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THE ROLE OF KISMET IN MAINTAINING TRANSCRIPTIONAL STATES IN  
*DROSOPHILA*

A dissertation submitted in partial satisfaction  
of the degree requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MOLECULAR, CELL AND DEVELOPMENTAL BIOLOGY

by

Kristel M. Dorigi

December 2012

The Dissertation of Kristel Dorigi is  
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Vice Provost and Dean of Graduate Studies



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## **Abstract**

### **The Role of Kismet in Maintaining Transcriptional States in *Drosophila***

Kristel Dorigi

Polycomb and trithorax group proteins play highly conserved roles in cell fate maintenance by affecting gene expression during development. Acting at the level of chromatin regulation, Polycomb group proteins repress transcription while trithorax group proteins promote transcription. My research has focused on the function of the trithorax group protein Kismet (KIS) in *Drosophila*. KIS is a member of the CHD family of ATP-dependent chromatin-remodeling factors and is related to CHD7, a human protein linked to CHARGE syndrome. KIS maintains HOX gene transcription and facilitates global transcription elongation in *Drosophila*. In this work, I examined how KIS interacts with other trithorax group proteins to maintain active states of transcription. I found that KIS promotes the localization of the trithorax group histone methyltransferases ASH1 and TRX to chromatin, which antagonizes the methylation of histone H3 on lysine 27 (H3K27) by Polycomb group proteins. KIS recruits ASH1 to chromatin and antagonizes H3K27 methylation independently of its role in facilitating transcription elongation. Finally, I examined the mechanism by which ASH1 counteracts H3K27 methylation and found evidence that ASH1 dimethylates histone H3 on lysine 36 (H3K36) *in vivo*. My work indicates that KIS plays an important role in coordinating the function of trithorax group histone methyltransferases to antagonize Polycomb group repression by counteracting H3K27 methylation.

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## **Chapter 1**

### **Introduction**

Most multicellular organisms originate from a single fertilized egg (GILBERT 2000). All the information necessary to initiate development is contained within this zygote. It will divide many times - its daughter cells acquiring unique patterns of gene expression and differentiating according to the developmental program encoded in their genome and epigenome (OSTRUP *et al.* 2012). The fundamental challenge of developmental biology is to understand how this program is specified, maintained, regulated and ultimately carried out to give rise to a fully formed individual. My dissertation research has focused on addressing a single aspect of this vast topic: how developmental programs once specified are maintained across multiple cell divisions. To address this question I employed an excellent genetic and developmental model organism, the fruit fly *Drosophila melanogaster*.

*Drosophila* development involves an approximately 10-day progression from an embryo through larval and pupal stage transitions to form an adult organism (ASHBURNER *et al.* 2005). During this process, embryonic pluripotent progenitor cells commit to specific fates, undergo many rounds of cell division and ultimately differentiate to form the highly specialized cells of the adult. This process requires dramatic changes in the gene transcription program of the cell, as well as maintenance of that program over time and throughout cell divisions (DILLON 2012). Interestingly, many of the proteins involved in maintaining patterns of gene expression during development function by regulating chromatin structure.

### **Modulation of chromatin structure**



Chromatin structure is comprised of DNA wrapped around histone proteins, forming nucleosomes, which pack densely together to form higher-order structure (LUGER *et al.* 2012). Eight core histones bind together to form a nucleosome, containing two copies each of H2A, H2B, H3 and H4 molecules around which approximately 150 base pairs of DNA is wrapped. Densely packed chromatin structure restricts access to the DNA template presenting an inherent block to the binding of transcription factors, transcription elongation and other nuclear events (RYAN and OWEN-HUGHES 2011). Chromatin-remodeling factors use the energy of ATP to alter the structure, positioning and composition of nucleosomes, which can modulate DNA accessibility and facilitate the interaction between DNA and protein factors (HARGREAVES and CRABTREE 2011). Chromatin remodeling is particularly important during transcription, where RNA Polymerase II is recruited to promoters by transcription factors that bind specific DNA sequences and must transcribe through many base pairs of nucleosome-bound DNA (BROWN *et al.* 2007).

Chromatin structure is also regulated through the covalent modification of N-terminal histone tails. Histone proteins contain unstructured N-terminal domains that can be covalently modified through methylation, acetylation, ubiquitination, phosphorylation and sumoylation (KOUZARIDES 2007). These modifications are often associated with transcriptionally active or repressed regions and are thought to affect nucleosome stability and mediate the binding and activity of chromatin-associated proteins. For example, the N-terminus of histone H3 contains many lysine residues, which can be mono-, di- or tri-methylated by different histone-modifying enzymes. The specific lysine residues that are methylated, for example lysines 4, 9, 27 or 36

are each associated with different chromatin states and transcriptional activities (BANNISTER and KOUZARIDES 2011; LACHNER and JENUWEIN 2002).

### **Polycomb and trithorax group genes**

The importance of chromatin structure regulation during development was first established in *Drosophila* from studies of a gene family known as the Homeotic (Hox) genes. Hox genes encode homeodomain transcription factors whose expression contributes to cell fate specification, body patterning and segment identity in all metazoans (LEWIS 1994). The differential expression of Hox genes along the anterior to posterior axis of the *Drosophila* embryo gives rise to the segmented body plan seen in adult flies. Hox genes function as master transcriptional regulators driving the expression of many downstream target genes that determine cellular and segmental identity (GRABA *et al.* 1997). Patterns of Hox gene expression are established in the embryo by the gap and pair rule genes in response to gradients of maternally supplied mRNAs. As development proceeds, expression of the gap and pair rule genes is lost and maintenance of Hox gene expression becomes the responsibility of a different set of genes: the Polycomb and trithorax group genes (GRIMAUD *et al.* 2006).

Polycomb and trithorax group genes play highly conserved roles in the maintenance of Hox gene expression during development (GELLON and MCGINNIS 1998). In *Drosophila*, mutations in Polycomb and trithorax group genes can result in transformation of one body segment into another (homeotic transformations) stemming from the gain or loss of Hox gene expression. Polycomb and trithorax group genes are ubiquitously expressed during development but have antagonistic effects on transcription; Polycomb group proteins repress transcription, while

trithorax group proteins maintain active states of transcription. Both groups of proteins achieve this regulation through effects on chromatin structure (GRIMAUD *et al.* 2006).

Work over the last two decades has greatly illuminated the roles that Polycomb and trithorax group proteins play in transcription, although the picture is far from complete. Roughly a dozen Polycomb group genes have been identified in *Drosophila*, and the majority of these genes encode subunits of two protein complexes known as Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2) (LEVINE *et al.* 2004; OTTE and KWAKS 2003). PRC1 contains the chromodomain protein Polycomb (PC), the ubiquitin ligase dRING and two other Polycomb group proteins, Polyhomeotic (PH) and Posterior sex combs (PSC). PRC2 contains the histone methyltransferase Enhancer of zeste [E(Z)] which methylates histone H3 on lysine 27 (H3K27) and two other Polycomb group proteins Extra sex combs (ESC) and Suppressor of zeste 12 [Su(Z)12]. Methylation of H3K27 is critical for Polycomb group repression and may function to recruit or stabilize the binding of PRC1 or other repressors to chromatin (KOHLENER and VILLAR 2008).

The trithorax group proteins comprise a more heterogeneous and less well-characterized set of proteins (KINGSTON and TAMKUN 2007; SCHUETTENGRUBER *et al.* 2011). Histone methyltransferases, such as Trithorax (TRX) and Absent, small or homeotic 1 (ASH1) contain SET domains, which catalyze the methylation of nucleosomal histones. TRX, the founding member of the trithorax group, methylates lysine 4 of histone H3 (H3K4) (SMITH *et al.* 2004). H3K4 methylation is found near the promoters of many active genes and is thought to directly or indirectly counteract Polycomb group repression (SCHMITGES *et al.* 2011). ASH1 also methylates

histones. However, its specificity is more controversial. ASH1 has been reported to methylate a variety of residues including H3K4, H3K9 and H4K20 and H3K36 (BEISEL *et al.* 2002; BYRD and SHEARN 2003; TANAKA *et al.* 2007; TANAKA *et al.* 2011). Both ASH1 and TRX have been shown to associate with a histone acetyltransferase, CBP, which is thought to acetylate H3K27, directly interfering with Polycomb repression (BANTIGNIES *et al.* 2000; SMITH *et al.* 2004; TIE *et al.* 2009).

Another set of trithorax group proteins are involved in chromatin remodeling. The trithorax group proteins Brahma (BRM), Moira (MOR), Osa (OSA) are all subunits of the SWI/SNF ATP-dependent chromatin-remodeling complex in *Drosophila* (COLLINS *et al.* 1999; REISMAN *et al.* 2009). BRM contains a highly conserved ATPase domain and is the catalytic subunit of the complex. BRM also contains a bromodomain thought to interact with acetylated histones present at gene promoters (ELFRING *et al.* 1998; TAMKUN *et al.* 1992). Most BRM complex members are essential for viability and play important roles in regulating chromatin structure at a large number of genes, including those involved in development, cell division and cell signaling (HO and CRABTREE 2010).

The trithorax group protein Kismet (KIS) is another example of a chromatin-remodeling factor in *Drosophila*. The *kis* gene encodes two large nuclear proteins called KIS-L (574 kDa) and KIS-S (225 kDa) (DAUBRESSE *et al.* 1999; SRINIVASAN *et al.* 2005). Similar to BRM, KIS-L contains a highly conserved ATPase domain. However, unlike BRM, KIS-L does not seem to function in a multi-protein complex (SRINIVASAN *et al.* 2005; SRINIVASAN *et al.* 2008). KIS-L also contains two chromodomains, which are short domains implicated in binding methylated histone tails (FLANAGAN *et al.* 2007). KIS-S, which lacks chromodomains and an ATPase

domain, contains only a BRK domain that is also found in KIS-L and in BRM. However, the function of KIS-S and the BRK domain is currently unknown (DAUBRESSE *et al.* 1999).

Despite the importance of Polycomb and trithorax group proteins in development, their precise mechanisms of action and the ways in which they interact to bring about stable patterns of gene expression are not well understood. The goal of my dissertation research has been to shed light on how gene expression patterns are maintained through development. I decided to focus my efforts on further characterizing the function of the trithorax group protein, Kismet (KIS) and how it interacts with other trithorax group proteins to promote active states of gene expression.

### **Function of the trithorax group protein KIS**

Like many other trithorax group genes, *kis* was first identified in a genetic screen for extragenic suppressors of *Polycomb* (*Pc*) mutations, suggesting that it directly or indirectly counteracts Polycomb group repression (KENNISON and TAMKUN 1988). In support of this, *kis* has a highly dosage sensitive effect on *Pc* mutant homeotic phenotypes. Mutations in *kis* suppress *Pc* mutant phenotypes, while duplications of *kis* enhance them (KENNISON and RUSSELL 1987; KENNISON and TAMKUN 1988). Loss of zygotic *kis* function causes recessive lethality at the first or second instar larval stage of development, but does not cause obvious patterning defects (DAUBRESSE *et al.* 1999). Elimination of the maternal contribution of *kis* through the generation of germ line clones causes embryonic pair-rule segmentation defects, while creation of somatic clones later in development results in homeotic transformations. The homeotic phenotypes resulting from *kis* loss include

transformation of the fifth abdominal segment to a more anterior segment, characteristic of reduced expression of the Hox gene *Abdominal-B* and transformation of the first leg to second leg, due to reduced expression of the Hox gene *Sex combs reduced* (DAUBRESSE et al. 1999).

KIS is a member of a large family of CHD proteins that is conserved from flies to humans. KIS is homologous to mammalian CHD6, CHD7, CHD8 and CHD9 (FLANAGAN *et al.* 2007; MURAWSKA and BREHM 2011). Heterozygosity for loss of function mutations in one of these KIS homologues, CHD7, leads to CHARGE syndrome, a serious developmental disorder affecting approximately one in 10,000 births (JANSSEN *et al.* 2012). CHARGE syndrome affects tissues derived from the neural crest, including the eyes, ears, craniofacial structures, heart and genitalia. Approximately 60% of CHARGE syndrome patients have a mutation in CHD7, strongly linking the varied phenotypes of this complex disease to the function of a single gene (JANSSEN *et al.* 2012). The association of CHD7 with CHARGE syndrome has generated significant interest in the biochemical and developmental functions of CHD7 and its counterpart in *Drosophila*, KIS.

Genetic studies of KIS-L function in *Drosophila* have shown that it is required to antagonize Polycomb repression at Hox genes during development. Recent studies of KIS-L, however, suggest it might play a more global role in transcription; KIS-L localizes to virtually all RNA Pol II transcribed genes on polytene chromosomes (SRINIVASAN *et al.* 2005). Polytene chromosomes from the third instar larval salivary gland are excellent tools for studying the localization of proteins to chromatin on a genome-wide scale. Cells of the salivary gland undergo successive rounds of DNA replication without cytokinesis, generating huge polytene

chromosomes containing thousands of copies of DNA arrayed in tandem. These chromosomes can be fixed and stained with antibodies to visualize the distribution of proteins across the genome (JOHANSEN *et al.* 2009; STEPHENS *et al.* 2004). Interestingly, a hypomorphic *kis* allele was identified that survives to the late third instar larval stage, enabling isolation of polytene chromosomes from homozygous *kis* mutants (ROCH *et al.* 1998; SPRADLING *et al.* 1999). Characterization of larvae homozygous for this hypomorphic *kis* allele by immunostaining revealed that they lack detectable KIS-L protein on their polytene chromosomes (SRINIVASAN *et al.* 2005). This enabled characterization of the role of KIS-L in the RNA Pol II transcription cycle.

Eukaryotic transcription involves a highly ordered series of events beginning with the binding of transcription factors to cis-regulatory elements; followed by the assembly of the pre-initiation complex and recruitment of Pol II to promoters; initiation; promoter clearance; elongation and finally termination (BURATOWSKI 2009). Each step in the transcription cycle is highly regulated and the phosphorylation state of the RNA Pol II C-terminal Domain (CTD) is correlated with the progression through each step (BARTKOWIAK *et al.* 2011). The RNA Pol II CTD is a large unstructured domain containing dozens of repeats of the basic seven amino acid sequence  $Y_1S_2P_3T_4S_5P_6S_7$ . The CTD is unphosphorylated when RNA Pol II binds to the promoter and initiates transcription. As RNA Pol II clears the promoter the kinase CDK7 phosphorylates the CTD on serine 5 and serine 7. When RNA Pol II switches into productive elongation tyrosine 1 is phosphorylated and the p-TEFb complex, comprised of the kinase CDK9 and CycT, phosphorylates the CTD on serine 2 (MAYER *et al.* 2012; ZHANG *et al.* 2012). Serine 2 phosphorylation is required for

productive elongation and the recruitment of transcription elongation factors, such as SPT6, and other protein factors that aid in processing the nascent mRNA (Ni *et al.* 2008; ZHOU *et al.* 2012).

KIS is not required for early stages of the transcription cycle, including recruitment of Pol II to promoters and promoter clearance (SRINIVASAN *et al.* 2005) as evidenced by the normal levels of unphosphorylated and serine 5 phosphorylated Pol II on the polytene chromosomes of *kis* mutant larvae. However, the loss of *kis* did lead to a dramatic reduction in the levels of elongating serine 2 phosphorylated Pol II, as well as loss of the elongation factors SPT6 and CHD1 (SRINIVASAN *et al.* 2005). These findings suggested that KIS-L activates transcription by promoting an early stage of transcription elongation.

I joined John Tamkun's laboratory shortly after it was discovered that KIS plays a role in transcription elongation. As the goal of my research was to understand how patterns of gene expression are maintained throughout development, I immediately became interested in KIS and its role in promoting active states of gene expression in *Drosophila*. In my dissertation I chose to focus on two main aspects of KIS function. The first involves how KIS is recruited to actively transcribed genes; the second addresses the question of how KIS antagonizes Polycomb group repression.

### **How is KIS recruited to actively transcribed genes?**

When I started my research, I was very interested in the question of how KIS and other trithorax group proteins are targeted to active gene promoters. Work in other systems had discovered that protein domains called chromodomains could



bind methylated histone tails. It was hypothesized that these interactions might mediate the recruitment and activity of chromodomain-containing proteins (BANNISTER *et al.* 2001; BREHM *et al.* 2004; FLANAGAN *et al.* 2005; JACOBS and KHORASANIZADEH 2002; MIN *et al.* 2003; PRAY-GRANT *et al.* 2005; SIMS *et al.* 2005). The presence of two chromodomains in KIS-L suggested that its ability to bind chromatin and stimulate transcription might be regulated by the methylation of nucleosomal histones.

The best candidate for a chromatin modification that targets and regulates KIS-L function was H3K4 methylation. Numerous H3K4 methyltransferases have been identified, including yeast SET1, its relatives in *Drosophila* and mammals, and the trithorax group proteins ASH1 and TRX (SHILATIFARD 2012). H3K4 methylation is found near the promoters of many active genes and is thought to stimulate transcription by promoting the association of multiple regulatory proteins with chromatin. For example, CHD1, a CHD protein related to KIS-L, directly binds methylated H3K4 (H3K4me) via its chromodomains (SIMS *et al.* 2005). Thus, the prevailing hypothesis was that perhaps KIS-L is recruited to actively transcribed genes through the association of its chromodomains with methylated H3K4. I set out to test this hypothesis in collaboration with Shrividhya Srinivasan, another graduate student in the lab. Our published results are detailed in Chapter 2 of my dissertation (SRINIVASAN *et al.* 2008).

### **How does KIS antagonize Polycomb group repression?**

In addition to characterizing how KIS is recruited to active genes, I was also interested in the antagonism between Polycomb and trithorax group proteins. An

elegant study of the trithorax group histone methyltransferases ASH1 and TRX had shown that these proteins function as Polycomb anti-repressors (KLYMENKO and MULLER 2004). Loss of ASH1 or TRX in the presence of Polycomb group proteins resulted in loss of Hox gene expression. However, in double mutants that lose both trithorax and Polycomb group function, Hox gene transcription was restored. These studies suggested that ASH1 and TRX, though necessary to prevent Polycomb repression, are not strictly required for transcription itself.

These results prompted me to investigate how KIS antagonizes Polycomb repression. I wondered whether the loss of transcription elongation seen in *kis* mutants was a direct consequence of KIS loss or a secondary consequence of failure to antagonize Polycomb repression. In collaboration with Shrividhya Srinivasan I began to address this question by testing the hypothesis that KIS is required to counteract Polycomb group protein binding and activity. The results of this study were published and are presented in Chapter 2 of my dissertation (SRINIVASAN *et al.* 2008). The question of how KIS and other trithorax group proteins counteract Polycomb repression has since become a major focus of my thesis research. In my subsequent work, I specifically tested whether KIS antagonizes Polycomb repression by promoting transcription elongation. I also investigated whether KIS antagonizes Polycomb repression by promoting histone modifications and recruiting other trithorax group proteins. The results of these studies are presented in Chapter 3 of my dissertation.

## Chapter 2

### ***Drosophila* Kismet Regulates Histone H3 Lysine 27 Methylation and Early Elongation by RNA Polymerase II<sup>1</sup>**

#### **Summary**

Polycomb and trithorax group proteins maintain heritable states of transcription mainly through covalent modification or remodeling of chromatin. However, how Polycomb and trithorax group proteins interact with each other and the general transcription machinery to regulate transcription is not well understood. The trithorax group protein Kismet-L (KIS-L) is a member of the CHD subfamily of chromatin-remodeling factors that plays a global role in transcription by RNA polymerase II (Pol II). We aimed to determine how KIS-L is recruited to active gene promoters in order to shed light on how KIS-L maintains active gene expression and counteracts Polycomb group silencing. The presence of two chromodomains in KIS-L suggested that its recruitment or function might be regulated by the methylation of histone tails. We hypothesized that the trithorax group proteins ASH1 and TRX might be involved in recruiting KIS to active genes by methylating histone H3 on lysine 4 (H3K4). However, when we tested this hypothesis, the chromodomains of KIS-L did not bind methylated histone tails *in vitro* and loss of TRX or ASH1 function did not alter the association of KIS-L with chromatin. By contrast, loss of *kis* function led to a dramatic reduction in the levels of TRX and ASH1 associated with chromatin and

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<sup>1</sup> The text and figures for this chapter include contributions by S. Srinivasan (figures 2-1, 2-2, 2-4, 2-5, 2-9, 2-11) and are excerpted from the following previously published material: Srinivasan S., Dorigi K.M. and Tamkun, J.W. (2008) *Drosophila* Kismet Regulates Histone H3 Lysine 27 Methylation and Early Elongation by RNA Polymerase II. *PLoS Genetics* 4 (10): e1000217 doi:10.1371/journal.pgen.1000217

was accompanied by increased histone H3 lysine 27 methylation, a modification required for Polycomb group repression. A similar increase in H3 lysine 27 methylation was observed in *ash1* and *trx* mutant larvae. Our findings suggest that KIS-L counteracts Polycomb group repression by recruiting the ASH1 and TRX histone methyltransferases to chromatin.

