Molecular Analysis of Components in *Drosophila* Polycomb Group Silencing

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Neal Richard Jahren

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Jeffrey A Simon, Advisor

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Advisor: Jeff Simon

Committee Members (past and present):
Thomas Hays, Chair
Vivian Bardwell
Steve Ekker
Michael Flickinger
Robert Herman
Thomas Neufeld
Michael O'Connor

Simon Lab Members (past and present):
Erica Andersen
Preeti Joshi
Carrie Ketel
Daniel Mallin
Sarah Malmquist
Ellen Miller
Aswathy Nair
Alicia Trossen
Liangjun Wang

Collaborators:
Rick Jones Laboratory
Lori Wallrath Laboratory
especially Diane Cryderman

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Dedication

This dissertation is dedicated to my father, who has encouraged all of my endeavors, scientific and otherwise.

Abstract

The aim of this research is to describe the molecular mechanism used by a set of regulatory proteins, called Polycomb Group proteins, to silence genes. In general, the function of the Polycomb Group is to silence cell differentiation factors in the course of embryonic development by operating on chromatin. First, we investigate a recently discovered Polycomb protein to determine its expression profile during development and to establish its role within a complex of proteins that methylates chromatin during Polycomb repression. Second, we target the methyltransferase complex to artificial loci on chromosomes by fusing one of its subunits to a heterologous DNA-binding domain. Third, we investigate the recruitment of the SCM Polycomb Group protein to chromatin during silencing. We perform molecular epistasis experiments to determine the dependencies between SCM and the other Polycomb Group proteins for arriving at chromatin. These studies use the *Drosophila* model system. The experiments include the observation of phenotypes resulting from genetic crosses and modifications, immunostaining of tissues and chromosomes, and chromatin immunoprecipitation. The Polycomb Group of proteins is highly conserved from *Drosophila* to humans, so the results of this research should yield insights about gene regulation in all higher organisms.

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Chapter One

Introduction

I. Developmental Functions of Polycomb Proteins

The Polycomb Group is a set of transcriptional repressors that silence target genes by interacting with and modifying chromatin. Multicellular organisms with specialized cell types require differential gene silencing because different cell types must be distinguished despite having essentially the same genome. The solution to this problem is that each cell expresses only a subset of genes relevant to its particular functions. The Polycomb system contributes to this solution by imposing a stable state of repression on genes that should not be expressed in a particular cell. Observations on the polytene chromosomes of larval *Drosophila* (DECAMILLIS *et al.* 1992; ZINK and PARO 1989) and in genome-wide chromatin immunoprecipitation experiments (NEGRE *et al.* 2006; SCHWARTZ *et al.* 2006) indicate that Polycomb proteins control hundreds of target genes. As these target genes have been identified in *Drosophila* and in mammalian genomes, it has become clear that a major developmental function of the Polycomb Group (PcG) is to silence cell differentiation factors (BOYER *et al.* 2006; BRACKEN *et al.* 2006; LEE *et al.* 2006).

PcG genes were discovered in *Drosophila* because loss-of-function mutations can produce homeotic phenotypes (LEWIS 1978; reviewed in RINGROSE and PARO 2004). A homeotic phenotype involves a transformation where one body part assumes characteristics of a body part from a different segmental position in the organism. Further investigation revealed that PcG genes produced these phenotypes because they regulate *Homeotic* (*Hox*) genes (MCKEON and BROCK 1991; SIMON *et al.* 1992; STRUHL and AKAM 1985). *Hox* genes encode a set of transcription factors that determine segmental identity along the anterior-posterior axis (reviewed in MCGINNIS and KRUMLAUF 1992). The combination of *Hox* genes expressed in a given segment initiates a specific developmental program appropriate to that segment (LEWIS 1978; PEIFER *et al.* 1987). In the case of PcG loss-of-function, homeotic phenotypes result from the mis-expression of *Hox* genes along the anterior-posterior axis, reflecting the breakdown of the silencing mechanism. The most severe *Drosophila* PcG mutants die at late embryonic stages with all body segments having taken on the identity of the most posterior abdominal segment. In contrast, the phenotypes of PcG hypomorphs can be quite mild and lead only to minor segmental transformations of adult structures, such as wing-to-haltere or extra sex combs (see Chapter Two, Figure 3 for an example). Although PcG proteins have a variety of targets, their regulation of *Hox* genes has been most intensively studied and is still their best understood function.

