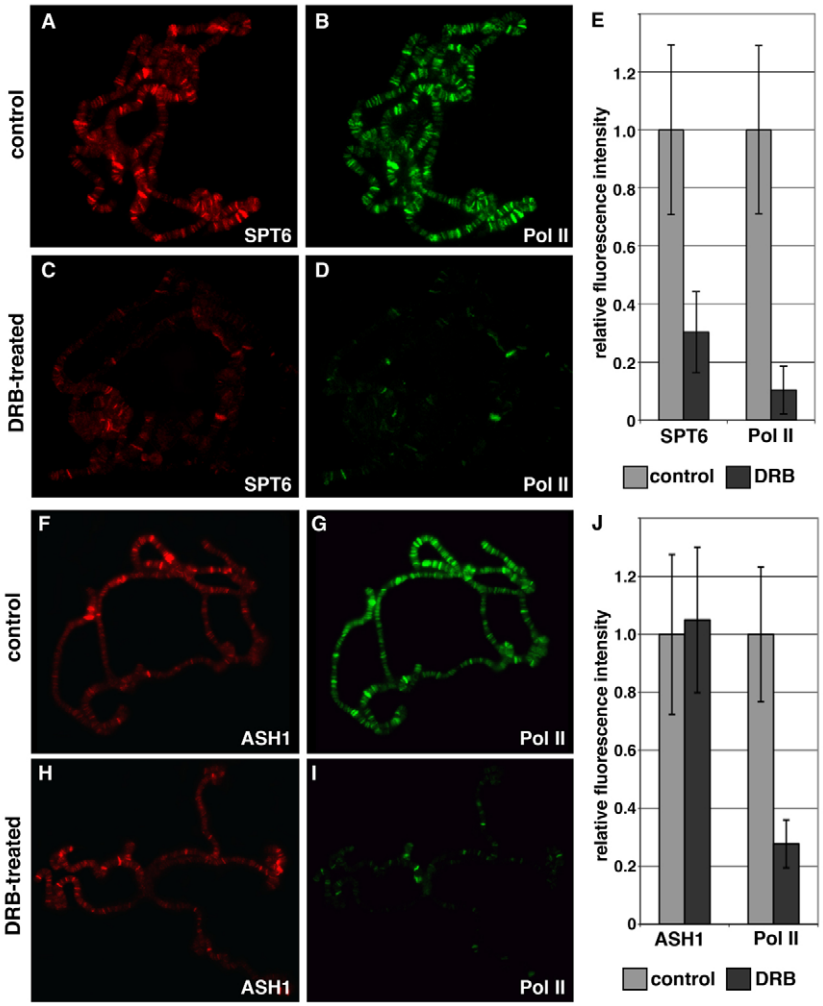

Fig. 3. ASH1 does not require elongating RNA Pol II to localize to chromatin. (**A-D**,**F-I**) Polytene chromosomes were treated with DMSO (A,B,F,G) or DRB (C,D,H,I) and stained with antibodies against RNA Pol IIo^{ser2} (green, B,D,G,I) and either SPT6 (red, A,C) or ASH1 (red, F,H). (**E**,**J**) The 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).