## **Materials and Methods**

### ***Drosophila* stocks**

Flies were raised on cornmeal/molasses/yeast/agar medium containing Tegosept and propionic acid. Strains are described in FlyBase (<http://www.flybase.org>) unless otherwise indicated. *kis*<sup>k13416</sup> is a recessive loss of function allele; homozygotes survive until late larval or early pupal stages, but express undetectable levels of KIS-L in salivary gland nuclei (SRINIVASAN *et al.* 2005). Oregon R was used as the wild-type strain for all experiments.

### **Immunostaining of polytene chromosomes**

Indirect immunofluorescence microscopy was used to examine the distribution of proteins on salivary gland polytene chromosomes (CORONA *et al.* 2004; SRINIVASAN *et al.* 2005). Primary antibodies used included goat antibodies against PC and KIS-L (Santa Cruz Biotech); rat antibodies against KIS-L (SRINIVASAN *et al.* 2005); rabbit antibodies against ASH1 (TRIPOULAS *et al.* 1996), E(Z) (CARRINGTON and JONES 1996), KIS-L (SRINIVASAN *et al.* 2005), TRX (KUZIN *et al.* 1994), Histone H3, H3K4me2, H3K4me3 and H3K27me3 (Upstate Signaling); and mouse antibodies against Pol IIa (8WG16), RPB1 (CTD4H8) (Covance) and His

epitope tag (Anaspec). Salivary gland polytene chromosomes from third instar larvae were fixed for 5 minutes in 45% acetic acid/1.85% formaldehyde and stained with antibodies against ASH1, TRX, RPB1, Pol IIa, Pol Ilo<sup>ser5</sup>, Pol Ilo<sup>ser2</sup> and KIS-L. To stain polytene chromosomes with antibodies against KIS-L, CycT, Pol Ilo<sup>ser2</sup>, E(Z), PC, H3K4me2, H3K4me3 and H3K27me3, glands were dissected in 0.7% NaCl and fixed in 6 mM MgCl<sub>2</sub>, 1% citric acid and 1% Triton X-100 for 2 minutes. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Samples were mounted in Vectashield containing DAPI (Vector Laboratories). Images were captured using a Zeiss Axioskop 2 plus microscope equipped with an Axioplan HRm camera and Axiovision 4 software (Carl Zeiss, Germany). Merged and split images were generated using Adobe Photoshop CS3 software as previously described (CORONA *et al.* 2004).

The levels of proteins associated with wild-type and mutant polytene chromosomes were compared by processing, capturing and analyzing the samples at the same time under identical conditions as described in Srinivasan *et al.* (2005). To quantify the increase in H3K27me3 levels in *kis* mutants, polytene chromosomes from wild-type and mutant larvae stained with antibodies against H3K27me3 were photographed using exposure times that yielded images of comparable intensity. The fold increase in H3K27me3 was calculated as a ratio of the average exposure times for the wild-type and mutant samples.

### **Protein expression and binding assays**

Standard techniques were used to analyze proteins by SDS-PAGE and Western blotting (HARLOW and LANE 1988). To produce recombinant

chromodomains, DNA encoding KIS-L chromodomain 2 (amino acids 1937-1997) was amplified using the primers 5'-GGAATTCCATATGCAGGACTTTACTGAAGT-3' and 5'-CGGGATCCGATTTTGTAAAGCGCAGGTA-3'. DNA encoding the HP1 chromodomain (amino acids 22-75) was amplified using the primers 5'-GGAATTCCATATGGAGGAGTACGCCGTGGA-3' and 5'-CGGGATCCCTTGCGGCTCGCCTCGTACTG-3'. The amplified sequences were cloned between the *Nde* I and *Bam*H I sites of pET-16b (Novagen). A pET-16b construct encoding chromodomains 1 and 2 of human CHD1 (amino acids 268 to 443) was generously provided by Sepidah Khorasanizadeh (FLANAGAN *et al.* 2005). Chromodomains were expressed as His-tagged proteins in BL21pLysS (Stratagene) and purified by Ni<sup>2+</sup> affinity chromatography under native conditions using the manufacturer's protocol (Qiagen). The binding of purified chromodomains to biotinylated peptides corresponding to N-terminal histone tails (Upstate) immobilized on strepavidin agarose (Upstate) was assayed as described in Pray-Grant *et al.* (2005).

### **Chromatin immunoprecipitation (ChIP) and quantitative-PCR**

Chromatin was isolated from salivary glands of wild-type and *kis*<sup>k13416</sup> larvae (DANZER and WALLRATH 2004) and analyzed by ChIP (KURAS and STRUHL 1999) using quantitative PCR as reported (SRINIVASAN *et al.* 2008).

## **Results**

**The chromodomains of KIS-L do not bind methylated histone peptides *in vitro***

It has been reported that both ASH1 and TRX methylate H3K4 (BEISEL *et al.* 2002; BYRD and SHEARN 2003; SMITH *et al.* 2004); this covalent modification of chromatin is enriched near the promoters of many genes and is thought to recruit factors required for early events in the transcription cycle (RUTHENBURG *et al.* 2007). Like other CHD proteins, KIS-L has two adjacent chromodomains (CD1 and CD2) suggesting that it might directly interact with methylated histone tails. The CD2 of KIS-L is highly related to chromodomains that directly bind methylated histone tails (Figure 2-1 A), including CD2 of yeast CHD1, which binds both di- and tri-methylated H3K4 (PRAY-GRANT *et al.* 2005). This similarity suggested that KIS-L might directly bind methylated H3K4.

To investigate this possibility, we examined the ability of recombinant KIS-L proteins to bind immobilized synthetic peptides corresponding to N-terminal histone tails. A recombinant protein corresponding to KIS-L CD2 (residues 1937 to 1997) did not bind unmodified histone H3 tails or a variety of methylated H3 tails (including H3K4me2, H3K4me3, and H3K9me2), even at relatively low (150 mM) salt concentrations (Figure 2-1 B). By contrast, we were able to detect the binding of the *Drosophila* HP1 chromodomain to H3K9me3 using this assay (Figure 2-1 B), as previously observed by others (BANNISTER *et al.* 2001; LACHNER *et al.* 2001). Recent studies of the human CHD1 protein have shown that both CD1 and CD2 are required for binding of methylated H3K4 *in vitro* (FLANAGAN *et al.* 2005; SIMS *et al.* 2005). While we were able to reproduce this result (Figure 2-1 B), a comparable recombinant protein spanning CD1 and CD2 of KIS-L – as well as full-length KIS-L proteins from embryo extracts – bound both unmodified and methylated H3 and H4 tails (data not shown), presumably due to non-specific ionic interactions with the

positively charged tails. We were therefore unable to determine if regions outside of CD2 enable KIS-L to bind methylated histone tails. Thus, although KIS-L CD2 failed to interact with methylated histone tails *in vitro*, it remains possible that the full-length KIS-L protein recognizes one or more histone modifications *in vivo*.

### **H3K4 methylation is not the primary determinant for the recruitment of KIS-L to chromatin**

As an alternative approach for studying potential interactions between KIS-L and methylated histone tails, we compared the distributions of KIS-L and both di- and tri-methylated H3K4 on salivary gland polytene chromosomes. As expected for modifications associated with transcriptionally active regions, there is a high degree of overlap between KIS-L and both H3K4me2 and H3K4me3 (Figure 2-1 C and D). However, there are many sites where KIS-L and these methyl marks do not overlap as well as considerable differences in the relative levels of KIS-L and H3K4 methylation at many sites (Figure 2-1 C and D). These observations suggest that H3K4 methylation is not sufficient to recruit KIS-L to chromatin.

We next examined the relative distributions of H3K4 methylation and KIS-L at higher resolution using chromatin immunoprecipitation (ChIP) assays. We chose the *forkhead (fkh)* gene for these studies for several reasons. First, *fkh* is a relatively simple gene that is expressed in the salivary gland at high levels. A single enhancer located 9 kb upstream of the transcription start site activates *fkh* expression in this tissue (ZHOU *et al.* 2001). Furthermore, TRX has been implicated in *fkh* expression (KUZIN *et al.* 1994), and we had demonstrated that KIS-L is associated with *fkh* by immuno-FISH (data not shown).



We examined the binding of KIS-L to the *fkh* gene by ChIP using chromatin isolated from the salivary glands of wild-type third instar larvae. Consistent with a role in early elongation, KIS-L is associated with the transcriptional start site of the *fkh* gene (Figure 2-2 A). The enrichment of KIS-L with the transcriptional start site is about 3-fold over a control region upstream of *fkh* (Figure 2-2 A, primer P vs. primer C1). This binding is reduced to background levels in chromatin isolated from *kis* mutant larvae, suggesting that the association of KIS-L with *fkh* is specific (Figure 2-2 A). This finding is consistent with a recent study showing that KIS-L is associated with the *Ultrabithorax (Ubx)* promoter (PAPP and MULLER 2006). H3K4me3 is present at the transcription start site as well as the body of the *fkh* gene and does not precisely mirror the distribution of KIS-L (Figure 2-2 B). These observations provide additional evidence that H3K4 methylation is not sufficient to recruit KIS-L to chromatin.

As an alternative approach for investigating the role of H3K4 methylation in KIS-L recruitment, we examined the effect of *little imaginal discs (lid)* mutations on the association of KIS-L with chromatin. *lid* encodes a H3K4me3 demethylase (EISSENBERG *et al.* 2007a; LEE *et al.* 2007; LLORET-LLINARES *et al.* 2008; SECOMBE *et al.* 2007); larvae homozygous for the hypomorphic *lid*<sup>10424</sup> allele survive until the third larval instar and display elevated levels of H3K4me3 on their polytene chromosomes (LLORET-LLINARES *et al.* 2008). Increased trimethylation of H3K4 resulting from the loss of *lid* function has no obvious effect on the level of KIS-L associated with polytene chromosomes (Figure 2-3), suggesting that this covalent modification of chromatin does not mediate interactions between KIS-L and chromatin *in vivo*.

**Neither ASH1 nor TRX is required for the association of KIS-L with chromatin**

The above results led us to question our hypothesis that ASH1 and TRX recruit KIS-L to chromatin by methylating H3K4 in the vicinity of promoters. To clarify this issue, we examined whether the loss of ASH1 or TRX function alters the association of KIS-L with salivary gland polytene chromosomes. Individuals trans-heterozygous for the hypomorphic *ash1<sup>22</sup>* and *ash1<sup>17</sup>* alleles survive until the third larval instar and display significantly reduced levels of ASH1 on polytene chromosomes (Figure 2-4 A and C) (TRIPOULAS *et al.* 1996). No obvious changes in the level or distribution of KIS-L were observed in these mutants relative to wild-type (Figure 2-4 B and D), indicating that ASH1 is not required for the association of KIS-L with chromatin. Similar results were obtained using a conditional *trx* allele, *trx<sup>1</sup>*. At 29°C, *trx<sup>1</sup>* homozygotes survive until the third larval instar and display significantly reduced levels of TRX on polytene chromosomes (Figure 2-4 E and G) (KUZIN *et al.* 1994). We failed to detect obvious changes in the level or distribution of KIS-L on salivary gland chromosomes in *trx<sup>1</sup>* mutants (Figure 2-4 F and H). Thus, neither the ASH1 nor TRX histone methyltransferase is required for the association of KIS-L with chromatin *in vivo*.

### **KIS-L is required for the association of ASH1 and TRX with chromatin**

In some cases, chromatin-remodeling factors stimulate transcription by recruiting histone-modifying enzymes to promoters (COSMA *et al.* 1999; KREBS *et al.* 1999). We therefore examined if KIS-L is required for the association of ASH1 and TRX with chromatin. The loss of *kis* function resulted in a significant reduction in the levels of both ASH1 and TRX associated with polytene chromosomes (Figure 2-5 A,C,E,G). A few residual bands of relatively strong ASH1 and TRX staining were observed in the mutants (Figure 2-5 C and G), suggesting that the recruitment of the

two trithorax group proteins to a small number of chromosomal sites may be independent of KIS-L. These results demonstrate that KIS-L is required for the recruitment of ASH1 and TRX to the majority of their target genes *in vivo*.

Although the substrate specificity of ASH1 is controversial, at least one previous study reported it to be responsible for the bulk of H3K4 methylation in the larval salivary gland (BYRD and SHEARN 2003). This observation, together with our finding that KIS-L recruits ASH1 and TRX to actively transcribed genes, led us to investigate whether KIS-L is a global regulator of H3K4 methylation. Surprisingly, we did not observe a significant decline in either H3K4 di- or trimethylation on the polytene chromosomes of *kis*<sup>k13416</sup> mutant larvae (Figure 2-6). Consistent with these data, the loss of KIS-L function had no effect on the level of H3K4 methylation over the promoter and body of the *fkh* gene, as assayed by CHIP (Figure 2-2 B). We also examined the level of H3K4me2 and H3K4me3 on the salivary gland polytene chromosomes of *ash1* and *trx* mutant larvae. As observed in *kis* mutants, there was no significant decrease in H3K4 di- or trimethylation in either *ash1*<sup>22</sup>/*ash1*<sup>17</sup> or *trx*<sup>1</sup> larvae relative to wild type (Figure 2-7 and Figure 2-8). These data strongly suggest that ASH1, TRX and KIS-L are not required for the bulk of H3K4 methylation in *Drosophila*.

### **Loss of *kis* function does not alter the level or distribution of Polycomb group proteins on polytene chromosomes**

Genetic studies have suggested that KIS-L and other trithorax group proteins counteract Polycomb group repression (KENNISON and TAMKUN 1988; KINGSTON and TAMKUN 2007). Two complexes of Polycomb group proteins have been identified:

PRC1 and PRC2 (LEVINE *et al.* 2004). The E(Z) subunit of PRC2 methylates lysine 27 of histone H3; this modification is thought to promote the association of PRC1 with chromatin, thereby leading to heritable gene silencing (MULLER *et al.* 2002; WANG *et al.* 2004). Does KIS-L prevent the binding of either PRC1 or PRC2 to chromatin? As reported previously, the level of the PC subunit of PRC1 associated with salivary gland polytene chromosomes is similar in wild-type and *kis*<sup>k13416</sup> mutant larvae (Figure 2-9 A and B) (SRINIVASAN *et al.* 2005). Similar results were obtained when we compared the level of E(Z) on salivary gland chromosomes of wild-type and *kis*<sup>k13416</sup> mutant larvae (Figure 2-9 C and D). The loss of KIS-L function did not alter the number or distribution of PC binding sites (Figure 2-9 G and H), and extensive co-localization of PC and E(Z) was observed in both wild-type and *kis* mutant larvae (Figure 2-9 E and F). Thus, KIS-L does not appear to influence the association of either PRC1 or PRC2 with chromatin.

### **KIS-L counteracts H3 lysine 27 trimethylation *in vivo***

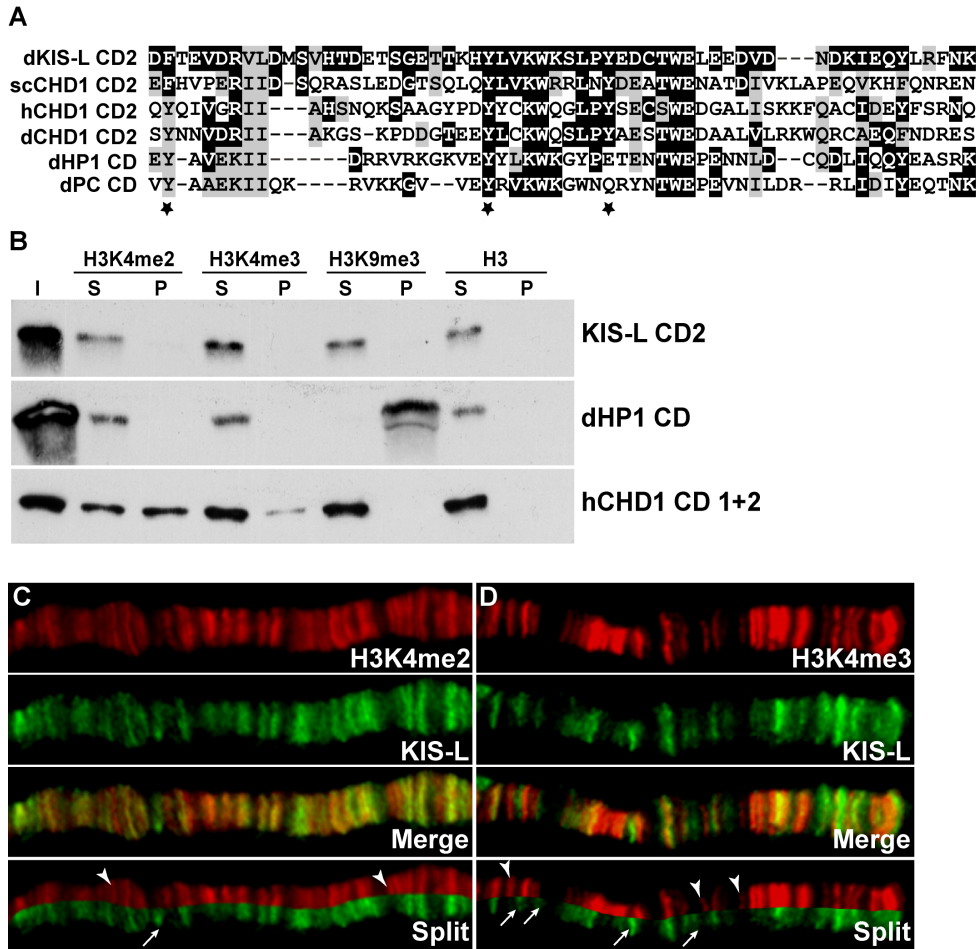
We previously noted that the majority (>80%) of PC binding sites in salivary gland polytene chromosomes are adjacent to sites of KIS-L binding (SRINIVASAN *et al.* 2005). The majority of sites of H3K27 methylation are also flanked on one or both sides by KIS-L (Figure 2-10). While it might be expected that KIS-L, which localizes to transcriptionally active regions, would be found adjacent to transcriptional repressed regions, these observations together with the lack of obvious changes in the level or distribution of PRC1 and PRC2 in *kis* mutants, suggested that KIS-L might counteract Polycomb group repression by antagonizing H3K27 methylation. To investigate this possibility, we stained salivary gland polytene chromosomes of wild-type and *kis*<sup>k13416</sup> mutant larvae with an antibody that specifically recognizes this

histone modification. Loss of *kis* function results in a large (~7 fold) increase in the level of H3K27me3 on polytene chromosomes (Figure 2-11 A and B) without altering the level or distribution of PC (Figure 2-11 C and D). A similar increase in H3K27 trimethylation was observed over the entire *fkf* gene of *kis*<sup>k13416</sup> mutant larvae by ChIP (Figure 2-11 G).

E(Z) is responsible for the majority of H3K27 methylation in wild-type *Drosophila* (CAO and ZHANG 2004) and likely catalyzes the additional trimethylation of H3K27 observed in *kis* mutants. In both wild-type and *kis* mutants, E(Z) colocalizes extensively with PC (Figure 2-9 E and F) and the number of PC binding sites remains unchanged (Figure 2-9 G and H). Thus, to determine whether the additional H3K27 methylation in *kis* mutants is caused by an increase in H3K27 methylation at existing sites or the appearance of ectopic bands of H3K27 methylation, we examined the chromosomal distributions of PC and H3K27me3 in both wild type and *kis*<sup>k13416</sup> mutant larvae (Figure 2-11 E and F). Although the level of H3K27 methylation is elevated in *kis*<sup>k13416</sup> mutants, the chromosomal distribution of Polycomb and H3K27me3 are virtually identical (>90% overlap), suggesting that this increase is not due to the appearance of H3K27me3 at ectopic sites but rather to a global increase in the levels of H3K27 methylation at existing sites (Figure 2-11 E and F). These findings suggest that KIS-L antagonizes Polycomb group repression by counteracting H3K27 methylation catalyzed by the E(Z) subunit of PRC2.