Multiple criteria can be used to define members of the PcG. Since the Polycomb system was originally investigated with genetic studies (BREEN and DUNCAN 1986; JÜRGENS 1985; LEWIS 1978), the PcG was defined as the set of genes where loss-of-

function causes the spread of *Hox* gene expression outside of their normal ranges (MCKEON and BROCK 1991; SIMON *et al.* 1992; STRUHL and AKAM 1985). As researchers have moved to biochemical studies of Polycomb repression, we can also define PcG proteins as the group of proteins that work together in chromatin complexes to achieve Polycomb repression. Thus, PcG proteins are defined functionally rather than by shared motifs or evolutionary origin. In some circumstances, these definitions do not precisely overlap. Because some proteins contribute to other developmental processes in addition to Polycomb repression, their loss can produce lethality at a very early stage where *Hox* misexpression cannot yet be observed. Table 1 shows a list of PcG proteins bearing a wide variety of protein motifs represented in different members of the group.

Soon after a *Drosophila* embryo is fertilized, a cascade of transcription factors divides it into a series of segments. A subset of these early-acting transcription factors is directly involved in delimiting the range of expression for the various *Hox* genes (CARROLL *et al.* 1988; IRISH *et al.* 1989; SHIMELL *et al.* 1994). Once the spatial pattern of *Hox* gene expression has been established, PcG proteins are deployed to maintain this pattern of expression, even after the transcription factors that initiated the pattern have decayed. Thus, PcG proteins can be considered "maintenance factors" because they maintain a pattern of expression that was determined previously by a distinct and transiently-acting system. Conversely, an antagonistic system consisting of Trithorax Group proteins maintains the active state of *Hox* genes within the range where they are normally expressed (MULLER and KASSIS 2006; PAPP and MULLER 2006). The Trithorax Group

proteins have been reviewed elsewhere (KENNISON 1995; SCHUETTENGRUBER *et al.* 2007; SIMON and TAMKUN 2002) and are not further detailed here. Recent studies suggest that at least some Trithorax Group proteins function more to protect genes from Polycomb repression than to activate genes directly (KLYMENKO and MULLER 2004).

Hox gene expression in a given cell will vary depending on that cell's position along the anterior-posterior axis. Often spatial control of gene expression is accomplished by restricted spatial accumulation of the trans-acting factor that controls the target gene. However, any given cell will require some of its *Hox* genes to be expressed and others to be silent, so PcG proteins must be present in every cell. Thus, position-specific Polycomb repression is accomplished by selective engagement of Polycomb proteins at specific target loci as development proceeds, rather than by spatially delimiting PcG protein expression itself. As the Polycomb system engages, it establishes a stable repressive state on whichever *Hox* genes are not actively transcribed within a particular cell by making modifications to chromatin at those specific loci. The stable state of transcriptional repression produced by the Polycomb system persists through the cell cycle, so that entire tissues maintain their proper patterns of *Hox* expression as their constituent cells proliferate. Genes subject to Polycomb repression can remain silent for long periods of developmental time unless the chromatin at affected loci is reprogrammed to relieve the repression (SCHWARTZ and PIRROTTA 2007).

II. The Molecular Mechanism of Polycomb Silencing

A. Polycomb Complexes

Genetic studies revealed that several different PcG genes had similar mutant phenotypes (reviewed in SIMON *et al.* 1995; SIMON *et al.* 1992), suggesting a system of proteins that was working together. Further evidence came from observations that different PcG proteins co-localize on polytene chromosomes (DECAMILLIS *et al.* 1992; FRANKE *et al.* 1992; LONIE *et al.* 1994; RASTELLI *et al.* 1993). Subsequently, *in vitro* interactions between PcG proteins and biochemical fractionations of *Drosophila* nuclear extracts (JONES *et al.* 1998; NG *et al.* 2000; SHAO *et al.* 1999; TIE *et al.* 2001), provided further evidence that PcG proteins form physical complexes.

Figure 1 shows core subunit compositions of the three PcG complexes that have been biochemically characterized from *Drosophila* nuclear extracts. Their subunits are also listed individually in Table 1. These complexes have typically been discovered by engineering an epitope tag on a PcG protein of interest, and then using that tag in affinity purification. This process recovers the target protein and any stably associated partners, which are then identified by mass spectroscopy (KLYMENKO *et al.* 2006; MULLER *et al.* 2002; SHAO *et al.* 1999).