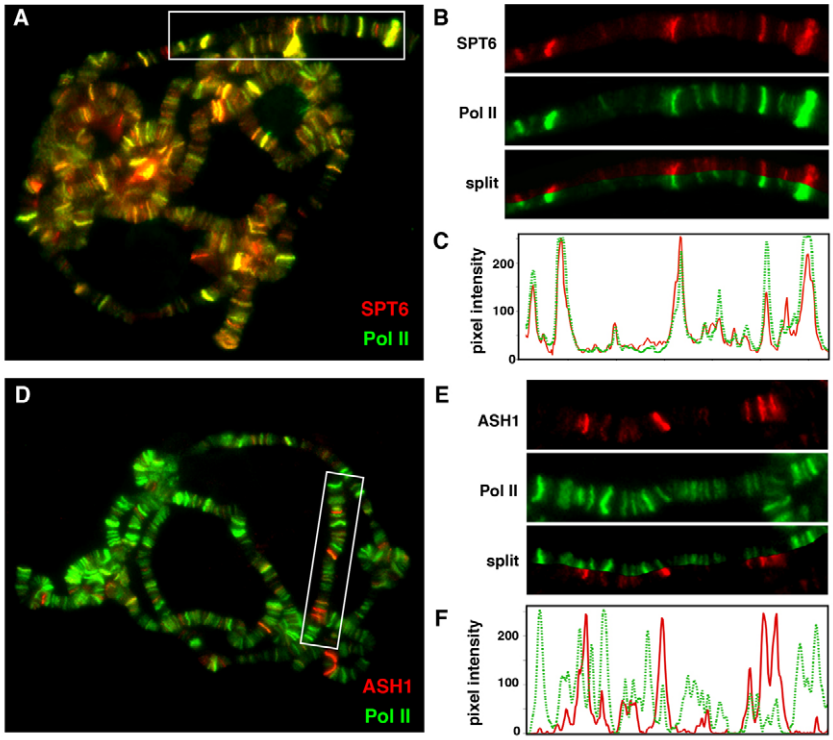

Fig. 4. SPT6 but not ASH1 colocalizes with elongating RNA Pol II on polytene chromosomes. (**A-C**) Merged image (A) of polytene chromosomes stained with antibodies against SPT6 (red) and RNA Pol IIo^{ser2} (green). The chromosome arm in A (boxed) is magnified in B, showing the banding patterns of SPT6 and Pol IIo^{ser2}, alone and split. A comparison of the band size and intensity for SPT6 and Pol IIo^{ser2} (C) confirms the highly coincident pattern. (**D-F**) Merged image (D) of polytene chromosomes stained with antibodies against ASH1 (red) and RNA Pol IIo^{ser2} (green). The chromosome arm in D (boxed) is magnified in E, showing the banding pattern of ASH1 and Pol IIo^{ser2}, alone and split. The band intensity distribution of ASH1 and Pol IIo^{ser2} (F) shows that although Pol IIo^{ser2} is present at many sites of ASH1, the intensities of each are not well correlated.

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 IIo^{ser2} on polytene chromosomes were examined after shifting third instar larvae from 18° 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. S5D-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).

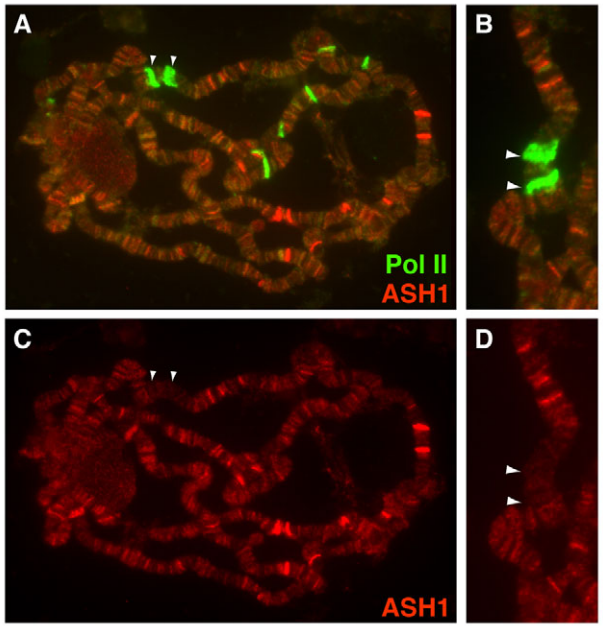

Fig. 5. ASH1 is not recruited to heat-shock puffs by elongating RNA Pol II. (**A**) Merged image of polytene chromosomes from heat shocked third instar larvae stained with antibodies against ASH1 (red) and Pol IIo^{ser2} (green). (**B**) Magnification of the region encompassing the 87A/C heat-shock puffs (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).