A recent study showed that loss of *ash1* function in the haltere discs of third instar larvae results in the spread of H3K27me3 into the coding region of the actively transcribed *Ubx* gene (PAPP and MULLER 2006). Thus, KIS-L may indirectly counteract H3K27 methylation by promoting the association of ASH1 with chromatin.

To investigate this possibility, we compared the level and distribution of H3K27me3 on the salivary gland polytene chromosomes of wild-type and *ash1* mutant larvae. As observed in *kis* mutants, the level of H3K27me3 was dramatically elevated on the salivary gland polytene chromosomes of *ash1<sup>22</sup>/ash1<sup>17</sup>* larvae relative to wild-type (Figure 2-12 A and B). A similar effect was observed in *trx<sup>1</sup>* homozygotes reared at 29° (Figure 2-12 C and D). These findings suggest that KIS-L counteracts Polycomb group repression by promoting the association of ASH1 and TRX with chromatin. These results and those described in the following chapter are discussed together in Chapter 4.

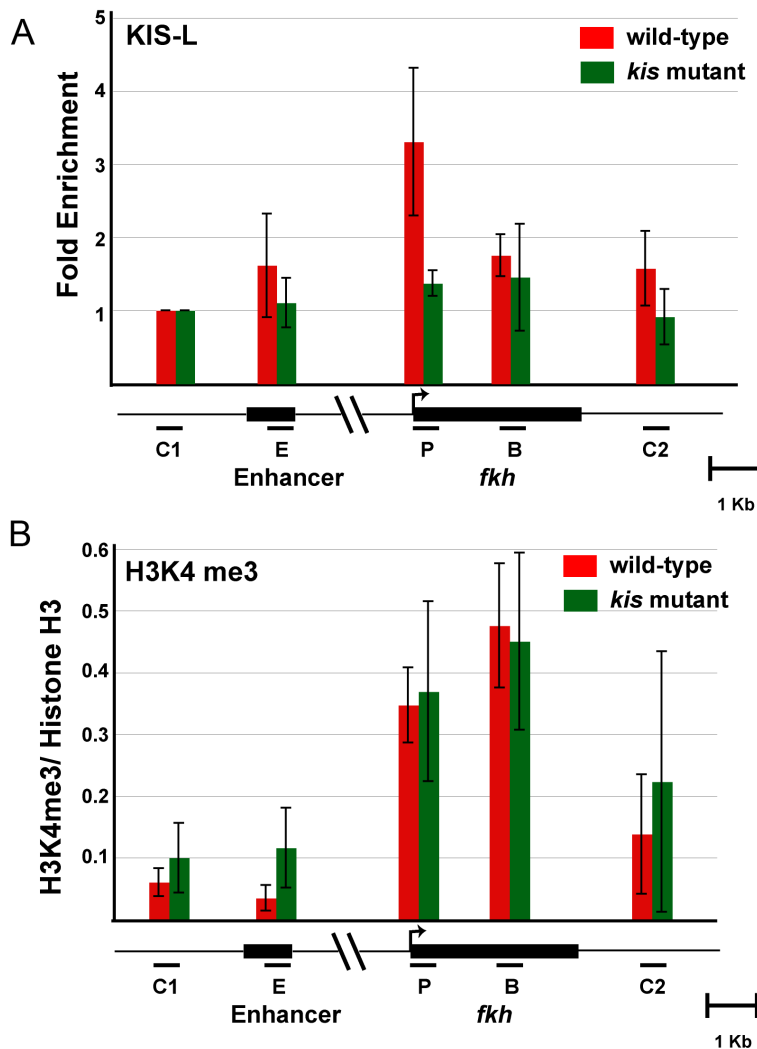


**Figure 2-1: H3K4 methylation is not sufficient for the recruitment of KIS-L to chromatin.**

A: CD2 of *Drosophila* KIS-L is aligned with CD2 of the *Saccharomyces cerevisiae* and human CHD1 proteins and the chromodomains of *Drosophila* HP1 and PC. Identical and conserved amino acids are highlighted in black and grey, respectively. Aromatic amino acids that are important for binding of methylated histone tails by the CD2 of yeast CHD1 are marked by stars.

B: Tests of the *in vitro* binding of HIS-tagged CD2 of KIS-L, CD1 and 2 of human CHD1 and the *Drosophila* HP1 chromodomain to histone H3K4me2, H3K4me3, H3K9me2 and histone H3 peptides. Input (I), unbound protein (S) and the bound proteins (P) were detected by western blotting using anti-HIS tag antibody. Note that the chromodomains of HP1 and human CHD1, but not KIS-L, specifically bound methylated H3K9 and H3K4 peptides, respectively.

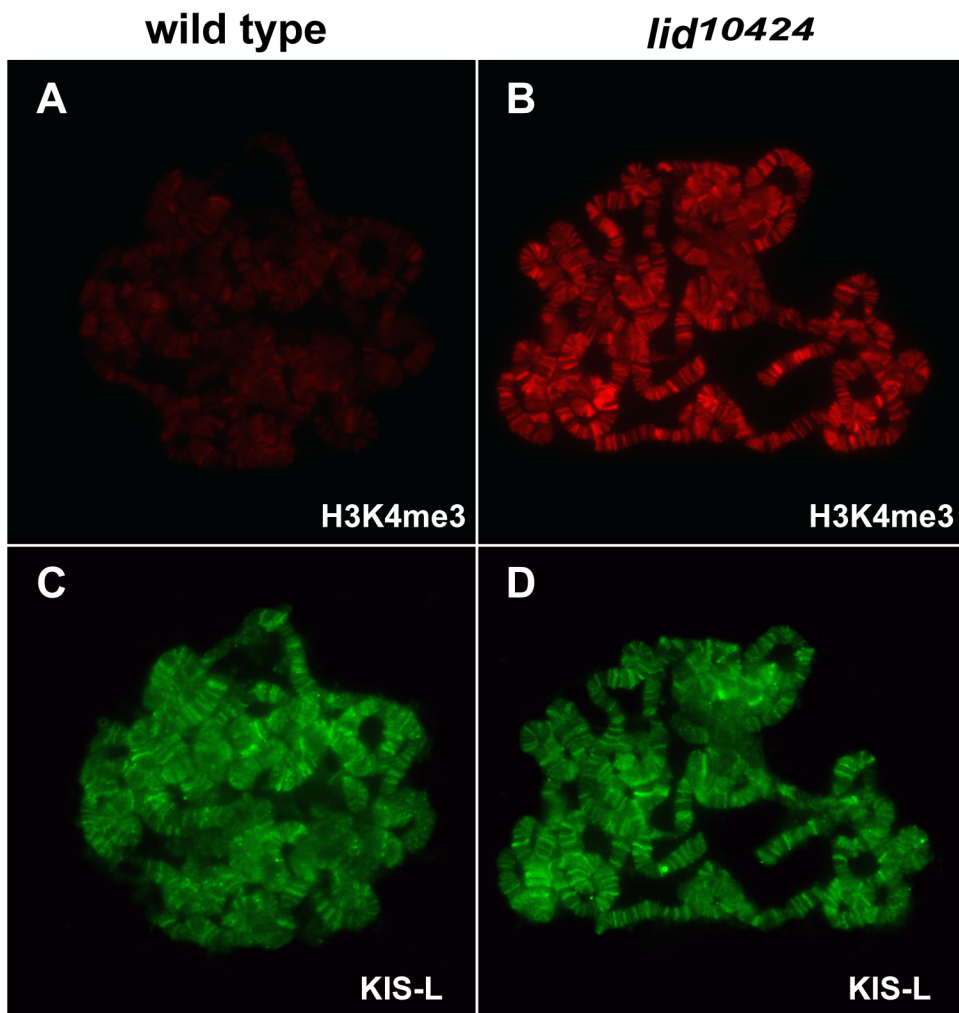
C-D: The distributions of H3K4me2 (C, red) and H3K4me3 (D, red) were compared to that of KIS-L (C and D, green) on a representative region of wild-type polytene chromosomes. The arrowheads represent H3K4me2 and H3K4me3 bands that do not overlap with KIS-L, while the arrows represent bands of KIS-L that do not overlap with H3K4me2 and H3K4me3 bands.



**Figure 2-2. Localization of KIS-L and H3K4 trimethylation within the *fkh* gene in wild-type and *kis* mutants.**

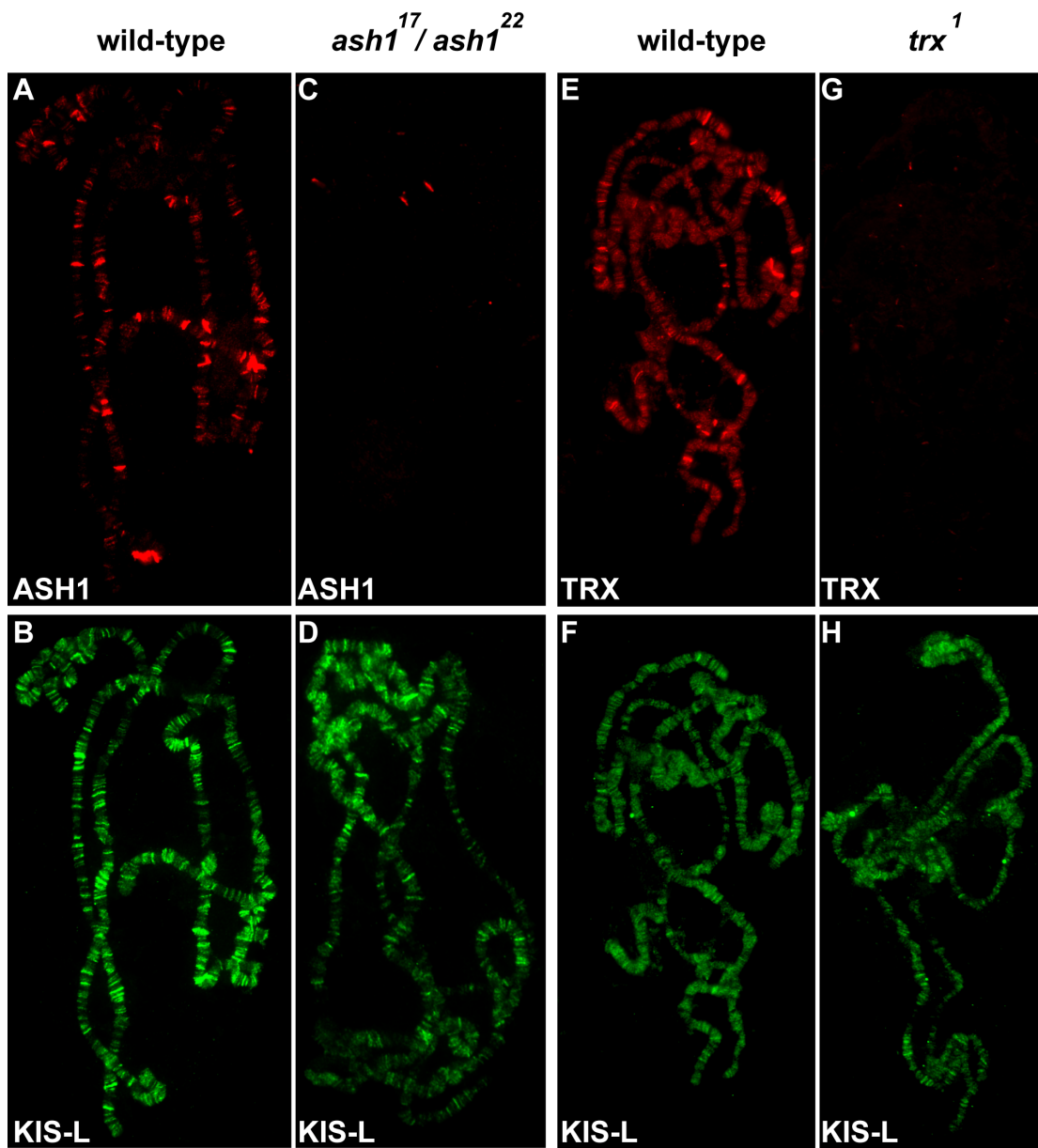
The distributions of KIS-L and H3K4me3 over the *fkh* gene were determined by ChIP using chromatin isolated from the salivary glands of wild-type (red bars) or *kis*<sup>k13416</sup> (green bars) larvae. A map of the *fkh* gene is shown below the X axis; black bars represent the primers used to amplify the following regions: C1: region upstream of *fkh*, E: *fkh* enhancer, P: *fkh* transcription start site, B: *fkh* body, C2: region downstream of *fkh*. For KIS-L, the percentages of DNA immunoprecipitated for regions E, P, B and C2 were normalized to the percentage of DNA immunoprecipitated for region C1 (A). The ratio of DNA immunoprecipitated with antibodies against H3K4me3 and histone H3 are shown for each region (B). Note that KIS-L is enriched over the transcription start site of *fkh* while H3K4me3 is enriched over both the transcription start site and the body of the *fkh* gene. The bars represent the average of independent biological experiments (n=4 for H3K4me3 and n=5 for KIS-L) with the corresponding standard deviations.





**Figure 2-3: The association of KIS-L with chromatin is not altered in *lid* mutants.**

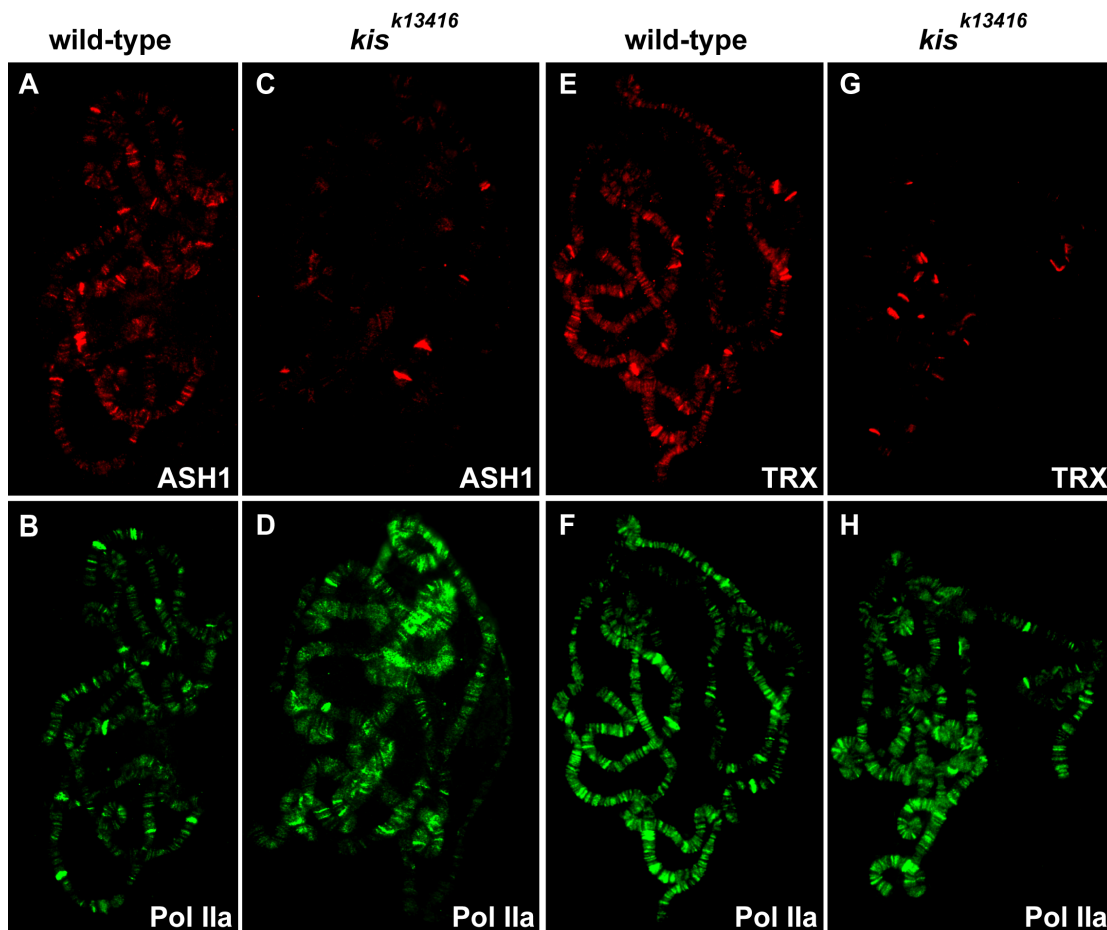
A-D: The levels of H3K4me3 (A, B, red) and KIS-L (C, D, green) on polytene chromosomes isolated from wild-type (A, C) and *lid*<sup>10424</sup> (B, D) larvae were examined by indirect immunofluorescence microscopy. Loss of *lid* function led to a dramatic increase in H3K4me3 without affecting the level of KIS-L associated with chromatin.



**Figure 2-4: The association of KIS-L with chromatin is not altered in *ash1* and *trx* mutants.**

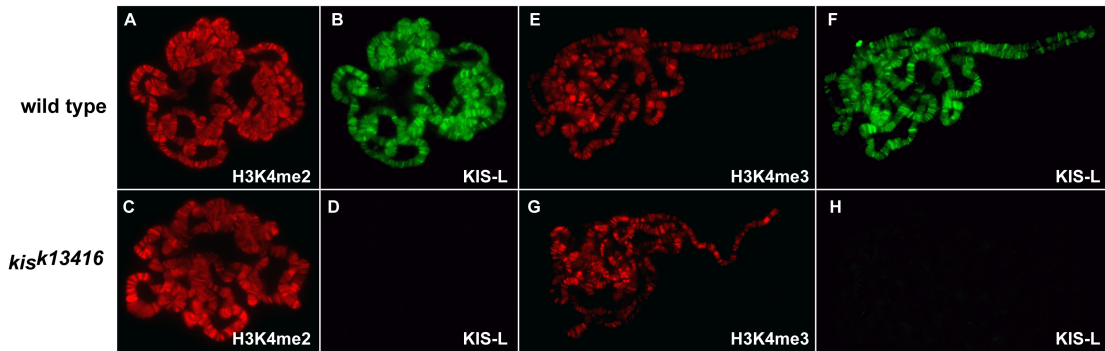
A-D: The association of ASH1 (A, C, red) and KIS-L (B, D, green) on salivary gland polytene chromosomes of wild-type (A, B) and *ash1*<sup>22</sup>/*ash1*<sup>17</sup> (C, D) larvae were detected by indirect immunofluorescence microscopy.

E-H: The association of TRX (E, G, red) and KIS-L (F, H, green) on polytene chromosomes isolated from wild-type (E, F) and *trx*<sup>1</sup> (G, H) larvae were detected by indirect immunofluorescence microscopy. Neither ASH1 nor TRX is required for the binding of KIS-L to polytene chromosomes.



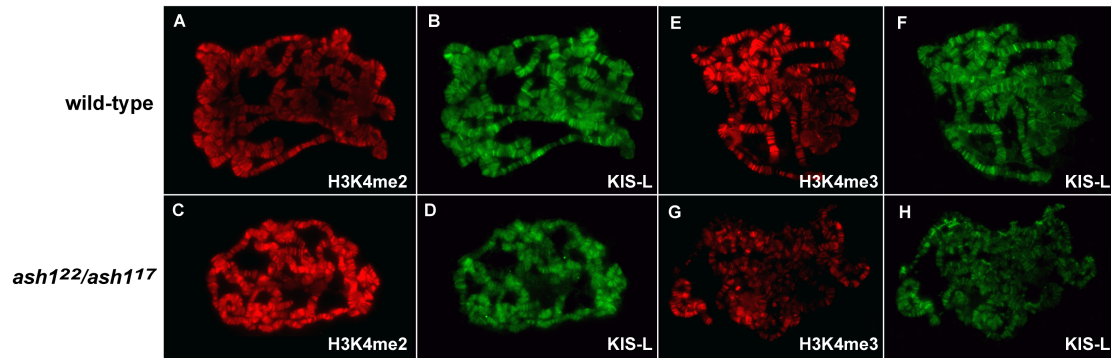
**Figure 2-5: KIS-L is required for the association of ASH1 and TRX with chromatin.**

The distributions of ASH1 (A, C, red) and TRX (E, G, red) on salivary gland polytene chromosomes isolated from wild-type and *kis*<sup>k13416</sup> larvae were detected by indirect immunofluorescence microscopy. The chromosomes were also stained with an antibody against Pol IIa (B, D, F, H, green) as an internal control. The loss of KIS-L function dramatically reduced the levels of ASH1 and TRX, but not Pol IIa, associated with polytene chromosomes.