The complexes shown in Figure 1 are the Pleiohomeotic Repressive Complex (PhoRC), Polycomb Repressive Complex 2 (PRC2), and Polycomb Repressive Complex 1 (PRC1). The individual subunits of each complex are considered in more detail in

Figure 1: Complexes of Polycomb Group Proteins. The core components of the three biochemically characterized PcG complexes from *Drosophila* are shown, in association with a chromatin template (in gray). Pho Repressive Complex (PhoRC) contains the DNA-binding protein Pleiohomeotic (PHO) and dSFMBT. Polycomb Repressive Complex 2 (PRC2) contains the SET-domain protein Enhancer of Zeste (E(Z)), along with Extra Sex Combs (ESC), Suppressor of Zeste-12 (SU(Z)12), and Nucleosome Remodelling Factor-55 (NURF55). Polycomb Repressive Complex 1 (PRC1) contains Polycomb (PC), Polyhomeotic (PH), Posterior Sex Combs (PSC), and Sex Combs Extra (SCE, also known as dRING1). The cartoon represents their major described biochemical functions: PhoRC recognizes specific DNA sequence, the set domain of E(Z) methylates lysine-27 of histone H3 (H3-K27), and PRC1 binds to methyl-H3-K27 through the chromodomain of PC. In reality, all three complexes are present simultaneously at chromatin targets during Polycomb repression. In addition, many models of Polycomb repression hypothesize that protein-protein interactions between these complexes, and/or with additional factors not represented here, are required to recruit PRC2 and PRC1 to target sites.

Figure 1

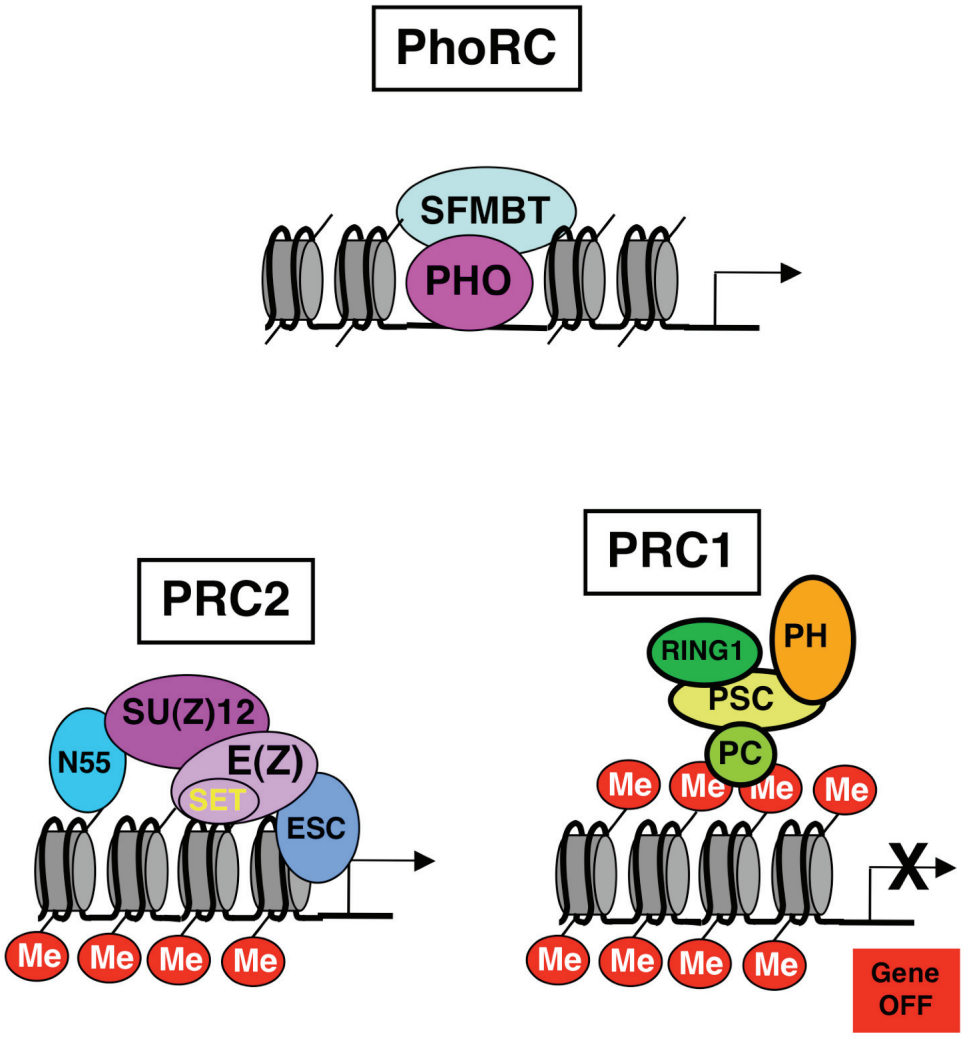
PhoRC
SFMBT
PHO
PRC2
SU(Z)12
N55
E(Z)
SET
ESC
Me
Me
Me
Me
PRC1
RING1
PH
PSC
PC
Me
Me
Me
Me
Me
Me
Me
Me
X
Gene
OFF