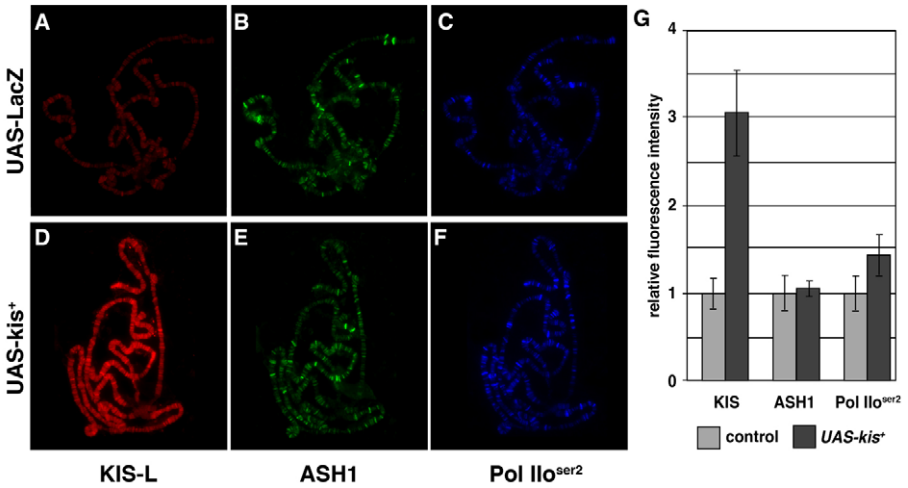

Fig. 6. KIS does not directly recruit ASH1 to chromatin. (**A-F**) Polytene chromosomes from larvae expressing *lacZ* (A-C) or KIS-L (D-F) in the salivary gland were stained with antibodies against KIS-L (red, A,D), ASH1 (green, B,E) or Pol IIo^{ser2} (blue, C,F). (**G**) The average fluorescence intensity 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 IIo^{ser2} staining is only slightly increased (P=0.01) on polytene chromosomes from KIS-L-expressing larvae.

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 IIo^{ser2} 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 IIo^{ser2} 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*^{k13416} mutant larvae with antibodies against H3K36me2 and H3K36me3. In *Drosophila*, H3K36 trimethylation is catalyzed by SET2 (also known as HYPB), which is known to associate with RNA Pol II (Bell et al., 2007; Stabell et al., 2007). As expected, the levels of both total Pol II and H3K36me3 were significantly reduced (65% and 70%, respectively) on polytene chromosomes from *kis* mutant larvae (Fig. 8A-D,I). The loss of *kis* function caused a similar decrease in H3K36me2 levels relative to controls (65%) (Fig. 8E,G,I). The decrease in H3K36me2 and H3K36me3 levels in *kis* mutants coincided with the previously reported increase in chromosomal levels of H3K27me3 (Fig. 8F,H,I). These data suggest that KIS might antagonize H3K27me3 by promoting H3K36 tri- and di-methylation.