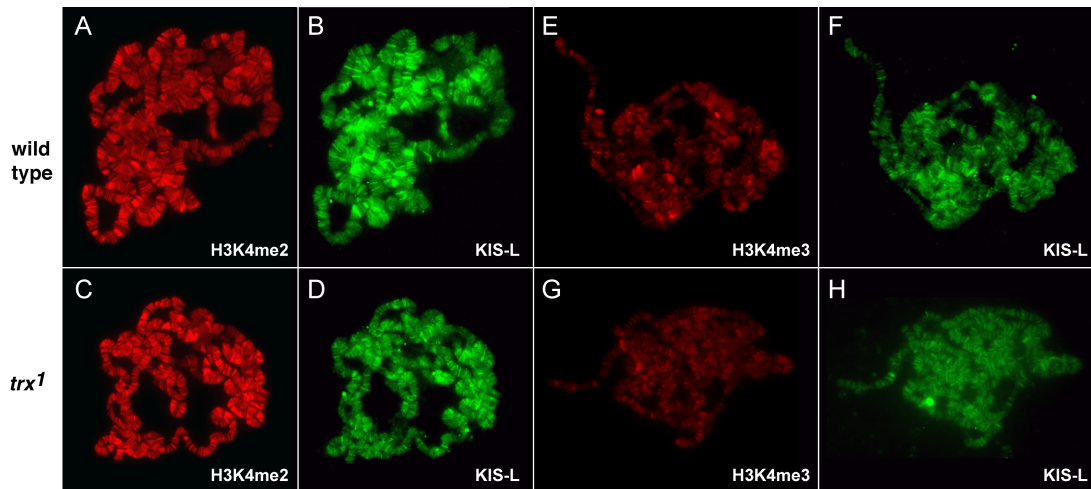
**Figure 2-6: KIS-L is not a global regulator of H3K4 methylation.**

The distributions of H3K4me2 (A, C, red), KIS-L (B, D, F and H, green), and H3K4me3 (E, G, red) on salivary gland polytene chromosomes isolated from wild-type (A, B, E and F) and *kis<sup>k13416</sup>* (C, D, G and H) larvae were detected by indirect immunofluorescence microscopy. The loss of KIS-L function did not cause obvious changes in the overall level or distribution of either H3K4me2 or H3K4me3.



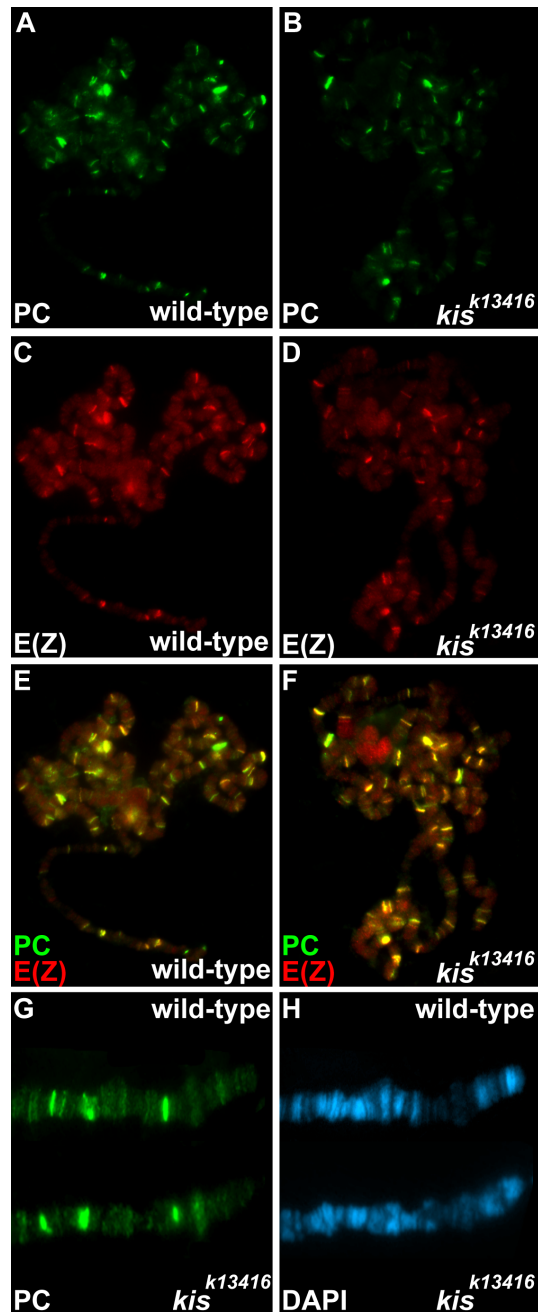
**Figure 2-7: Loss of ASH1 function does not dramatically alter H3K4 methylation *in vivo*.**

The distributions of H3K4me2 (A, C, red), KIS-L (B, D, F and H, green), and H3K4me3 (E, G, red) on salivary gland polytene chromosomes isolated from wild-type (A, B, E and F) and *ash1<sup>22</sup>/ash1<sup>17</sup>* (C, D, G and H) larvae were detected by indirect immunofluorescence microscopy. The loss of ASH1 function did not cause obvious changes in the overall level or distribution of either H3K4me2 or H3K4me3.



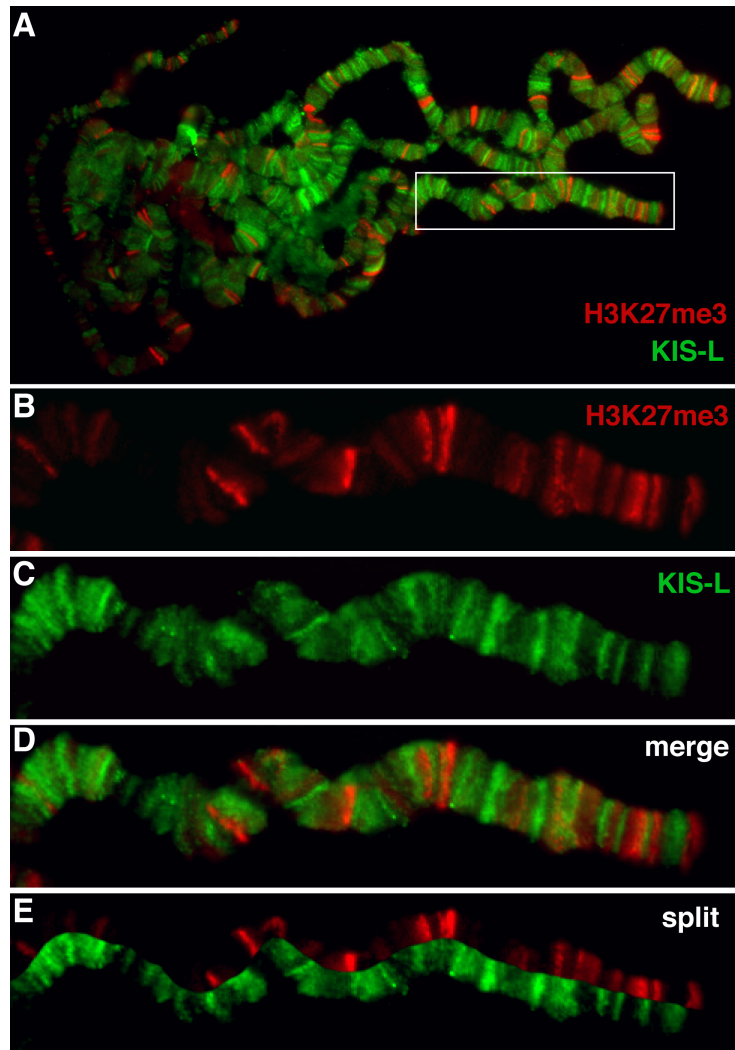
**Figure 2-8: Loss of TRX function does not dramatically alter H3K4 methylation *in vivo*.**

The distributions of H3K4me2 (A, C, red), KIS-L (B, D, F and H, green), and H3K4me3 (E, G, red) on salivary gland polytene chromosomes isolated from wild-type (A, B, E and F) and *trx<sup>1</sup>* (C, D, G and H) larvae were detected by indirect immunofluorescence microscopy. The loss of TRX function did not cause obvious changes in the overall level or distribution of either H3K4me2 or H3K4me3.



**Figure 2-9: Loss of *kis* function does not alter the distribution or level of Polycomb group proteins.**

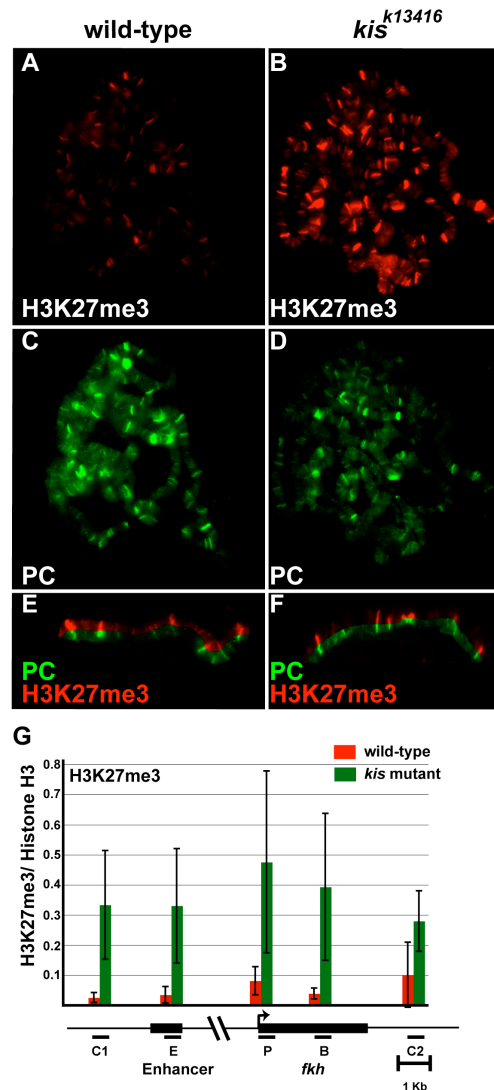
A-F: The distributions of PC (A, B, green) and E(Z) (C, D, red) and the merged images of PC and E(Z) (E, F) on polytene chromosomes isolated from wild-type (A, C, E) and *kis*<sup>k13416</sup> (B, D, F) larvae are shown. G-H: Comparison of the distribution of PC on the distal tip of the X chromosome of wild-type and *kis*<sup>k13416</sup> larvae (G), together with the corresponding DAPI staining (H). The loss of *kis* function did not lead to obvious changes in the level or distribution of PC or E(Z).



**Figure 2-10: Bands of KIS flank regions of H3K27 methylation.**

A. The distributions of KIS-L (green) and H3K27me3 (red) on wild-type salivary gland polytene chromosomes were compared by double-label immunofluorescence microscopy. B-E: The distributions of H3K27me3 (B), KIS-L (C), merged (D) and split images corresponding to the chromosome arm region bounded by the white rectangle are shown. KIS-L flanks many sites of H3K27me3 staining on polytene chromosomes.

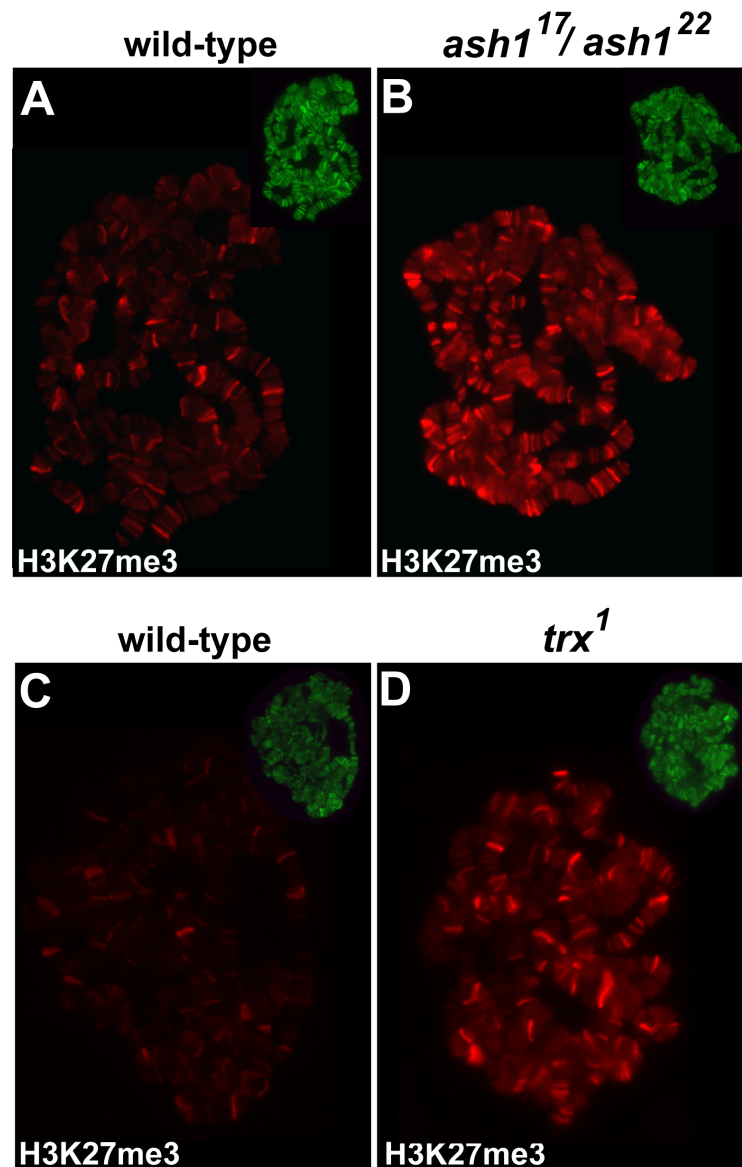




**Figure 2-11: Loss of *kis* function leads to increased H3K27 methylation.**

A-D: The level and distribution of H3K27me3 (A, B, red) and PC (C, D, green) on salivary gland polytene chromosomes of wild-type (A, C) and *kis*<sup>k13416</sup> (B, D) larvae were examined by double-label indirect immunofluorescence microscopy. Split images of the distributions of H3K27me3 (red) and PC (green) on wild-type (E) and *kis*<sup>k13416</sup> (F) polytene chromosomes are shown. For E and F, the levels of H3K27me3 were independently processed using Adobe Photoshop software to facilitate the comparison of the methyl mark and PC.

G: The distribution of H3K27me3 over the *fkh* gene was determined by ChIP using chromatin isolated from the salivary glands of wild-type (red bars) or *kis*<sup>k13416</sup> (green bars) larvae. The map of the *fkh* gene is the same as described in Figure 2-2. The ratio of DNA immunoprecipitated with antibodies against H3K27me3 and histone H3 are shown for each region. The bars represent the average of three independent biological experiments with the corresponding standard deviations.



**Figure 2-12: Loss of TRX and ASH1 function leads to increased H3K27 methylation.**

The levels of H3K27me3 (A-D, red) on polytene chromosomes isolated from wild-type (A, C), *ash1*<sup>22/ash1</sup><sup>17</sup> (B) and *trx*<sup>1</sup> (D) larvae were detected by double-label indirect immunofluorescence microscopy. H3K27me3 levels are higher on polytene chromosomes isolated from *ash1*<sup>22/ash1</sup><sup>17</sup> and *trx*<sup>1</sup> mutants as compared to wild-type chromosomes. As an internal control, the chromosomes were simultaneously stained with antibodies against the RPB1 subunit of RNA Pol II (inset in upper right corner of A-D, green).

## **Chapter 3**

### **Coordinated action of *Drosophila* trithorax group proteins Kismet and ASH1 counteract Polycomb group repression**

#### **Summary**

Polycomb and trithorax group proteins function antagonistically to maintain heritable states of gene transcription during development. How Polycomb and trithorax group proteins interact and exert their effects on gene expression is not entirely clear. One trithorax group protein, Kismet (KIS), facilitates transcription elongation and is related to the SWI/SNF and CHD families of chromatin-remodeling factors. As shown in the preceding chapter, KIS recruits the trithorax group histone methyltransferases ASH1 and TRX and counteracts the methylation of H3K27 by Polycomb group proteins. To determine the mechanism by which KIS functions, we examined the dependency relationships between transcription elongation, the trithorax group protein ASH1, and H3K27 methylation. Here we demonstrate that KIS facilitates transcription elongation independently of its role in recruiting ASH1 and counteracting H3K27 methylation. Additionally we present evidence that KIS promotes H3K36 methylation by recruiting ASH1, which we show dimethylates H3K36 *in vivo*. Our results indicate that KIS plays an important role in coordinating the function of trithorax group histone methyltransferases to antagonize Polycomb group repression.

#### **Materials and Methods**

##### **Fly culture**

Flies were raised on cornmeal, agar, yeast and molasses medium, supplemented with methylparaben (Tegosept) and propionic acid. Strains including the *kis*<sup>k13416</sup>, *ash1*<sup>22</sup> and *ash1*<sup>17</sup> recessive loss of function alleles used in this manuscript are described in FlyBase (<http://www.flybase.org>). Oregon R was used as the wild-type strain for all experiments.

### **Immunostaining of polytene chromosomes**

*Drosophila* salivary glands from 3<sup>rd</sup> instar larvae were fixed for 5 minutes in 45% acetic acid/1.85% formaldehyde and stained with rabbit polyclonal antibodies against ASH1 (TRIPOULAS *et al.* 1996) and KIS-L (SRINIVASAN *et al.* 2005), mouse monoclonal antibodies against Pol IIa, Pol IIo ser5 and Pol IIo ser2 (Covance) and guinea pig antibodies against SPT6 (KAPLAN *et al.* 2000). To stain polytene chromosomes with rabbit antibodies against H3K27me3 (Millipore), H3K36me2 (Abcam) and mouse antibodies against H3K36me2 (Wako) and 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. The secondary antibodies used in this study are from Jackson ImmunoResearch Laboratories. Chromosome preparations were mounted in Vectashield containing DAPI (Vector Laboratories).

For DRB treatment, salivary glands from wild-type third instar larvae were dissected in PBS and incubated for 1 hour at room temperature in Schneider's Insect Media (Sigma-Aldrich) treated with 65µM DRB (Sigma-Aldrich Cat.# D1916) dissolved in DMSO or DMSO alone as a control. Images were captured using a Zeiss Axioskop 2 plus microscope equipped with an Axioplan HRm camera and Axiovision 4 software (Carl Zeiss, Germany). Images were processed and split and merged images were generated using Adobe Photoshop CS4 software (CORONA *et*

*al.* 2004). Pixel intensities along polytene chromosome arms were measured using Image J and peak plots generated using Microsoft Excel.

When comparing the levels of proteins associated with wild-type and mutant polytene chromosomes, all images were captured with 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 fluorescence 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 Software (PerkinElmer). Average values were recorded in Microsoft Excel, normalized to the control values and plotted using standard deviation for error bars.

## **Results**

### **Establishing DRB treatment conditions**

One of the central functions of KIS is to facilitate transcription elongation. However, the role of transcription elongation itself in promoting the association of trithorax group proteins and counteracting Polycomb repression has not been established. Thus, the first goal of our study was to determine whether the loss of transcription elongation causes the concomitant loss of ASH1 from chromosomes

and increase in H3K27me3 observed in *kis* mutants. To do this, we needed to establish conditions in which transcription elongation was blocked, but the levels of KIS were unaffected. Thus, we took advantage of the commercially available transcription elongation inhibitor 5,6-dichlorobenzimidazole 1-beta-D-ribofuranoside (DRB).

DRB is a drug that has been used extensively to block transcription elongation in human cell lines (FRASER *et al.* 1978; LI *et al.* 2010; TAMM and KIKUCHI 1979), *Drosophila* cell culture and extracts (GIARDINA and LIS 1993; KELLNER *et al.* 2012; MARSHALL and PRICE 1992), *Drosophila* salivary glands (EGYHAZI *et al.* 1998; EGYHAZI *et al.* 1996) and *in vitro* transcription assays (CHODOSH *et al.* 1989; WADA *et al.* 1998). DRB is a nucleoside analog that binds to and inhibits CDK9, the RNA Pol II CTD kinase subunit of p-TEFb (BENSAUDE 2011). In our experiments, we treated salivary glands with DRB and then fixed, squashed and stained their polytene chromosomes with antibodies to investigate potential changes in the binding of chromatin factors upon loss of transcription elongation. To do this, we incubated dissected salivary glands for an hour in 65 $\mu$ M DRB. This concentration has been used in previous studies and was found in our own optimization experiments to strongly reduce the staining of Pol IIo ser2 on polytene chromosomes, with only modest effects on Pol IIo ser5 (Figure 3-3 M). DRB has been reported to have a 3-fold lower effect on CDK7 (MANCEBO *et al.* 1997), the serine 5 CTD kinase, than on CDK9. This is similar to our own observations, as upon treatment with 65 $\mu$ M DRB, the staining of initiating Pol II (Pol IIa) did not change (Figure 3-1 A-D), and though the levels of promoter proximal Pol II (Pol IIo ser5) were reduced (approximately 2.5 fold) (Figure 3-1 E-H), we saw a dramatic (7.5 fold) reduction in the levels of

elongating Pol II (Pol IIo ser2) (Figure 3-1 I-L) on polytene chromosomes relative to control salivary glands.