Table 1: Core members of the characterized PcG complexes. This table presents the PcG proteins that are mentioned frequently in the text, along with the PcG complexes they associate with. The Sex Combs on Midleg (SCM) protein has not been incorporated into the current framework of PcG complexes. Protein motifs are listed to demonstrate the wide variety of domains found in PcG proteins. Human homologs are listed to demonstrate conservation of the core subunits for each complex. References are provided in the text.

Table 1

PcG Proteins	Subunit of PcG complex	Protein Motifs	Human Homologs
Polycomb (Pc)	PRC1	chromodomain "shadow" domain	HPC1, HPC2, HPC3
Polyhomeotic (Ph)	PRC1	SPM domain C_2-C_2 Zn finger	HPH1, HPH2, HPH3
Posterior sex combs (Psc)	PRC1	RING finger (C_3HC_4 Zn finger)	BMI1, MBLR MEL-18, NSPC1
Sex combs extra (Sce = dRing)	PRC1	RING finger (C_3HC_4 Zn finger)	RING1, RING2
Enhancer of zeste [E(z)]	PRC2	SET domain, CXC domain, Homology domains I and II	EZH2, EZH1
Suppressor of zeste-12 (Su[z]12)	PRC2	C_2-H_2 Zn finger VEFS box	SUZ12
extra sex combs (Esc)	PRC2	7 WD repeats	EED
extra sex combs-like (Escl)	Alternate in PRC2	7 WD repeats	EED
Nucleosome Remodelling Factor-55 (NURF55)	PRC2	7 WD repeats	RbAp46 RbAp48
pleiohomeotic (Pho)	PHO-RC	4 C_2H_2 DNA-binding Zn fingers	YY1
Scm-related with four mbt repeats (Sfmbt)	PHO-RC	SPM domain, 4 mbt repeats 1 C_2-C_2 Zn finger	SFMBT
Sex comb on midleg (Scm)	?	SPM domain, 2 mbt repeats 2 C_2-C_2 Zn fingers	SCML1, SCML2

subsequent sections. The cartoons in Figure 1 present an oversimplified picture. Precise subunit stoichiometry has not been established for any of these complexes, and some purifications yield sub-stoichiometric quantities of protein that might be impurities or might represent alternate species of complex being copurified. Figure 1 represents the canonical complexes purified from *Drosophila* extracts, but additional variations also occur. *Drosophila* complex compositions can also vary over developmental stages (FURUYAMA *et al.* 2004; SAVLA *et al.* 2008). Mammalian genomes have a greater number of PcG orthologues, with a corresponding increase of heterogeneity in the collections of mammalian PcG complexes (reviewed in LEVINE *et al.* 2004; LEVINE *et al.* 2002).

Moreover, Figure 1 does not show a complete picture of the PcG because several PcG proteins are not yet assigned to particular characterized complexes. Researchers have identified a total of approximately 18 Polycomb Group proteins (GAYTAN DE AYALA ALONSO *et al.* 2007; SCHWARTZ and PIRROTTA 2007). The known physical complexes account for only 14, and in only a few cases can specific biochemical activities be assigned to a specific subunit (SIMON 2009). Active areas of Polycomb research seek to integrate the remaining PcG components into the overall framework of PcG complexes and to further elaborate the role of each individual protein.