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

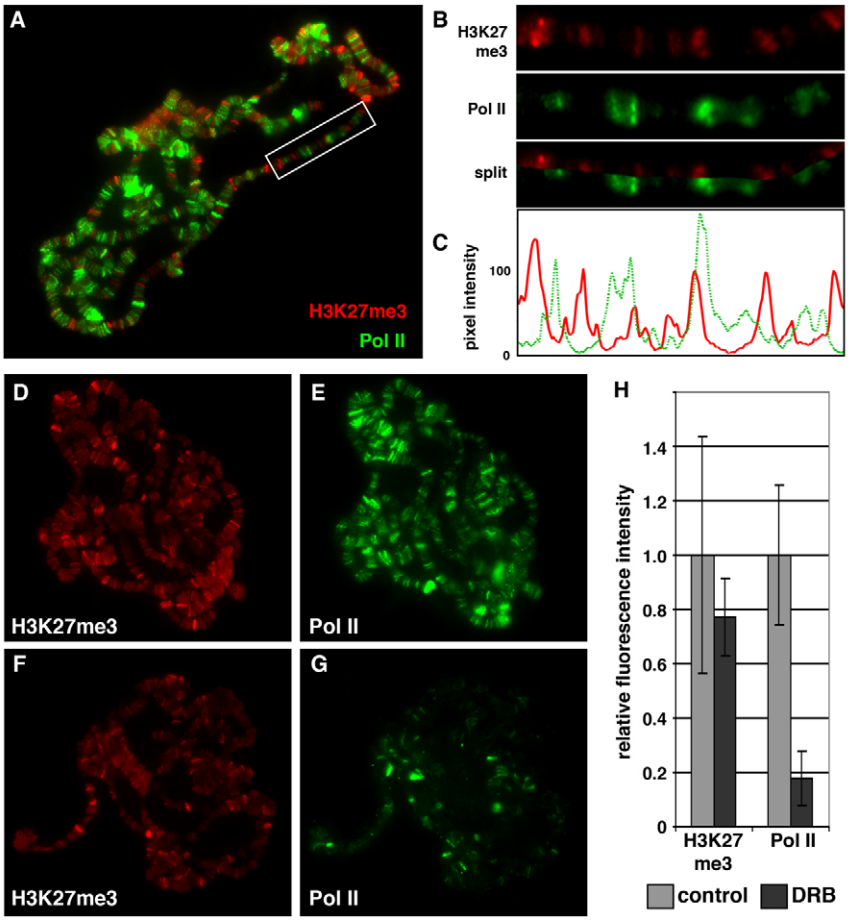

Fig. 7. DRB treatment does not affect the trimethylation of histone H3 on lysine 27. (**A-C**) Colocalization of the histone modification H3K27me3 (red) and Pol IIo^{ser2} (green) is shown on polytene chromosomes (A). The chromosome arm in A (boxed) is magnified in B. Bands of H3K27me3 and Pol IIo^{ser2} are often adjacent and occasionally partially overlapping, as shown in the split image and in the distribution of band intensities (C). (**D-G**) Polytene chromosomes treated with DMSO (D,E) or DRB (F,G) and stained with antibodies against H3K27me3 (D,F) and elongating Pol II (E,G). (**H**) No significant difference (P=0.08) in the relative fluorescence intensity of H3K27me3 was observed on polytene chromosomes from salivary glands treated with DRB.

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

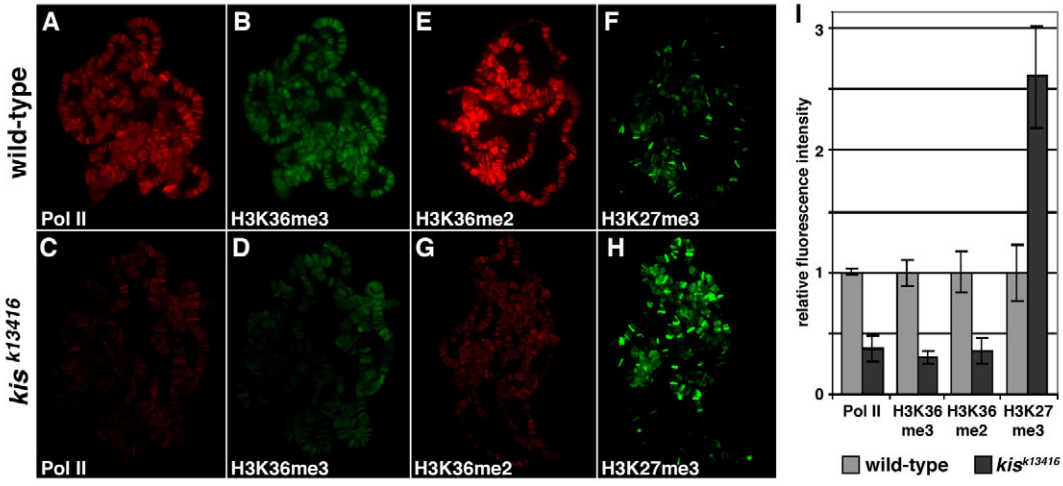

Fig. 8. Tri- and di-methylation of histone H3 on lysine 36 are reduced in *kis* mutants. (**A-H**) Polytene chromosomes from wild-type (A,B,E,F) and *kis* mutant (C,D,G,H) larvae were stained with antibodies against total RNA Pol II (A,C), H3K36me3 (B,D), H3K36me2 (E,G) and H3K27me3 (F,H). (**I**) The relative fluorescence intensity is significantly reduced for total Pol II (P=1.2×10^{-6}), H3K36me3 (P=9×10^{-7}) and H3K36me2 (P=0.0007) and significantly increased for H3K27me3 (P=0.0006).