The transcription elongation factor SPT6 has been shown to bind to and travel with the elongating Pol II CTD (YOH *et al.* 2007). Consistent with this, SPT6 is highly co-localized with the elongating form of RNA Pol II (Pol IIo ser2) on polytene chromosomes (Figure 3-2 A-C). We had previously shown that SPT6 is lost from the chromosomes of *kis* mutant larvae (SRINIVASAN *et al.* 2005). Given the reported association between SPT6 and the serine 2 phosphorylated CTD, we anticipated that this loss was due to a block in transcription elongation in *kis* mutants. If true, we would expect treatment of salivary glands with DRB to result in loss of SPT6 from chromosomes. Indeed, SPT6 staining was strongly reduced upon treatment with DRB (Figure 3-2 D-H), consistent with SPT6 targeting by the serine 2 phosphorylated CTD.

In contrast to SPT6, the levels of KIS on chromosomes treated with DRB are unchanged relative to the control (Figure 3-1). This suggests that KIS is recruited to chromatin independent of transcription elongation. Importantly, this also provides the conditions necessary to test whether the effects of KIS loss are indirect (due to the block in transcription elongation) or direct (due to loss of KIS alone). Thus, if DRB treatment results in changes also observed in *kis* mutants, it would suggest these effects are due specifically to the loss of transcription elongation.

#### **DRB treatment does not affect ASH1 binding**

Our previous studies showed that the trithorax group histone methyltransferase ASH1 is lost from the chromosomes of *kis* mutant larvae (SRINIVASAN *et al.* 2008). The mechanisms underlying how ASH1 and many other

trithorax group proteins are targeted to actively transcribed genes are largely unknown. However, one logical hypothesis is that RNA Pol II helps recruits these factors. In support of this, ASH1 has been observed to bind downstream of promoters of actively transcribed genes, including the *Ubx* gene in *Drosophila* and the poly(A) binding protein, cytoplasmic 1 gene in HeLa cells (GREGORY *et al.* 2007; PAPP and MULLER 2006). Since ASH1 is lost in *kis* mutants where transcription elongation is blocked as well, we asked the question whether serine 2 phosphorylation of the Pol II CTD is responsible for recruiting ASH1 to actively transcribed genes. To address this question, we first examined the colocalization of ASH1 and Pol II ser2 on polytene chromosomes. Although the majority of ASH1 bands also have Pol II ser2 present, the intensities of the signals are not well correlated, such that the brightest bands of ASH1 do not correspond to the brightest bands of Pol II ser2 (Figure 3-3 A-C). This suggests that Pol II ser2 is unlikely to be the major determinant in the targeting of ASH1 to chromatin. We next examined the levels of ASH1 on salivary glands treated with DRB and found that the levels of ASH1 were not reduced relative to control control chromosomes (Figure 3-3 D-H). This further supports the notion that transcription elongation does not target ASH1 to actively transcribed genes. Taken together, these data strongly suggest that ASH1 is able to bind chromatin independently of transcription elongation.

### **DRB does not affect the levels of H3K27me3 on salivary gland polytene chromosomes**

One of the most striking phenotypes observed in *kis* mutants is the dramatic increase in the chromosomal levels of H3K27 trimethylation. Laid down by the Polycomb Repressive Complex PRC2, H3K27me3 is a histone modification



characteristic of transcriptionally repressed chromatin (CAO *et al.* 2002; CAO and ZHANG 2004). Many models exist for how methylation of H3K27 is held in check by trithorax group proteins. Active transcription has been implicated in the exchange of histone H3 for histone H3.3, which is enriched for active marks such as H3K4me3 and depleted for repressive marks such as H3K27me3 (HENIKOFF *et al.* 2004; MCKITTRICK *et al.* 2004; MITO *et al.* 2007). Interestingly, the chromatin-remodeling complex PBAP in conjunction with the transcription elongation factor FACT are recruited to chromatin boundaries by GAGA factor where they are required for H3.3 exchange and boundary function in *Drosophila* (NAKAYAMA *et al.* 2012). There is also evidence that active transcription brings in factors that remove H3K27me3 such as the demethylase UTX, which has been shown to co-localize with elongating Pol IIo ser2 (SMITH *et al.* 2008). Thus, it seemed plausible that KIS might counteract H3K27me3 by promoting transcription elongation. To determine whether transcription elongation counteracts H3K27me3, we first looked at whether Pol IIo ser2 is present at H3K27me3 boundaries. If transcription elongation is functioning as a barrier to prevent the spread of H3K27me3 into actively transcribed genes, we might expect bands of H3K27me3 to be located adjacent to regions of Pol IIo ser2. However, we found that while Pol IIo ser2 and H3K27me3 are largely non-overlapping there is not always a strict coincidence of Pol IIo ser2 flanking H3K27me3 (Figure 3-4 A-C). Furthermore, when we treated salivary glands with DRB, we did not observe an increase in the levels of H3K27me3. Instead, the levels of H3K27me3 on chromosomes from DRB-treated salivary glands were similar, if not slightly reduced, compared to controls (Figure 3-4 D-H). This finding is incompatible with a role for transcription elongation in limiting H3K27 trimethylation.

We cannot exclude the possibility that a more prolonged block to transcription elongation might ultimately affect H3K27me3 levels. However, we performed DRB incubations for up to 3 hours and never observed an increase in H3K27me3 (data not shown). After 3 hours *ex-vivo*, degeneration of the salivary gland makes immunostaining infeasible, so we were unable to test the effects of transcription elongation inhibition beyond 3 hours. However, taken together our data suggest that loss of transcription elongation is not likely responsible for the simultaneous loss of ASH1 and increase in H3K27me3 observed in *kis* mutants.

### **ASH1 is not required for transcription elongation**

ASH1 plays an important role in maintaining active states of gene transcription during *Drosophila* development (KLYMENKO and MULLER 2004; PAPP and MULLER 2006; TRIPOULAS *et al.* 1994). Though ASH1 is required for Hox gene transcription and is found downstream of active gene promoters, it is unclear what role if any ASH1 has in actively promoting transcription elongation. Given that ASH1 and transcription elongation are both impaired in KIS mutants and our data suggest that elongation is not responsible for recruiting ASH1, we decided to investigate whether ASH1 is involved in facilitating transcription elongation. To test this hypothesis, we stained chromosomes from *ash1* mutant and control larvae for ASH1, Pol IIo ser2 and SPT6. While the levels of ASH1 were dramatically reduced in the *ash1* mutant, the levels of Pol IIo ser2 and SPT6 did not change (Figure 3-5 A-G). This suggests that ASH1 is not required for transcription elongation. All together, these data indicate that ASH1 recruitment and function is not linked to transcription elongation. Our data suggest that KIS antagonizes Polycomb repression through two

independent mechanisms, the first being to promote transcription elongation and the second being to recruit ASH1 and counteract H3K27me3.

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

Our findings that KIS counteracts H3K27me3 independently of its role in transcription elongation prompted us to investigate other mechanisms by which KIS might antagonize H3K27me3. Recent studies have reported that methylation of H3K4 and H3K36 can inhibit the methylation of H3K27 by PRC2 (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 was not previously explored. To investigate whether KIS antagonizes H3K27me3 by promoting H3K36 methylation, we stained polytene chromosomes from wild-type and *kis* mutant larvae with antibodies recognizing H3K36 di- or tri-methylation. In *Drosophila*, H3K36 trimethylation is performed by Set2/HypB, which is known to associate with RNA Pol II (BELL *et al.* 2007; STABELL *et al.* 2007). As we have previously shown, loss of KIS function causes a reduction in the total levels of RNA Pol II on polytene chromosomes. Similarly, the chromosomal levels of H3K36me3 were dramatically reduced on polytene chromosomes from *kis* mutant larvae (Figure 3-6 A-D, I). We next examined the role of KIS in H3K36 dimethylation, a modification catalyzed by the histone methyltransferase dMes4 (BELL *et al.* 2007). Interestingly, H3K36 dimethylation can also be catalyzed *in vitro* by ASH1 (AN *et al.* 2011; TANAKA *et al.* 2007; YUAN *et al.* 2011). We observed a 2-3 fold reduction in H3K36me2 on polytene chromosomes from *kis* mutant larvae, coinciding with increased chromosomal levels of H3K27me3 as previously reported (Figure 3-6 E-H,I). These data suggest KIS may antagonize H3K27me3 by

promoting H3K36 tri- and di-methylation. Furthermore, the reduction of both H3K36me2 and ASH1 observed in *kis* mutants suggested ASH1 might methylate this residue. Therefore, we decided to directly examine whether ASH1 dimethylates H3K36 *in vivo*.

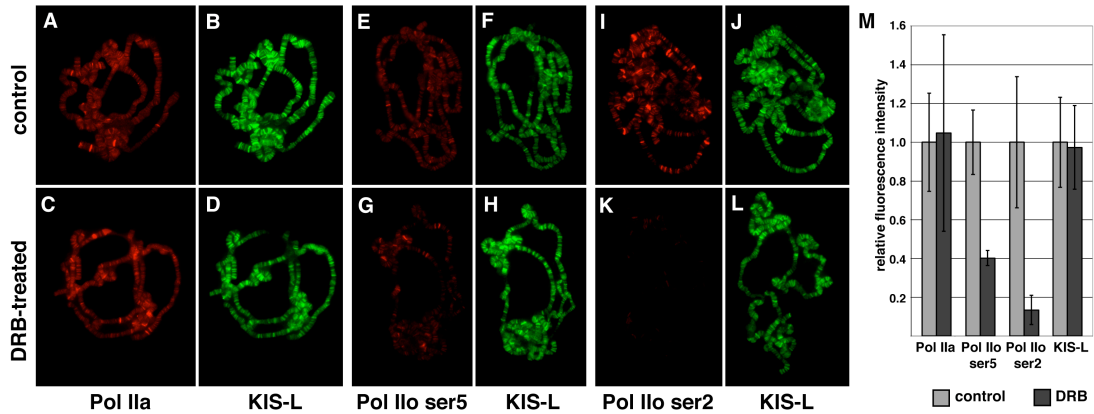
### **Ash1 dimethylates H3K36me2 *in vivo***

ASH1 has been reported to methylate a number of histone residues, including H3K4, H3K9, H4K20. However, there is not strong evidence that ASH1 catalyzes these modifications *in vivo* (BEISEL *et al.* 2002; BYRD and SHEARN 2003). Recently, two separate reports have suggested ASH1 dimethylates H3K36 *in vitro* (TANAKA *et al.* 2007; YUAN *et al.* 2011). Intriguingly, one of these reports also demonstrated that H3K36 methylation prevents PRC2 from methylating H3K27 *in vitro* (YUAN *et al.* 2011). These data suggest ASH1 may counteract Polycomb repression by dimethylating H3K36. We therefore decided to test 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, we should observe a significant overlap between the chromosomal distributions of ASH1 and H3K36me2. Consistent with this, ASH1 strongly colocalizes with H3K36me2 on polytene chromosomes (Fig 3-7 A-B). We next examined whether the loss of ASH1 function affects the chromosomal levels of H3K36me2. Strikingly, we observed a strong reduction in the levels of H3K36me2 on the polytene chromosomes of *ash1* mutant larvae compared to wild-type (Figure 3-7 D,G,I). This result indicates ASH1 catalyzes H3K36me2 *in vivo*. To determine whether ASH1 also contributes to H3K36me3, we examined the levels of H3K36me3 in the absence of ASH1 function.

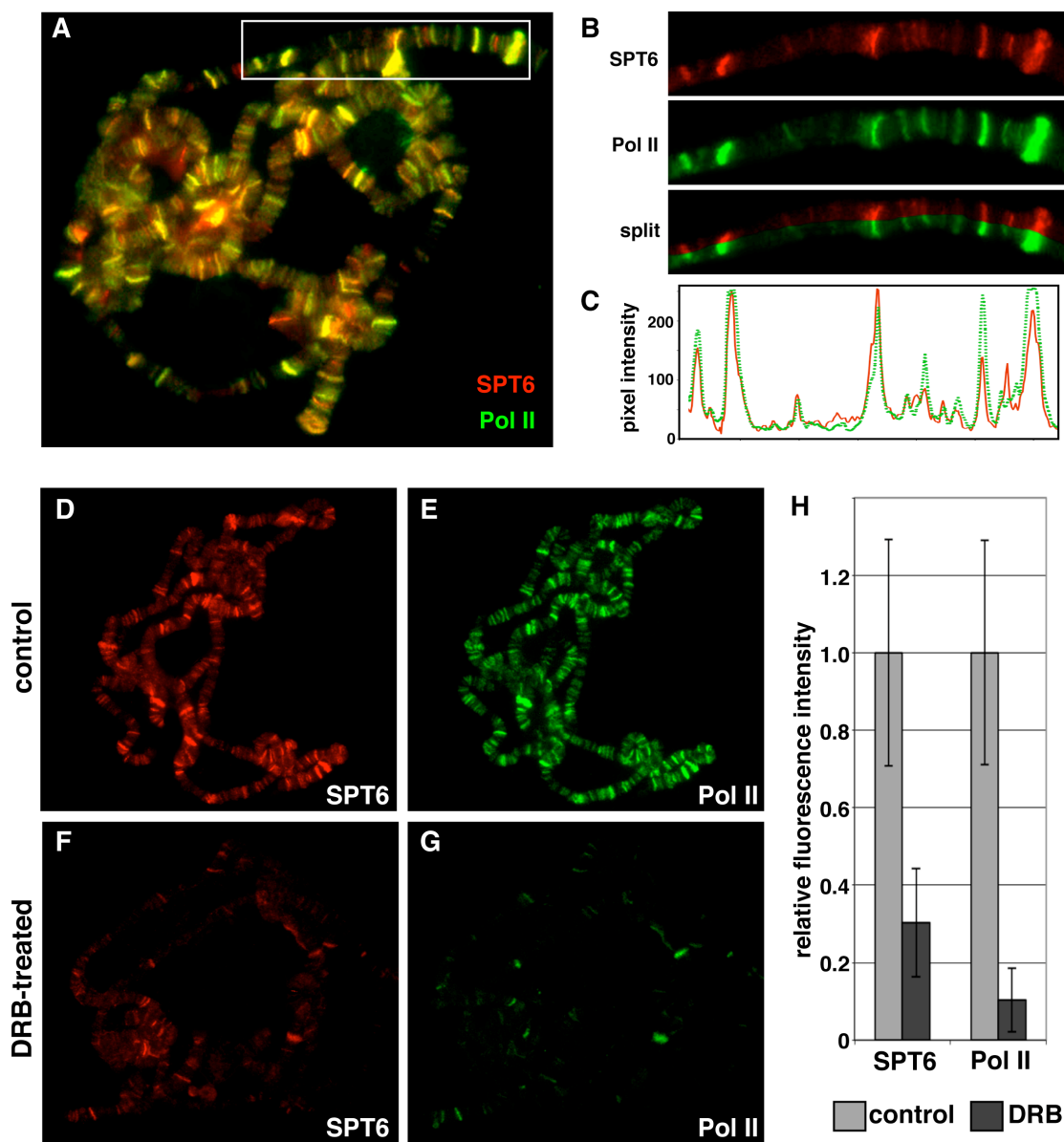
In contrast to H3K36me2, we did not observe a reduction in the levels of H3K36me3 in *ash1* mutant larvae relative to controls (Figure 3-7 E,H,I), suggesting ASH1 specifically dimethylates H3K36.

We next examined the distribution of H3K36me2 relative to H3K27me3 to investigate whether H3K36me2 inhibits the ability of PRC2 to methylate H3K27 *in vivo*. Consistent with this possibility, we observed very little overlap between these marks (Figure 3-8 A-B). Furthermore, we observed that H3K36me2 often flanks regions of H3K27me3 (Figure 3-8 A-B). This juxtaposed pattern suggests 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, increased levels of H3K27me3 were strongly correlated with reduced levels of H3K36me2 on polytene chromosomes from *ash1* mutant larvae relative to controls (Figure 3-8 C-G). Thus, our *in vivo* data, combined with the previously published *in vitro* data of others strongly suggest that ASH1 functions to dimethylate H3K36. Importantly, this may represent the primary mechanism by which ASH1 counteracts H3K27me3 and Polycomb-mediated transcriptional repression. Furthermore, it suggests KIS may antagonize Polycomb repression by promoting H3K36 dimethylation through the recruitment of ASH1 to chromatin.



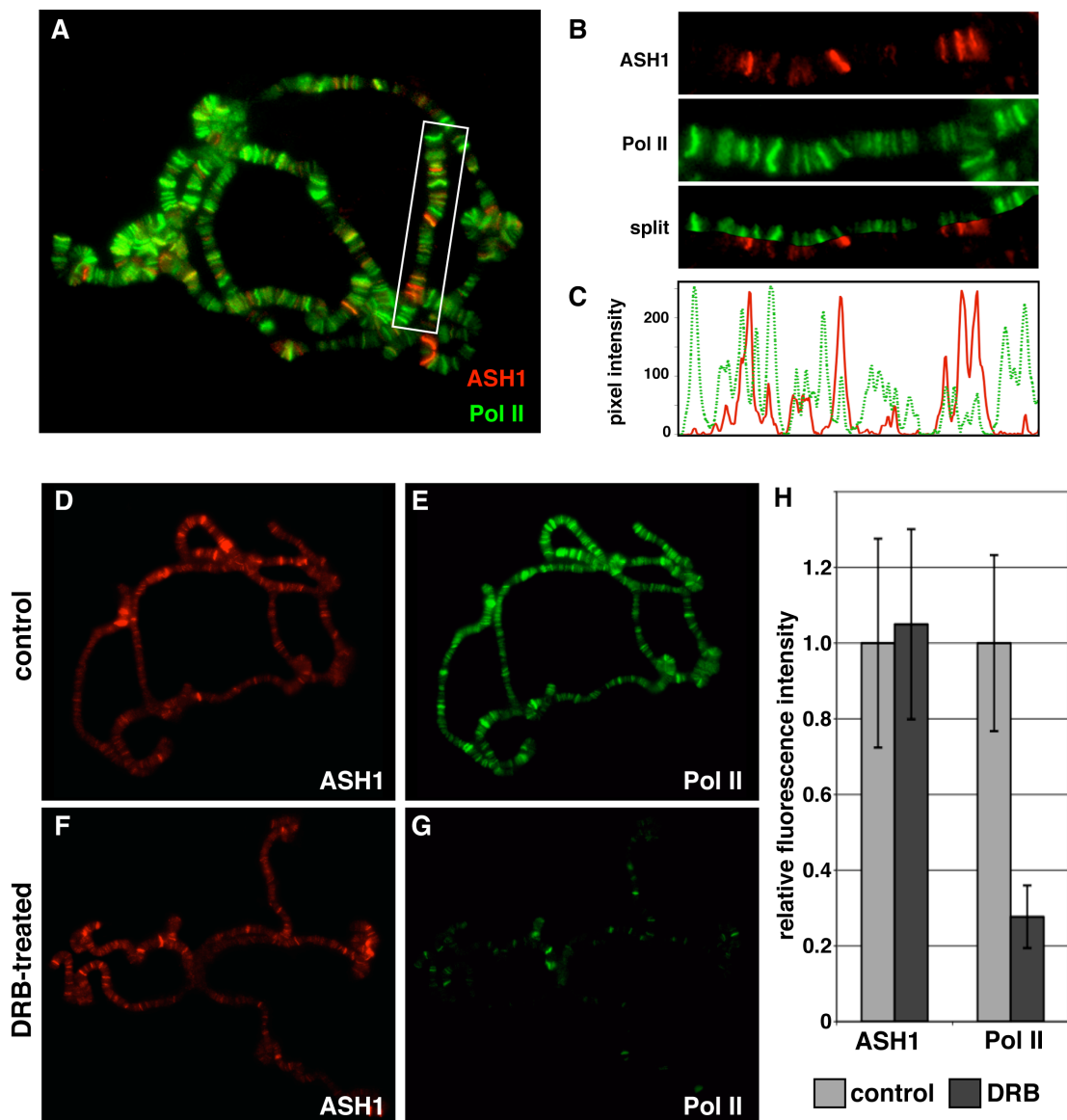
**Figure 3-1. DRB inhibits phosphorylation of RNA Pol II on serine 2.**

Polytene chromosomes were treated with DMSO as a control (A, B, E, F, I and J) or the drug DRB (65 $\mu$ M) (C, D, G, H, K and L) and stained with antibodies against RNA Pol IIa (A, C), RNA Pol IIo ser5 (E, G), RNA Pol IIo ser2 (I, J) and KIS-L (B, C, F, H, J, L). The average fluorescence intensity normalized to the DMSO control (M) shows an approximate 7.5-fold reduction in RNA Pol IIo ser2 fluorescence on the DRB treated chromosomes, but no change in KIS-L or RNA Pol IIa and only a 2.5-fold reduction in RNA Pol IIo ser5.



**Figure 3-2. SPT6 colocalizes with Pol II ser2 and requires elongating RNA Pol II to localize to chromatin.**

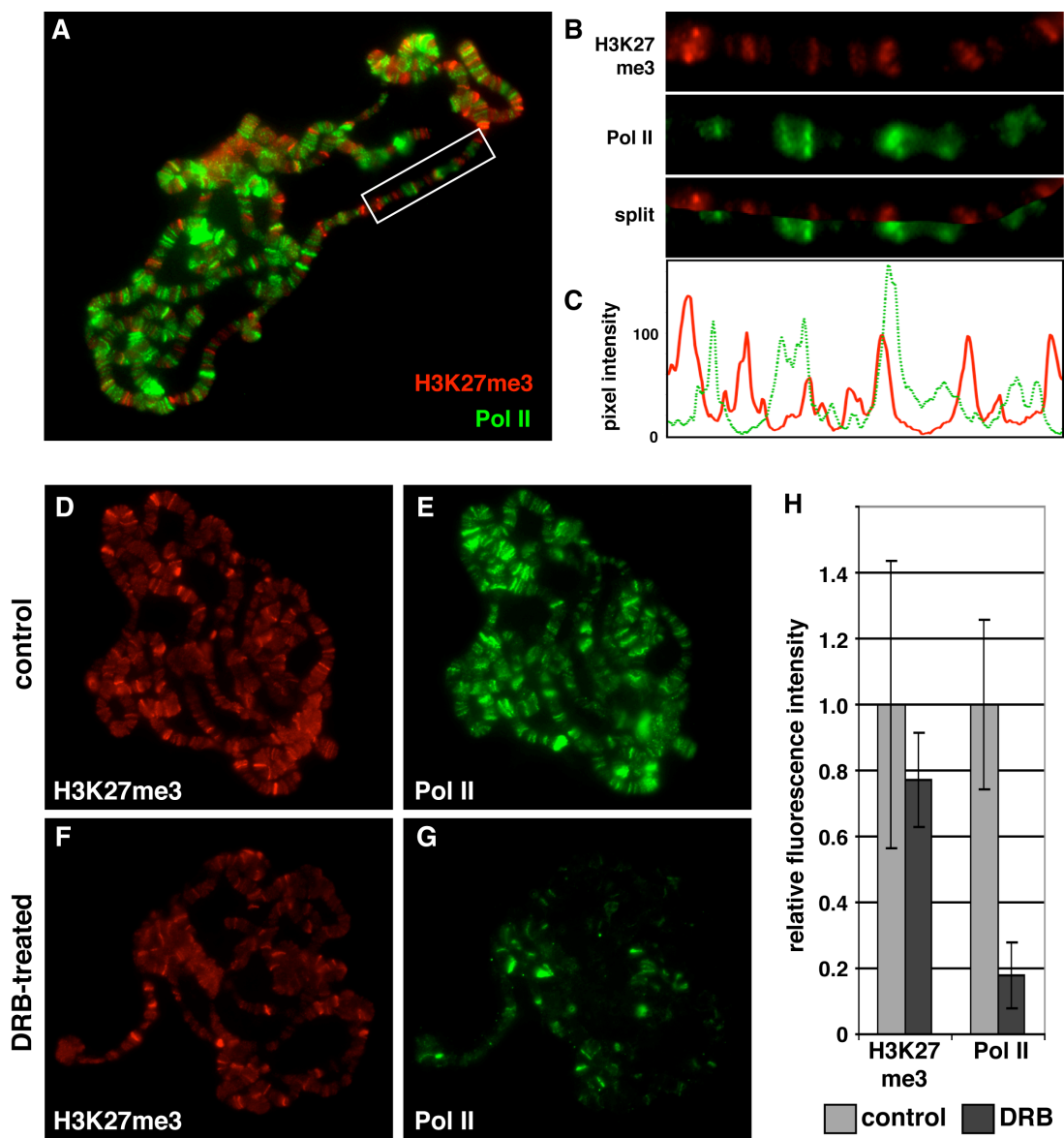
Merged image shows staining with antibodies against SPT6 (red) and RNA Pol II ser2 (green) on polytene chromosomes (A). Magnification of the chromosome arm bound by the white box in A is shown in B. The banding pattern of SPT6 (red) and Pol II ser2 (green) alone and split in conjunction with a comparison of the band size and intensity for both SPT6 and Pol II ser2 (C) show a highly coincident pattern. Polytene chromosomes were treated with either DMSO (D,E) or DRB (F,G) and stained with antibodies against SPT6 (D,F) and Pol II ser2 (E,G). The relative fluorescence intensity of SPT6 was reduced about 3-fold on the DRB treated chromosomes (H).



**Figure 3-3. ASH1 does not require elongating RNA Pol II to localize to chromatin.**

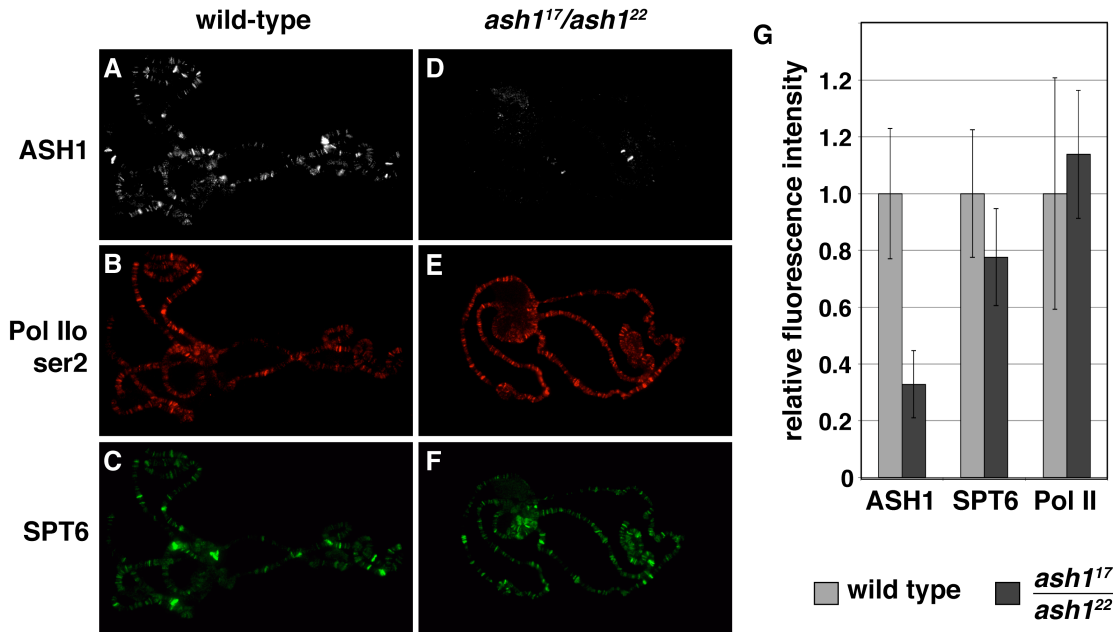
Merged image shows staining with antibodies against ASH1 (red) and RNA Pol II ser2 (green) on polytene chromosomes (A). Magnification of the chromosome arm bound by the white box in A is shown in B. The banding pattern of ASH1 (red) and Pol II ser2 (green) alone and split in conjunction with the band intensity distribution of ASH1 and Pol II ser2 (C) show that though Pol II ser2 is present at many sites of ASH1, the intensities of each are not well correlated. Polytene chromosomes were treated with either DMSO (D,E) or DRB (F,G) and stained with antibodies against ASH1 (D,F) and Pol II ser2 (E,G). The relative fluorescence intensity of ASH1 was not reduced on the DRB-treated chromosomes (H).



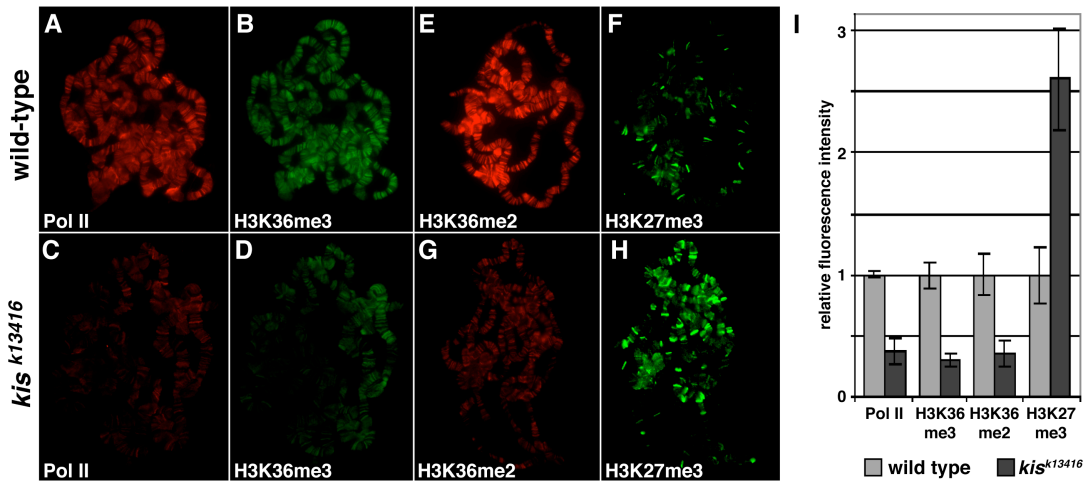


**Figure 3-4. DRB treatment does not affect the trimethylation histone H3 on lysine 27.**

Colocalization of the histone modification H3K27me3 (red) and Pol II ser2 (green) is shown on a polytene chromosome (A). The region of the chromosome arm bound by white box in A is magnified in B. Bands of H3K27me3 (red) and Pol II ser2 (green) are often adjacent and non-overlapping as shown in the split image and in the distribution of band intensities (C). Polytene chromosomes treated with either DMSO (D,E) or DRB (F,G) and stained with antibodies against H3K27me3 (D, F) and Pol II ser2 (E,G). No significant difference in the relative fluorescence intensity of H3K27me3 was observed on polytene chromosomes from salivary glands treated with DRB (H).

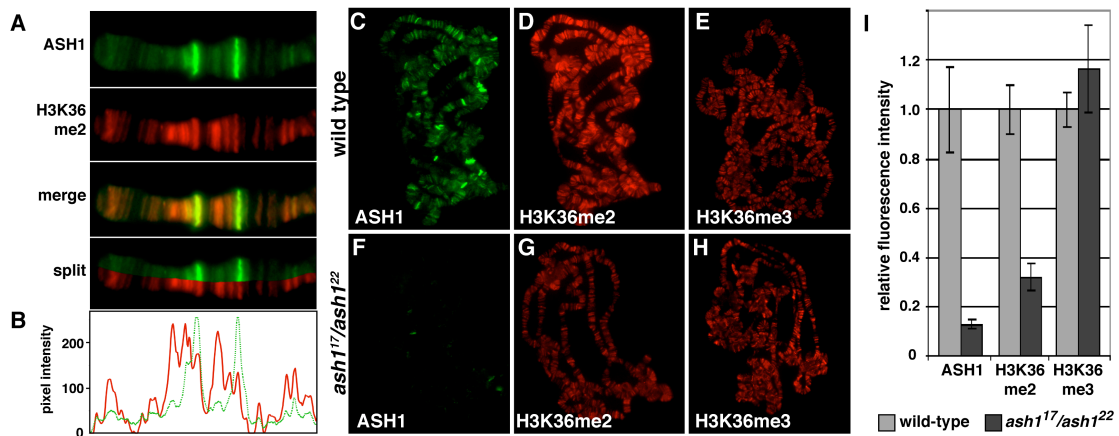


**Figure 3-5. Transcription elongation is not affected by loss of ASH1.** Polytene chromosomes from wild-type (A, B, C) and *ash1* (D, E, F) mutant larvae were stained with antibodies against ASH1 (A, D), Pol Ilo ser2 (B, E) and SPT6 (C, F). The relative fluorescence intensity for the staining of ASH1, SPT6 and Pol Ilo ser2 is shown in G.



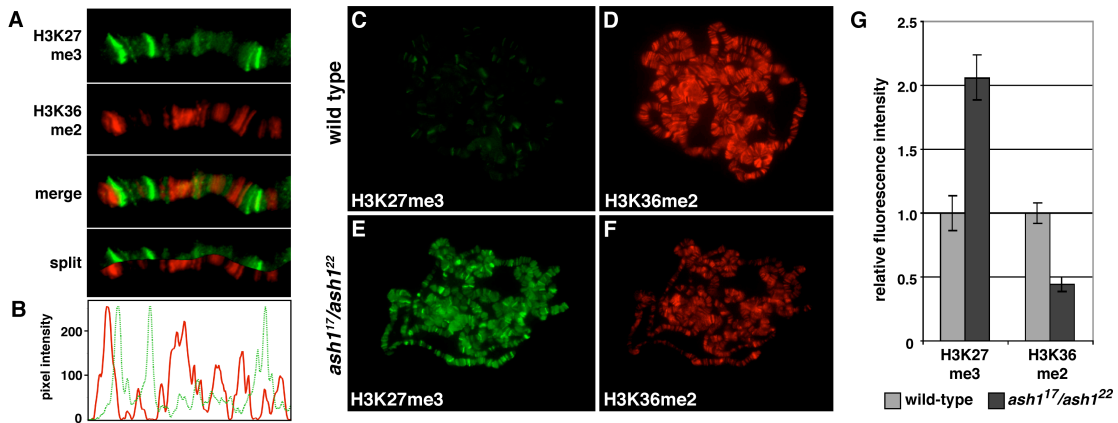
**Figure 3-6. Tri- and di-methylation of histone H3 on lysine 36 are reduced in *kis* mutants.**

Polytene chromosomes from wild-type (A, B, E, F) and *kis* mutant (C, D, G, H) were stained with antibodies against total RNA Pol II (A, C), H3K36me3 (B, D), H3K36me2 (E, G) and H3K27me3 (F, H). The relative fluorescence intensity of Total Pol II, H3K36me3, H3K36me2 and H3K27me3 staining is shown in I.



**Figure 3-7. ASH1 is a H3K36 dimethylase.**

Colocalization of ASH1 (green) and H3K36me2 (red) on the distal arm of a polytene chromosome is shown individually and in merged and split images, as well as in a comparison of the band intensities along the arm (B). Polytene chromosomes from wild-type (C,D,E) and *ash1* mutant larvae (F,G,H) were stained with antibodies against ASH1 (C,F), H3K36me2 (D,G) and H3K36me3 (E,H). An approximately 3-fold reduction in the average fluorescence intensity of H3K36me2 was observed in *ash1* mutants (I).



**Figure 3-8. Decrease of H3K36me2 is correlated with increase in H3K27me3 in *ash1* mutants.**

The banding pattern of H3K27me3 (green) and H3K36me2 (red) from a polytene chromosome arm shown individually and in merged and split images, as well as in the distribution of pixel intensities along an arm (B). Polytene chromosomes from wild-type (C,D) and *ash1* mutant larvae (E,F) stained with antibodies against H3K27me3 (C,E) and H3K36me2 (D,F). Relative fluorescence intensities of H3K27me3 and H3K36me2 are shown in G.

## Chapter 4

### Discussion

When I began my dissertation research I was very interested in how trithorax group proteins are recruited to active genes. Unlike many transcription factors, which bind a small subset of genes through interaction with specific DNA sequences, trithorax group proteins localize to many genes and lack sequence-specific recognition domains. How do trithorax group proteins get to their target genes and maintain active states of transcription? This is one of the questions I worked to address in my dissertation.

#### **ASH1 and TRX antagonize H3K27 methylation**

The trithorax group histone methyltransferases ASH1 and TRX are thought to function as Polycomb anti-repressors that indirectly promote transcription by counteracting Polycomb group repression. Neither ASH1 nor TRX are required for transcription of the Hox gene *Ubx* in the absence of Polycomb group function (KLYMENKO and MULLER 2004). Interestingly, recent genome-wide ChIP assays have revealed that Polycomb group proteins are associated with relatively specific regions of chromatin (Polycomb-response elements, or PREs) in contrast to H3K27me<sub>3</sub> which is found over broad chromatin domains adjacent to PREs, encompassing both the regulatory and coding regions of transcriptionally silent genes (SCHWARTZ *et al.* 2006; SCHWARTZ *et al.* 2010; TOLHUIS *et al.* 2006). Evidence in *Drosophila* suggests that ASH1 and TRX counteract Polycomb group silencing by interfering with H3K27 methylation. Loss of *ash1* in haltere discs leads to the spread of H3K27me<sub>3</sub> into the body of the *Ubx* gene, which is normally transcribed in this tissue (PAPP and MULLER 2006). In addition, our studies suggest that ASH1 and TRX counteract global H3K27

methylation. The levels of H3K27 methylation increase globally on polytene chromosomes in the absence of ASH1 and TRX (SRINIVASAN et al. 2008). Thus ASH1 and TRX each seem to play a role in counteracting H3K27 methylation.

### **KIS recruitment is independent of ASH1, TRX and H3K4 methylation**

At the time I began my research, the prevailing model was that ASH1 and TRX promote active transcription through the methylation of histone H3 on lysine 4. The presence of two chromodomains in KIS-L suggested that it might directly interact with nucleosomes in the vicinity of promoters that are methylated on H3K4. Our hypothesis was that H3K4 methylation by ASH1 and TRX might recruit KIS to chromatin or stimulate its remodeling activity via its chromodomains. We suspected the recruitment of KIS by ASH1 and TRX might function to antagonize H3K27 methylation. However, when we tested this hypothesis we found many lines of evidence to the contrary. The chromodomains of KIS-L did not interact with H3K4 methylated peptides *in vitro* and there was not a strong correlation between the distribution of KIS-L and methylated H3K4 on salivary gland polytene chromosomes. Levels of KIS binding did not increase in larvae lacking the H3K4 demethylase LID, which have higher levels of H3K4 methylation compared to wild-type. Furthermore, neither ASH1 nor TRX were necessary for the association of KIS-L with chromatin *in vivo* (SRINIVASAN et al. 2008). Thus, our data strongly suggested that ASH1 and TRX do not mediate interactions between KIS-L and chromatin via H3K4 methylation. However, it is worth noting that H3K4 methylation may still play a role in stimulating the remodeling activity of KIS after it has been recruited to chromatin.

A surprising discovery was that KIS-L is required for the recruitment of ASH1 and TRX. In the absence of KIS, ASH1 and TRX localization is lost at the majority of sites on polytene chromosomes. This finding suggested that the loss of KIS function would also result in increased H3K27 methylation as a consequence of reduced ASH1 and TRX binding. We therefore decided to determine whether KIS affects either the binding or activity of Polycomb group proteins. We found that the loss of KIS function did not affect PRC1 or PRC2 binding, but did lead to a significant increase in the levels of H3K27me3 on salivary gland polytene chromosomes (SRINIVASAN *et al.* 2008). This result supports our hypothesis that KIS-L may prevent the spread of H3K27 methylation in the vicinity of PREs by recruiting ASH1 and TRX. Importantly, these observations provide a potential molecular explanation for the genetic antagonism between Polycomb group genes and the trithorax group genes *kis*, *ash1* and *trx*.