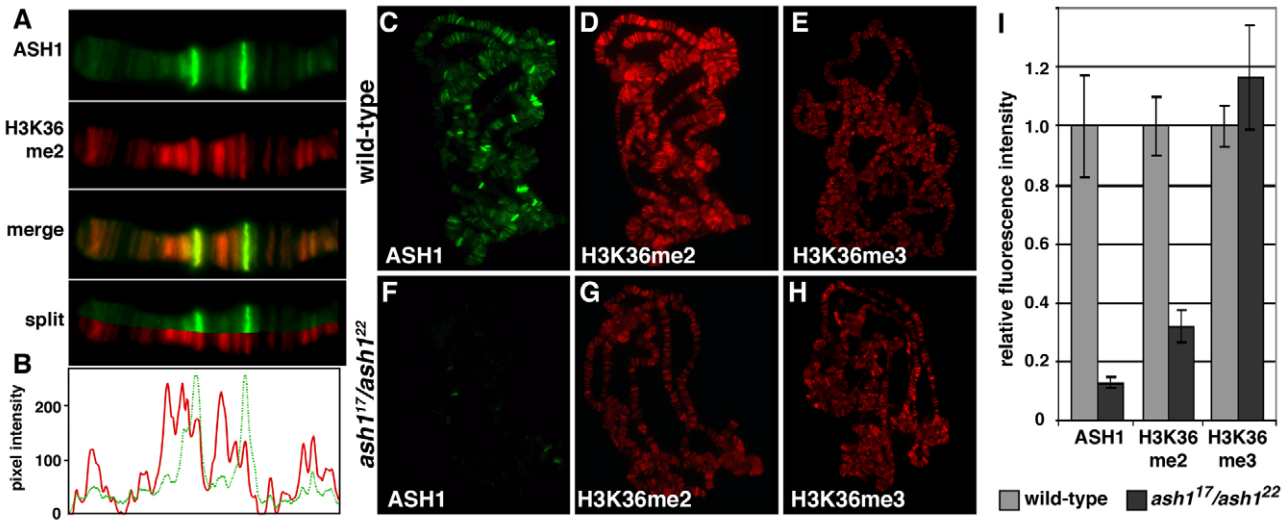

Fig. 9. ASH1 promotes H3K36 dimethylation *in vivo*. (**A**,**B**) Colocalization of ASH1 (green) and H3K36me2 (red) on the distal arm of a polytene chromosome (A), together with a comparison of the band intensities along the arm (B). (**C-H**) Polytene chromosomes from wild-type (C-E) and *ash1* mutant (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^{-7}) and no significant change in H3K36me3 (P=0.088) were observed in *ash1* mutants.

polytene chromosomes of *ash1*^{17}/*ash1*^{22} 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 *in vivo*.

To investigate whether H3K36me2 inhibits the ability of PRC2 to trimethylate H3K27 *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 H3K36me2 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 H3K36me2. Indeed, the increased levels of H3K27me3 on the polytene chromosomes of *ash1* mutant larvae were accompanied by a reduction in the levels of H3K36me2 relative to controls (Fig. 10C-G). These observations suggest that ASH1 antagonizes H3K27 methylation catalyzed by PRC2 by dimethylating H3K36.