### **KIS recruits ASH1 and counteracts H3K27 methylation independent of its role in transcription elongation**

The established role of KIS in promoting transcription elongation suggested that KIS recruits trithorax group proteins and antagonizes H3K27 methylation by promoting transcription elongation. Insights into the respective roles of TRX and ASH1 in counteracting Polycomb repression have come from recent genome-wide CHIP studies of TRX and ASH1 localization in *Drosophila* tissue culture cells (SCHUETTENGRUBER *et al.* 2009; SCHWARTZ *et al.* 2010). Whereas TRX binds to active gene promoters and the PREs adjacent to both transcriptionally active and repressed genes, ASH1 localizes more specifically at the promoters of



transcriptionally active genes. These results suggest ASH1 might function primarily in transcriptional activation and furthermore may be recruited by RNA Pol II.

Elongating RNA Pol II has a well-known role in recruiting protein factors that perform a variety of functions during the transcription cycle. A host of mRNA processing factors and histone modifying enzymes are recruited to RNA Pol II's phosphorylated CTD during transcription and are required for the appropriate transcription, splicing, capping and export of mRNAs (BARTKOWIAK *et al.* 2011; ZHANG *et al.* 2012). For example, the histone methyltransferase SET2 is recruited to elongating serine 2 phosphorylated RNA Pol II CTD and is responsible for methylating histone H3 on lysine 36. Elongating RNA Pol II also recruits the histone deacetylase RPD3, which it is activated by H3K36 methylation to orchestrate the closing of chromatin behind the elongating polymerase to prevent cryptic initiation (CARROZZA *et al.* 2005; DROUIN *et al.* 2010; KEOGH *et al.* 2005). Another example of CTD recruitment is the histone methyltransferase COMPASS/SET1, which is recruited to the 5' ends of genes by serine 5 phosphorylated RNA Pol II CTD where it methylates H3K4, a mark associated with transcriptionally active genes (NG *et al.* 2003). In addition, phosphorylated Pol II CTD has been hypothesized to recruit the histone demethylase UTX, which removes methyl groups from H3K27 to prevent transcriptional repression (SMITH *et al.* 2008). These findings suggest the CTD of RNA Pol II plays an important role in recruiting factors that maintain active states of transcription. Thus, we hypothesized that KIS might recruit ASH1 and counteract Polycomb repression by facilitating transcription elongation by RNA Pol II.

To test this hypothesis we used the drug DRB – which prevents phosphorylation of the Pol II CTD at serine 2 – to selectively block transcription

elongation. The association of KIS with chromatin was not affected upon treatment with DRB, which allowed us to determine which aspects of the *kis* mutant phenotype are caused by loss of elongation. We observed that the trithorax group protein ASH1 is not lost in the absence of transcription elongation, suggesting it is not recruited by the phosphorylated CTD. These data are consistent with studies in *Drosophila* showing that RNAi knock down of CDK9 does not affect ASH1 localization (EISSENBERG *et al.* 2007b) and studies in mammalian cells where ASH1 is still present at promoters upon DRB treatment (GREGORY *et al.* 2007). Taken together, the model that KIS recruits ASH1 by promoting transcription elongation is not supported by our data. However, we were still interested in whether KIS might play a role in counteracting H3K27 methylation by promoting transcription elongation.

Steady-state levels of H3K27 methylation on chromatin are determined by multiple factors. The activity of the E(Z) methyltransferase, the accessibility of its nucleosome substrate, the frequency of nucleosome eviction or exchange, and the level and activity of histone H3K27 demethylases all play a role in regulating H3K27 methylation. The *Drosophila* H3K27 demethylase UTX co-localizes with elongating Pol II suggesting H3K27 demethylation may be directly coupled to transcription elongation (SMITH *et al.* 2008). Replacement of histone H3 by the histone variant H3.3 occurs during transcription. H3.3 harbors covalent modifications associated with actively transcribed genes, including elevated H3K4 methylation and low levels of H3K27 methylation (MCKITTRICK *et al.* 2004); loss of KIS-L function leading to a block in transcription elongation could therefore lead to elevated levels of H3K27 methylation in the body of genes through loss of H3K27 demethylases and reduced histone H3.3 exchange.

Using the DRB assay we asked whether the levels of H3K27 methylation increase following inhibition of elongation. When we treated salivary glands with DRB we did not observe an increase H3K27me3. We looked for effects on H3K27 methylation for up to 3 hours and at a ten-fold higher concentration of DRB, but never observed an increase in H3K27 methylation levels. While we cannot rule out that long-term loss of transcription elongation may indirectly affect the levels of H3K27 methylation, our data suggest that active elongation is not required to directly counteract E(Z) activity. We therefore concluded that KIS does not antagonize H3K27 methylation by promoting transcription elongation. Taken together, our data suggest KIS does not recruit ASH1 or counteract H3K27 methylation by promoting transcription elongation.

This finding prompted us to investigate whether KIS might promote transcription as a consequence of recruiting the trithorax group histone methyltransferase, ASH1. We hypothesized that the failure to recruit ASH1 might be responsible for the elongation defects observed in *kis* mutants. Thus, we set out to test whether KIS facilitates transcription elongation by recruiting ASH1. When we looked at the levels of elongating RNA Pol II and SPT6 in *ash1* mutants, we found the levels were normal. Our observation that transcription elongation proceeds normally in the absence of ASH1 suggests that KIS-L does not facilitate transcription elongation by recruiting ASH1. These studies clearly indicate that transcription elongation is not linked to ASH1 recruitment, function or H3K27 methylation. This suggests KIS-L must play two mechanistically distinct roles in maintaining active states of transcription. KIS-L facilitates transcription elongation and independently promotes ASH1 recruitment and inhibits H3K27 methylation.

## **KIS coordinates the function of histone methyltransferases to antagonize H3K27 methylation**

A larger picture of H3K27me<sub>3</sub> regulation is beginning to take shape, based on our data and the work of others. It seems increasingly likely that many mechanisms exist to counteract methylation of H3K27 by PRC2. Interestingly, KIS seems to be at the nexus of many of these mechanisms.

The trithorax group protein and histone methyltransferase TRX has been proposed to counteract H3K27me<sub>3</sub> in at least two ways. One is through the methylation of H3K4, a modification that was recently shown to inhibit PRC2. H3K4 trimethylation directly interferes with the binding of the PRC2 subunit Nurf55 to nucleosomes and inhibits the catalytic activity of E(Z) allosterically through interactions with the PRC2 subunit Su(Z)12 (SCHMITGES *et al.* 2011). TRX-mediated inhibition of PRC2 via H3K4 methylation however is likely to be relevant only at a small subset of genes, as global H3K4 methylation in *Drosophila* is mediated primarily by another histone methyltransferase dSET1 (ARDEHALI *et al.* 2011; HALLSON *et al.* 2012). The other mechanism by which TRX may affect H3K27 methylation is through its interaction with the acetylase CBP and acetylation of H3K27 (TIE *et al.* 2009). H3K27 acetylation constitutes a direct physical block to methylation of the same residue by PRC2. Importantly, KIS is required to recruit TRX to many sites on polytene chromosomes, suggesting that the increase in H3K27me<sub>3</sub> observed in *kis* mutants may in part be due to the loss of TRX and CBP activity.

Another mechanism for counteracting H3K27me<sub>3</sub> is through the methylation of another histone residue, lysine 36 on histone H3 (H3K36). Two recent studies have suggested that both di- and tri-methylation of H3K36 can block the catalytic

activity of PRC2 *in vitro* (SCHMITGES et al. 2011; YUAN et al. 2011). H3K36 methylation-mediated inhibition of PRC2 *in vitro* suggests that H3K36 methyltransferases could play an important role in counteracting Polycomb repression *in vivo*. In *Drosophila*, H3K36 trimethylation is catalyzed by Set2, also known as HypB, which associates with the elongating RNA Polymerase 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). However, H3K36me3 may also play a role in preventing H3K27me3 from spreading into the 3' end of transcribed genes. Although we did not observe an increase in H3K27 methylation when we block transcription elongation with DRB, residual H3K36 trimethylation could persist possibly due to slow turnover of this mark. If H3K36 trimethylation does block PRC2 function *in vivo*, it may represent another mechanism by which KIS antagonizes H3K27me3, as we observed reduced H3K36me3 in *kis* mutants consistent with the role of KIS in facilitating transcription elongation. In *Drosophila*, the protein dMes-4 contributes to H3K36 dimethylation as well as trimethylation and plays a role in regulating H4K16 acetylation during transcription elongation (BELL *et al.* 2007). In *C.elegans*, the ability of MES-4 to trimethylate H3K36 at germline genes is required to prevent germline gene silencing by H3K27 methylation (GAYDOS *et al.* 2012). Thus, antagonism of H3K27 methylation by H3K36 trimethylation may represent a conserved mechanism for antagonizing PRC2 function to maintain appropriate patterns and steady-state levels of transcription.

In contrast to H3K36 trimethylation, H3K36 dimethylation is concentrated in the 5' coding region adjacent to regions of H3K4 trimethylation (BELL *et al.* 2007).

H3K36 dimethylation also antagonizes PRC2 function (SCHMITGES *et al.* 2011; YUAN *et al.* 2011). This is compelling given the recent reports that link ASH1 to H3K36 dimethylation *in vitro* (AN *et al.* 2011; TANAKA *et al.* 2007; YUAN *et al.* 2011) and the findings that ASH1 antagonizes H3K27 methylation *in vivo* (PAPP and MULLER 2006; SRINIVASAN *et al.* 2008). The histone specificity of ASH1 has been highly controversial over the years. ASH1 has been proposed to methylate H3K4, H3K9 and H4K20 *in vitro* and *in vivo* (BEISEL *et al.* 2002; BYRD and SHEARN 2003; SANCHEZ-ELSNER *et al.* 2006). However, these results have been called into question as we and others have failed to replicate them.

The findings that ASH1 antagonizes PRC2 function, that H3K36 dimethylation antagonizes PRC2 function, and that ASH1 dimethylates H3K36 *in vitro* prompted us to test whether ASH1 might dimethylate H3K36 *in vivo*. Indeed, we found that ASH1 not only significantly colocalizes with H3K36me<sub>2</sub>, but is also required for H3K36 dimethylation *in vivo*. Thus, our findings in conjunction with the reports that ASH1 dimethylates H3K36 *in vitro* have exciting implications for how ASH1 might antagonize Polycomb group protein function. The ability of ASH1 to dimethylate H3K36 could represent the major mechanism by which ASH1 counteracts H3K27 methylation by PRC2. Similarly, KIS may counteract H3K27 methylation through effects on H3K36me<sub>2</sub> mediated by the recruitment of ASH1, as we also find the levels of H3K36me<sub>2</sub> reduced in *kis* mutants.

Taken together, our findings suggest KIS plays a central role in coordinating the function of trithorax group histone methyltransferases to antagonize H3K27 methylation. Histone methylation by ASH1 and TRX may represent a significant mechanism for constraining the positive feedback loop by which PRC2 H3K27

methylation spreads. Inhibition of E(Z) histone methyltransferase activity by H3K4 and H3K36 methylation may function as a barrier to halt the spread H3K27 methylation and silencing of genes by Polycomb. The model that these marks inhibit H3K27 spreading may explain why we do not observe new bands of H3K27 methylation on polytene chromosomes in *kis* mutants despite global increases in H3K27 methylation.

### **KIS human homologue CHD7 is important for human development**

Our results demonstrating that KIS plays an important role in integrating the function of trithorax group histone methyltransferases and antagonizing Polycomb repression have potentially significant implications for human health. CHD7, a KIS homologue in humans, is required for normal human development and may have roles in cancer as well. CHD7 haplo-insufficiency is a common cause of CHARGE syndrome as 58% of affected children have a mutation in one copy of CHD7 (JANSSEN *et al.* 2012). CHARGE syndrome is a human developmental disorder involving tissues derived from the developing neural crest. Common symptoms of CHARGE syndrome are coloboma of the eye, cranial nerve abnormalities, ear defects and hearing loss, congenital heart defects, genital abnormalities and narrowing or blockage of the choanae (nasal passages) (JANSSEN *et al.* 2012; JONGMANS *et al.* 2006). CHARGE syndrome affects approximately 1 in 10,000 live births, and babies born with CHARGE syndrome often have severe health complications. Studies of CHD7 have shown that CHD7 loss impairs the migration of neural crest stem cells differentiated from human embryonic stem cells and disrupts the formation of the neural crest leading to CHARGE-like defects in *Xenopus* (BAJPAI *et al.* 2010). Interestingly, CHD7 interacts with the BRM-related chromatin-

remodeling complex PBAF in this process (BAJPAI *et al.* 2010). CHD7 was also reported to cooperate with Sox2 to regulate transcription in ES cells (ENGELEN *et al.* 2011) and to localize to ES cell active gene enhancers (SCHNETZ *et al.* 2010). CHD7 also binds to promoter regions and largely co-localizes with H3K4 monomethylation at enhancers (SCHNETZ *et al.* 2009). This is consistent with our data in *Drosophila* showing that KIS binds to the *forkhead* promoter and enhancer, and with other work showing KIS binds to PREs and promoters at the *Ubx* locus in imaginal discs (PAPP and MULLER 2006; SRINIVASAN *et al.* 2008). Our findings that KIS functions as a key modulator of Polycomb group protein repression and transcription elongation suggest that CHARGE syndrome may arise from the inability of CHD7 to maintain active states of gene expression during development.

Insight into the function of CHD7 was recently gained from biochemical studies illustrating that CHD7 has nucleosome-remodeling activity (BOUAZOUNE and KINGSTON 2012). *In vitro* nucleosome-remodeling assays demonstrated CHD7 has the ability to mobilize nucleosomes and expose nucleosomal DNA to restriction digestion. Both of these activities were dependent on ATP and the presence of a conserved lysine in the ATPase domain of CHD7. Furthermore, mutations commonly seen in CHARGE syndrome, including truncations and point mutations in the chromodomains, reduce or abolish CHD7 remodeling activity. These data suggest that the nucleosome-remodeling activity of CHD7 is highly relevant to CHARGE syndrome. Furthermore, these data implicate the chromodomains in potentially fine-tuning the remodeling activity of CHD7, possibly through the recognition of histone modifications such as H3K4 and H3K36 methylation. This was recently described for the chromatin-remodeling factor CHD1, where the chromodomains promote the



binding of nucleosomal substrates and regulate the activity of the ATPase motor (HAUK *et al.* 2010).

Finally, the role of KIS in antagonizing Polycomb repression is potentially relevant to cancer. Increased expression and deregulation of the Polycomb group proteins BMI-1 and EZH2 are associated with tumor formation, emergence of cancer stem cells and the occurrence of many different forms of cancer (MILLS 2010; RICHLIY *et al.* 2011). It is conceivable that manipulation of trithorax group proteins KIS/CHD7, ASH1 and TRX/MLL, which antagonize the action of Polycomb group proteins, could aid in the treatment of cancer.

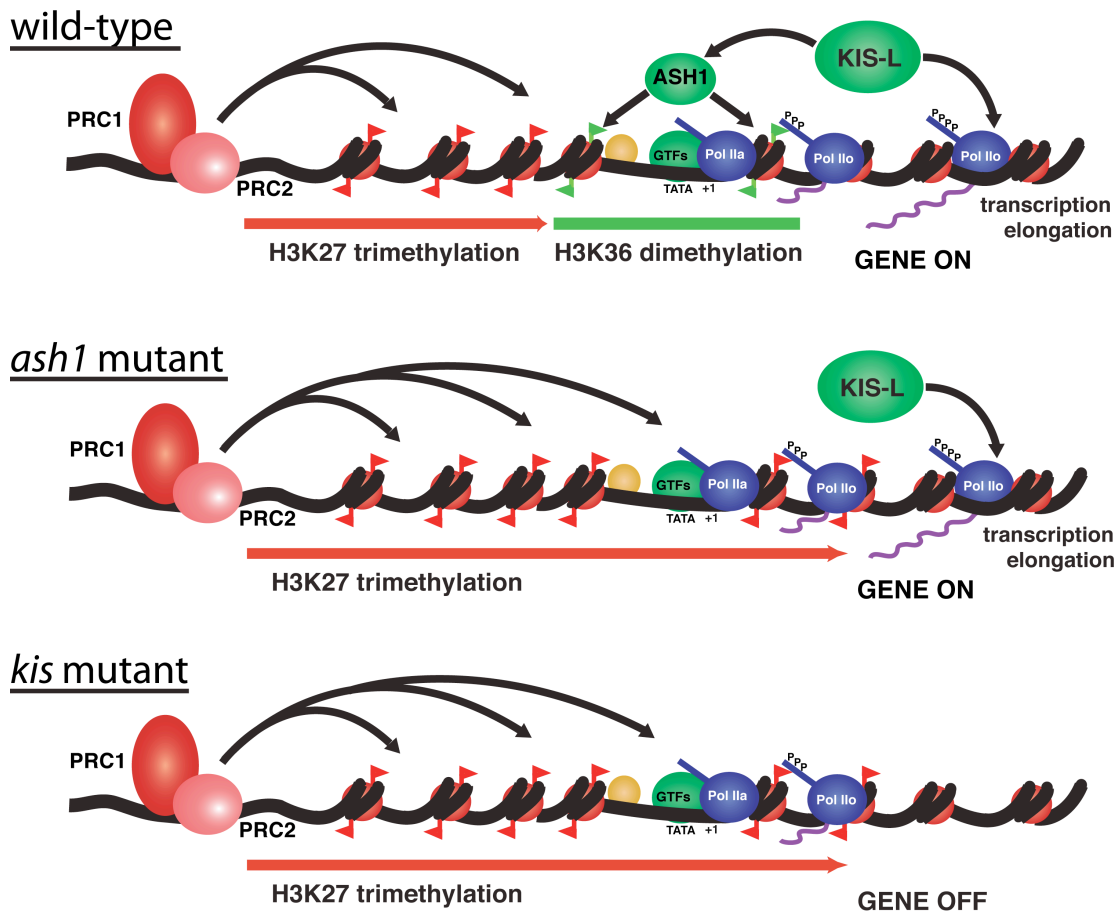
### **Conclusions and Future Directions**

The goal of my research has been to understand how gene expression programs once established are maintained throughout development. My studies have focused on elucidating the mechanism of action of Polycomb and trithorax group proteins, which have well-characterized roles in transcriptional maintenance. In this work, I have made great progress towards characterizing how trithorax group proteins interact to bring about stable patterns of gene expression. My work has demonstrated that the trithorax group protein KIS maintains active states of transcription through two separate and distinct mechanisms. First, KIS facilitates the elongation step of RNA Polymerase II. Second, KIS counteracts Polycomb repression in part by recruiting ASH1, which dimethylates H3K36 and prevents PRC2 from methylating H3K27 (Figure 4-1).

This study raises a number of key questions that merit future investigation. One unresolved issue is whether KIS recruits ASH1 to chromatin through direct or indirect interactions. Also unclear is whether the remodeling activity of KIS is

required for its role in transcription elongation, ASH1 recruitment and H3K27me3 antagonism. KIS, ASH1 and TRX all counteract H3K27me3, so one important question is whether these proteins function as barriers to prevent the spread of H3K27me3 into actively transcribed genes.

Finally, this study provides testable insights into the role of CHD7 in normal human development and CHARGE syndrome. Understanding how KIS interacts with other trithorax and Polycomb group proteins in *Drosophila* will help direct studies on the function of CHD7 in humans.



**Figure 4-1. KIS performs two independent functions in the maintenance of transcription.**

Model depicts the role of KIS in recruiting ASH1 which dimethylates H3K36 to prevent the spread of H3K27 methylation; and in promoting transcription elongation.

## Chapter 5

### Science Education Training Experiences in the ISEE Professional Development Program

A major component of my training and experience as a graduate student was in the field of science education through a program called the Professional Development Program (PDP), run by the Institute for Science and Engineering Educators (ISEE) at UC Santa Cruz (HUNTER *et al.* 2010). The main goal of the PDP is to provide teaching preparation and curriculum design experience for early career scientists and engineers to prepare them for their educational role as future faculty members or science and engineering professionals. Participants admitted to this program receive training in pedagogy and evidence based teaching methodologies with an emphasis on inquiry design at a 3-day intensive workshop. Following this, participants put the tools and ideas they have learned into practice by designing inquiry-based courses and activities as part of a team to be taught in various teaching venues.

Inquiry-based learning is an innovative method of engaging students in meaningful learning experiences which reflect the true processes of scientific research (CHINN and MALHOTRA 2002). As defined in the context of the PDP, inquiry is any activity that requires students to use reasoning skills while learning science content by engaging in processes that mirror authentic scientific research. Inquiry has been hailed as the future of science education due to its strong results in terms of student engagement and long-term retention (WILSON *et al.*). As such, inclusion of inquiry-based learning activities is a goal for many instructors who wish to engage their students more fully and see more meaningful learning in their courses.