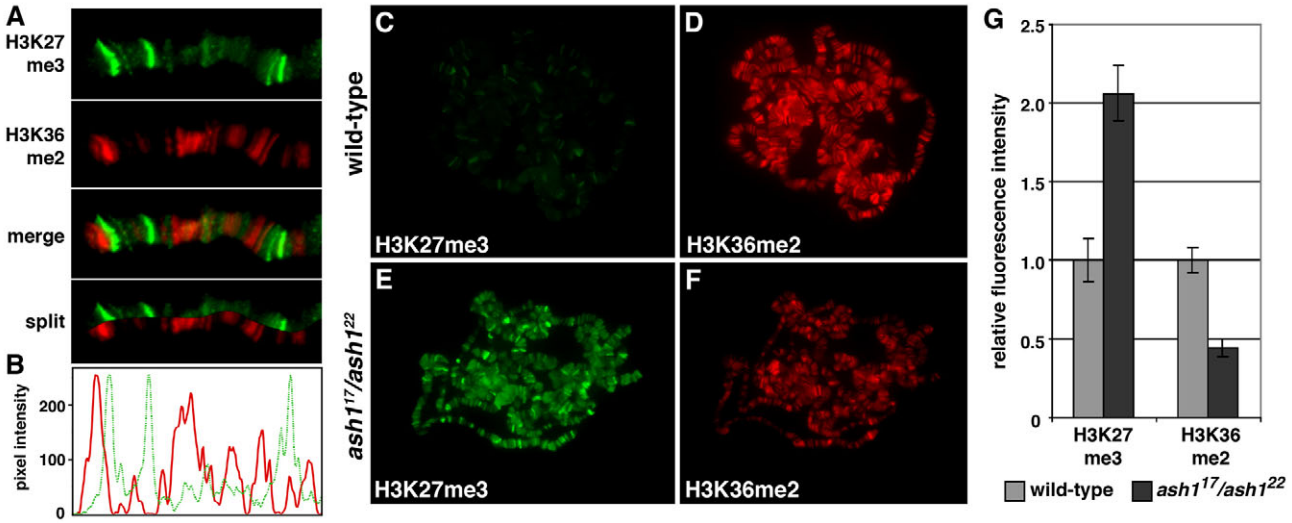

Fig. 10. Decrease in H3K36me2 is correlated with increase in H3K27me3 in *ash1* mutants. (**A**,**B**) The banding patterns of H3K27me3 (green) and H3K36me2 (red) from a polytene chromosome arm (A), together with the distribution of pixel intensities along an arm (B). (**C-F**) Polytene chromosomes from 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^{-6}) and decrease in H3K36me2 (P=1.2×10^{-6}).

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 polytene 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 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 tri-methylation 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 polytene 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 polytene 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 polytene 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*.

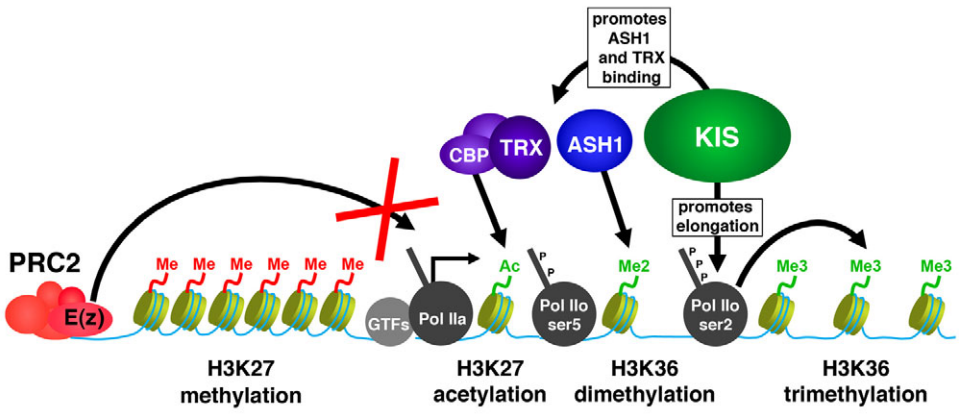

Fig. 11. KIS promotes histone modifications that antagonize H3K27 methylation. KIS promotes H3K36 dimethylation in the vicinity of active genes by promoting the association of ASH1 with chromatin. KIS also promotes TRX binding, which acetylates H3K27 through its association with CBP in the TAC1 complex (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 promotes multiple histone modifications that counteract H3K27 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 Polycomb 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 Polycomb group repression by promoting multiple histone modifications that inhibit H3K27 methylation by the E(Z) subunit of PRC2.

Implications for CHARGE syndrome

Haploinsufficiency for CHD7, a KIS homolog in humans, is the major cause of CHARGE syndrome, a serious developmental disorder affecting ~1 in 10,000 live births (Janssen et al., 2012). Infants born with CHARGE syndrome often have severe health complications due to defects in the development of tissues derived from the neural crest, including coloboma of the eye, cranial nerve abnormalities, ear defects and hearing loss, congenital heart defects, genital abnormalities and narrowing or blockage of the nasal passages (Janssen et al., 2012; Jongmans et al., 2006). Based on the phenotypes associated with *kis* mutations in *Drosophila*, it seems likely that some of these defects may stem from changes in gene expression resulting from loss of transcription elongation and inappropriate gene silencing by Polycomb group proteins. Our findings suggest that changes in histone H3 modifications resulting from the loss of CHD7 function might contribute to the broad spectrum of developmental defects associated with CHARGE syndrome.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

K.M.D. and J.W.T. conceived and designed the experiments. K.M.D. performed the experiments. K.M.D. and J.W.T. analyzed the data and wrote the paper.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.095786/-/DC1

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