ISEE has partnered with many different teaching venues, educational programs and institutions, in which these inquiry activities are implemented. Some examples of teaching venues include: Astronomy and Optics courses for native Hawaiian students; Biology, Chemistry and Earth Science workshops for transfer students to UCSC; Chemistry and Biology laboratory classes at UCSC; and science summer school programs for high school students. After PDP participants teach the inquiry activity or course at the teaching venue, the last part of the program is to evaluate the effectiveness of the activity they designed and the outcome of their teaching experience. Often multiyear participants in the program have the opportunity to go back and improve the activities and re-implement them the following year.

I participated in this program for four years and in that time designed and taught three different inquiry activities for three different venues and had the opportunity to improve and re-implement one of my activities. In my first year, I designed and taught an inquiry activity at the COSMOS science summer school for high school students focused on Astrobiology. In this inquiry students studied extremophiles, bacteria and archea that prefer high temperature or high saline growth conditions. In my second year, I designed and taught a weeklong inquiry-based Biology course in the SUMS Program, a summer bridge program for minority students at Hartnell Community College in Salinas. This course focused on teaching students to design experiments to investigate bacteria in their environment. In my third year, an NSF grant to ISEE funded the design of a unit taught in the UC Santa Cruz upper division laboratory class Eukaryotic Genetics Laboratory (Bio105L). The inquiry activity I designed for this course was on the polymerase chain reaction

(PCR). I redesigned and taught this activity again in my final year as an ISEE participant. To improve the course I used student-learning outcomes to redesign the composition and presentation of the activities and to better assess student learning.

In year three of the ISEE program, I was fortunate to take part in a conference organized by ISEE with the goal of highlighting and sharing the experiences and efforts of participants in the Professional Development Program. In order to record our achievements ISEE solicited papers from its participants to be published in a conference proceedings. The proceedings titled “Learning from Inquiry in Practice” published by the Astronomical Society of the Pacific describes the ISEE program and many of the inquiry-based activities and courses that have been designed over the years. I was the first author on two papers describing the design and teaching of the Hartnell SUMS Biology Course and the UCSC Eukaryotic Genetics PCR Inquiry (DORIGHI *et al.* 2010a; DORIGHI *et al.* 2010b). I also co-authored two additional papers, one describing the COSMOS Astrobiology activity and one reflecting on the use of inquiry-based learning in Biology curriculum (PETRELLA *et al.* 2010; QUAN *et al.* 2010). Through the Professional Development Program I also earned a Certificate demonstrating my completion of the program.

One of the major techniques I used in designing each inquiry activity was to define a clear set of learning goals, often called student learning outcomes and refer to them repeatedly throughout the design process. The technique of operationalizing learning goals is particularly important when designing assessments such as exam questions, lab reports and homework problems. “Operationalizing” involves specifying exactly what you want the students to learn and how you expect students to demonstrate that learning. In this chapter, I describe how I improved the PCR Unit

for the Eukaryotic Genetics Laboratory by operationalizing student learning outcomes and assessing content gains resulting from the inquiry activity. In the following sections, I provide evidence that inquiry activities can be highly successful at teaching scientific content and I demonstrate how student-learning outcomes can be useful tools for designing and assessing inquiry activities.

### **Using student learning outcomes to design and assess a laboratory inquiry on the polymerase chain reaction**

In this section I describe my use of student learning outcomes to design and assess a PCR Inquiry for the Eukaryotic Genetics course at UC Santa Cruz. The inquiry focuses on teaching specific scientific content, as well as scientific reasoning skills and lab skills. To facilitate the assessment of student learning gains and ensure a more objective assessment, I focused on linking the learning goals to the assessment of the activity. My approach relied on establishing very specific learning goals for students and then designing questions to assess whether students had attained these goals. Administering these questions prior to the start of the unit and at the end of the unit enabled me to determine the learning gains for each student. In the following pages, I describe the activity, its design and assessment of its effectiveness.

### **Eukaryotic genetics laboratory and the polymerase chain reaction inquiry**

The Eukaryotic Genetics Laboratory (BIOL105L) is an upper-division laboratory class offered by the Molecular, Cell and Developmental Biology Department at the University of California, Santa Cruz. This class teaches fundamental principles in genetics using a classic genetic model organism, the fruit

fly *Drosophila melanogaster*. The Eukaryotic Genetics Laboratory is a ten-week course consisting of two three-hour class meetings per week. The instructor and one teaching assistant guide approximately twenty students per section through laboratory activities resulting in student to teacher ratios of approximately 10 to 1. With the support of an NSF grant to the Institute of Science and Engineer Educators at UC Santa Cruz the lab unit on PCR for this class was replaced with an activity I designed with the help of three other PDP participants. Our goal in designing this new unit was to teach students about PCR, a widely used technique to amplify DNA, through inquiry-based teaching methods that reflect authentic research practices.

The PCR unit we designed was introduced into the Eukaryotic Genetics Laboratory in the fall of 2010. This activity encompassed five class meetings, spanning two and a half weeks, and involved an experimental planning component, followed by actually carrying out the experiment and analyzing the results. The full design of the activity is described in (DORIGHI *et al.* 2010a) but I will briefly describe the activity below, including slight modifications reflecting how it was improved the following spring.

### **Overview of PCR inquiry activity**

Day 1: The PCR Inquiry for the Eukaryotic Genetics Laboratory begins by asking the students how they might identify which of three possible genes is deleted in a mutant fruit fly line. In small groups, students brainstorm ideas and techniques to answer the question, weighing the pros and cons of each technique they devise. Ultimately, students vote on which technique is the best method for identifying the deleted gene given the constraints of limited time and money. Invariably the students choose to use PCR to identify the mutated gene. This is not by chance, however,



and comes about through careful facilitation by the instructors. Students are then told that the rest of the unit will be spent designing and carrying out this PCR experiment.

Day 2: In the next class meeting, students learn about PCR in greater detail by reinventing the PCR method. Students are asked to devise a method of replicating DNA given a set of materials (DNA polymerase, dNTPs, template DNA, primers) and their properties (melting temperature, enzyme kinetics, basepairing rules, etc). With this understanding of PCR, students are prompted to design the primers necessary to identify the deleted gene in the research scenario. Students are provided with the DNA sequence of the genes in question and must use them to design their own primers. The final activity for this class meeting is a discussion about the use of positive and negative controls in PCR. In this discussion, students are asked to draw conclusions from PCR data with and without positive and negative controls, in order to teach them the importance of controls in interpreting data.

Day 3-5: At the next class meeting, the students begin conducting the experiment to identify the deleted gene, which takes a total of three class meetings to complete. They first isolate genomic DNA from adult fruit flies. Then they set up the PCR reaction, determine and add the correct amount of each reagent to the PCR tube and start the reactions. Next, the students pour and run DNA agarose gels to determine the size of their PCR products and then photograph their data. Lastly, the students analyze their data and make conclusions with respect to which gene they think is deleted in the fruit fly. At the end of the inquiry, the instructors then reemphasize the most important concepts that we wanted them to learn from this activity.

### **Student learning outcomes**

This inquiry was designed to teach eight core student learning outcomes (learning goals/objectives). Over the course of the PCR activity we wanted students to demonstrate the ability to: 1. choose an experimental technique and justify that choice; 2. describe PCR at the molecular level; 3. choose relevant controls for a PCR experiment; 4. identify how primers bind to DNA and determine the product of a given set of primers; 5. mathematically describe the accumulation of PCR products during multiple rounds of PCR; 6. accurately follow an experimental protocol; 7. make conclusions using data; and 8. use evidence and reasoning to support their claim of which gene they believe is deleted in the mutant flies. We operationalized these student learning outcomes to facilitate the design and assessment of the inquiry. Operationalized learning outcomes are shown in Figure 5-1. We next assessed whether students had attained these outcomes primarily by grading short answer questions on the midterm exam and in a final report, with the exception of outcomes 1 and 6, which were assessed verbally and visually at specific points during the activity.

### **Assessment of student learning outcomes**

In order to assess the effectiveness of the inquiry at teaching outcomes 2 through 5, students completed a pre-inquiry survey, which included four questions aimed at assessing the students' prior knowledge and establishing a baseline with which to compare gains at the end of the inquiry. These same four questions were also asked on the midterm exam to determine how effectively the inquiry taught these learning objectives. The student learning outcomes, questions, average pre-inquiry and post-inquiry scores are presented in Figure 5-1.

The first question evaluates outcome 2 and requires students to understand

how PCR works mechanistically. For example, the two strands of DNA must first be separated (step 1) to allow the primers to bind (step 2) which then enables the replicating enzyme to bind and synthesize the complementary strand (step 3). The second question evaluates outcome 3 and requires that students understand the logic behind selecting certain controls for experiments - specifically, that you must have known samples to compare and reference to your unknown samples. The third question evaluates outcome 4 and requires students to understand that primers bind to DNA through base pairing interactions, and that both primers and intervening DNA sequences are amplified during PCR. Finally, the fourth question evaluates outcome 5 and requires students to understand that the number of PCR products grows exponentially from one cycle to the next.

Of the 37 students who took this pre-survey, there was a wide spread in responses on average yielding scores of 49.6% +/- 24% overall. We attribute the range in student performance on these questions to varying degrees of prior knowledge likely acquired through exposure to PCR in previous coursework. After the inquiry the same group of students was asked the same four questions as part of the midterm examination and students averaged 85.4% +/- 13% on these questions. This represents an average gain of +35.8%. However, due to the large range in initial student knowledge as evidenced by the pre-inquiry scores, we calculated normalized gains for each student. Normalized gains give an indication of student improvement as a result of the inquiry activity, regardless of their prior knowledge. When each student's final score is normalized against his or her pre-score, normalized gains of 65% on average are observed. This means that on average each student improved his or her score by approximately 65% of the available points as a result of the

activity. Normalized overall gains for each student are shown in Figure 5-2.

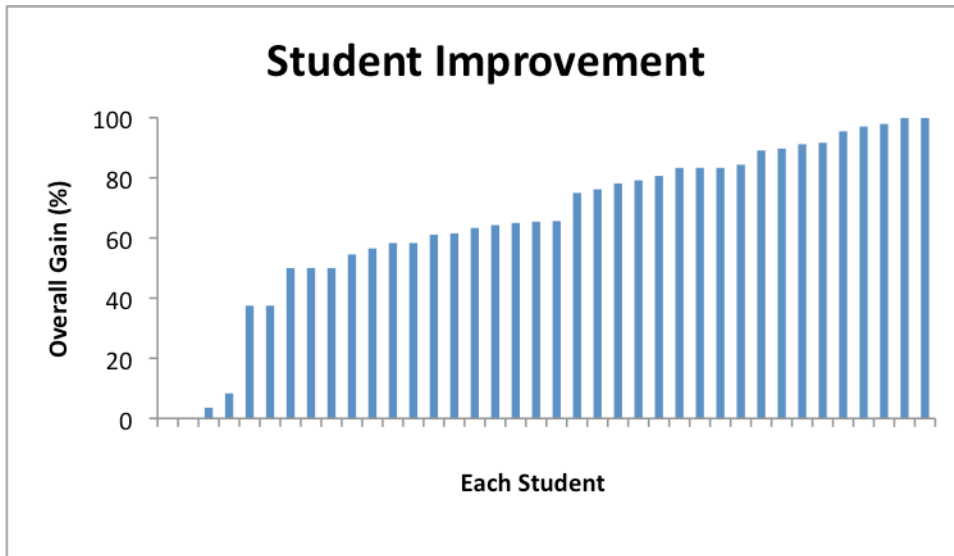
In addition to these four questions, a fifth question was added to the midterm, in order to assess outcome 7 (making conclusions from data) and to determine if students could apply this newly gained understanding of PCR to interpret data. Students averaged 81.2% on this transfer question, showing that the understanding gained was not simply from memorization, but instead could be synthesized and applied to analyze data.

Interestingly, we found that there was no statistically significant correlation between the pre-inquiry scores and post-inquiry scores. This can be seen in Figure 5-3, in which we compare pre-scores on the x-axis to post-scores on the y-axis for each student. Students who showed positive gains from pre- to post-inquiry are visible at the top of the chart. Although there are a few students who show little if any gains, the majority of students regardless of pre-score achieved high post-scores. This plot highlights a strength of the inquiry learning method – students with all levels of prior knowledge (varied pre-score/x-axis values) emerged from the inquiry experience with almost equally strong understandings of the PCR method as evidenced by their uniformly strong post-inquiry scores (y-axis values). Thus we can see that this inquiry activity allowed all students to learn the material presented.

Student Learning Outcome	Question	Average Score	
		Pre-Inquiry	Post-Inquiry
(2) Students should be able to name, describe and draw a graphic representation of what is happening at the molecular level during the three steps of PCR	Describe and draw a diagram of what is happening in each of the three steps of PCR	55%	93%
(3) Students should be able to describe verbally and in writing which controls are necessary for unambiguously determining the results of a PCR experiment and why	List two controls necessary to determine the results of the following PCR experiment	42%	74%
(4) Students should be able to identify how primers bind to template DNA and what the products of the PCR reaction will be using those primers	Given the following template DNA and pair of primers, what size PCR product will be formed?	43%	82%
(5) Students should be able to mathematically describe the exponential accumulation of PCR products during multiple rounds of PCR	What is a mathematical formula that expresses the number of DNA molecules produced at the end of 10 cycles of PCR?	58%	93%
Overall:		49%	85%

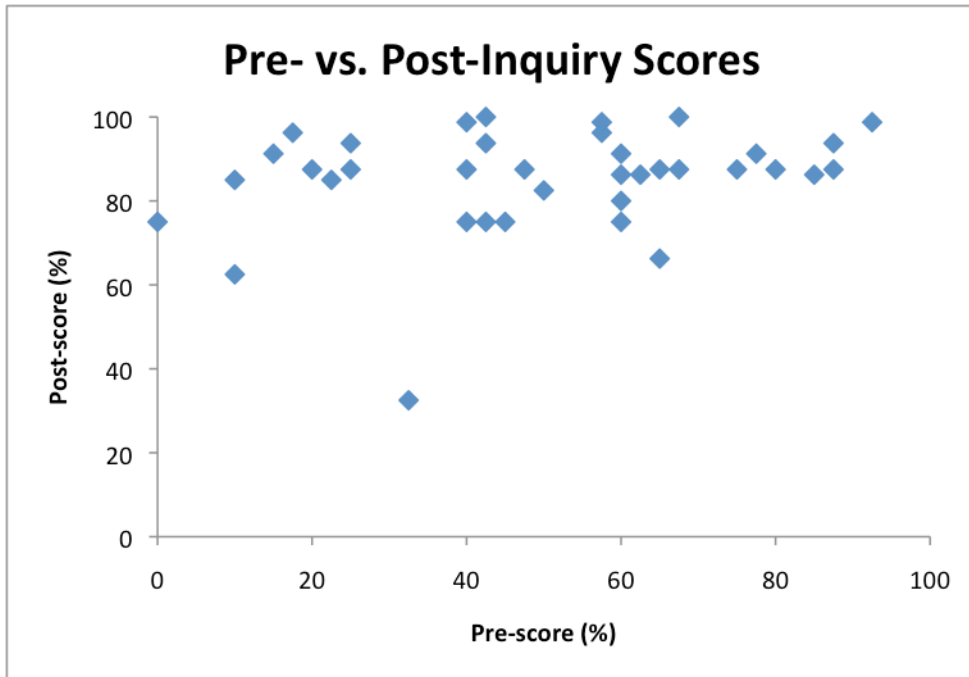
**Figure 5-1. Assessment materials and results for the Eukaryotic Genetics PCR Inquiry.**

The first column lists the operationalized student learning outcomes 2 through 5. The second column lists the questions asked on the pre-and post-inquiry test designed to assess student achievement of the outcomes. The final columns list the results of both pre-inquiry and post-inquiry assessment. N=37 students. Overall pre-inquiry standard deviation is +/- 24%. Overall post-inquiry standard deviation is +/- 13%.



**Figure 5-2. Normalized overall student gains.**

Shown are the normalized gains for each student, calculated by the formula  $(\text{Post-score} - \text{Pre-score}) / (100 - \text{Pre-score}) * 100$ . The majority of students improved their understanding as a result of the inquiry as evidenced by the distribution of normalized gains for each student in the class.



**Figure 5-3: Pre- and post-score distribution for the Eukaryotic Genetics PCR Inquiry.** After the inquiry experience the majority of students understood the material well (high post-scores on the y-axis) irrespective of their incoming knowledge level (wide variation in pre-scores on the x-axis).

## Discussion

The assessments we performed demonstrate that students who participated in the PCR inquiry-based activity achieved our student learning outcomes. As evidenced by the large student gains and high post-inquiry overall scores, we conclude that this inquiry was quite successful at teaching this particular Biology content. This suggests that a similar type of inquiry-based approach could be used to teach other topics in Biology as well. Inquiry-based methods can be challenging to incorporate into Biology laboratory courses due of the nature of biological research and biological organisms. Biology research frequently requires a high level of technical expertise in addition to expensive reagents and equipment. It also often involves lengthy experiments and utilizes model organisms with relatively long life cycles. However, there are ways get around these challenges which still enable instructors to engage students in meaningful biology-based inquiry experiences. Some approaches include using rapidly growing, inexpensive organisms such as bacteria and *Drosophila*. Other approaches involve using procedures that can be performed with inexpensive reagents and commonly available lab equipment, such as DNA preparations and restriction enzyme digests. Experiments that use these approaches and can be performed in 2-3 hour blocks of time can be highly successful in Biology laboratory classes.

The PCR Inquiry Activity and assessment strategies described in this chapter demonstrate that it is possible to teach students content using actual research methods. Clearly defining and using student learning outcomes while designing assessment materials, such as exam questions enables instructors to more accurately measure student understanding and achievement in relevant areas. The



success of this inquiry activity resulted in it being adopted by the lead instructor of the course and re-taught as part of the standard curriculum. I would like to emphasize that instructors need not redesign entire course curricula in order to incorporate inquiry-based teaching methods. A more practical approach for instructors wanting to use inquiry in their classrooms might be to revisit the goals of curricula and gradually build in new activities designed to achieve these goals. Combining this with assessment to monitor changes in student learning as a result of new activities is a sound strategy for more effective teaching.

Finally, I would like to highlight a report on undergraduate education in the Biological Sciences called BIO2010: Transforming Undergraduate Education for Future Research Biologists published by the National Academies Press. This report provides a list of recommendations for reforming undergraduate Biology curricula including an emphasis on interdisciplinary coursework, strong foundations in math and physical sciences and more active and engaging approaches to teaching and learning. These recommendations reflect the changing nature of biological research and are designed to better prepare students for graduate study and careers in biomedical research. Implementation of inquiry-based learning techniques in undergraduate Biology curricula is one way of addressing these goals. The report also lists faculty development as a crucial component in improving undergraduate education. This report underscores the importance of programs such as the ISEE PDP in training future faculty and bringing inquiry-based learning into curricula. Importantly, the report also offers suggestions for how departments, faculty, administrators, facilities, funding agencies and textbook companies can all play a role in modernizing and refocusing undergraduate biology education.

## **Conclusions**

Participation in the ISEE Professional Development Program has without a doubt revolutionized my approach to teaching. The theory behind inquiry-based teaching is that students learn better when actively participating in the creation of knowledge. This model is mirrored in the teaching strategy employed during the ISEE Program; a cycle that involves learning about education theory, designing activities for a purpose, implementing and finally evaluating the outcome of that activity. This cycle generates instructors that understand how to design courses and activities that are innovative, engaging and highly effective.

One of the key insights I gained from this program is that effective teaching utilizes a number of core principles. One is teaching with intention – establishing teaching priorities to guide instruction. Clearly defining a set of teaching goals and then using them to design teaching materials and activities provides a focus and structure that benefits both instructors and students. Another is using varied teaching methodologies. Having a diverse set of teaching tools allows instructors to use the most appropriate teaching strategy for that particular concept. Effective teaching adapts based on student feedback and assessment. Student learning, the ultimate goal of teaching, requires a dynamic partnership between students and instructors. Students are responsible for putting in the time and effort needed to learn the material. Instructors are responsible for facilitating learning by monitoring student progress and modifying their teaching accordingly. Finally, the most effective teaching makes learning engaging. People care about science when they are curious about natural phenomena and fascinated by the complexity that exists in the world.

As educators, if we are to raise science literacy and build the next generation scientists we need to cultivate a sense of scientific curiosity in our students.

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