In the remainder of this section, I describe the chromosomal features that these PcG complexes interact with, namely the specific DNA sequences and chromatin modifications that are recognized by the Polycomb system. Next, I consider hypotheses

for how these complexes arrive at chromatin targets. Following that, I describe each complex and its functions in greater detail. Finally, I introduce the SCM Polycomb repressor, which is one of the proteins that has not yet been incorporated into current molecular models for PcG silencing.

B. The Polycomb Response Element

Genes that are subject to Polycomb Repression are marked with a Polycomb Response Element (PRE). In *Drosophila*, the existence of cis-acting PREs has been verified experimentally by tracking expression from reporter transgenes fused to upstream sequences from PcG target genes (CHAN *et al.* 1994; SIMON *et al.* 1993). PREs are required continuously during development to maintain silencing (BUSTURIA *et al.* 1997; SENGUPTA *et al.* 2004) but can contain separable sequences that support Polycomb silencing at different developmental stages (SCHWARTZ and PIRROTTA 2007). Based on these experimental observations, (RINGROSE *et al.* 2003) developed a computational algorithm designed to predict the position of PREs in the *Drosophila* genome. When compared to a chromatin immunoprecipitation study in which samples were hybridized to microarrays in order to determine PcG target loci over the entire *Drosophila* genome (NEGRE *et al.* 2006; reviewed in RINGROSE 2007; SCHWARTZ *et al.* 2006), however, overlap with the predicted sequences was only about 20% (SCHWARTZ and PIRROTTA 2007). Moreover, while *Drosophila* PREs are often found immediately upstream of the genes they control, they can also be found 10-100kb upstream (MULLER and KASSIS 2006).

Analysis of PREs in *Drosophila* has revealed clusters of protein binding sequences arranged in highly complex and variable patterns (MULLER and KASSIS 2006). Binding sites for the Polycomb protein Pleiohomeotic (PHO) are critical functional elements of PREs (BROWN *et al.* 1998; SHIMELL *et al.* 2000), and point mutations in these binding sites can abolish Polycomb repression of reporter genes (FRITSCH *et al.* 1999). PHO has a great deal of functional overlap with a closely-related orthologue named PHO-Like, but removal of both proteins results in a strong homeotic phenotype associated with loss of Polycomb silencing (BROWN *et al.* 2003; KLYMENKO *et al.* 2006; WANG *et al.* 2004b). However, PHO binding by itself is not sufficient to support Polycomb repression of a target gene, and there are a variety of other binding motifs and factors observed at PREs. GA repeats and their corresponding binding proteins are particularly prevalent, but the connection of these additional sequences and factors to the mechanism of Polycomb repression remains unclear (reviewed in MULLER and KASSIS 2006; RINGROSE and PARO 2004). While there are still many open questions concerning PREs in *Drosophila*, in other species PREs are not nearly so well described (MULLER and KASSIS 2006; SIMON 2009).

C. Chromatin Structure

Genes subject to Polycomb repression are recognized through the DNA sequence elements of the PRE, but additional steps in Polycomb repression are accomplished through interactions with and modification to chromatin. Chromatin has multiple levels

of organization that package DNA (FELSENFELD and GROUDINE 2003; HORN and PETERSON 2002), however, most investigation into PcG mechanisms has focused on the lowest level of organization: the nucleosome. The nucleosome core particle consists of eight histone proteins wrapped by almost two turns of DNA. The low-compaction "beads on a string" conformation of chromatin involves approximately 200bp of DNA per nucleosome (HORN and PETERSON 2002).

Each histone protein consists of a globular domain and an N-terminal tail; the globular domains interact to form the protein "kernel" of the nucleosome, leaving the tails free to extend away from the core particle, where they can interact with other nucleosomes and the environment (LUGER *et al.* 1997). Chromatin was originally thought to be merely a packaging scaffold for DNA; we now know that it is a highly dynamic system and that changes to chromatin are an important part of regulating the transcriptional activity of nearby genes (LI *et al.* 2007). Among the most relevant chromatin changes in Polycomb repression are covalent modifications to the histone tails.

The histone tails can accept a variety of different covalent modifications, including lysine acetylation and methylation, serine phosphorylation, and lysine ubiquitylation. A huge recent literature implicates these covalent histone modifications in controlling the transcriptional status of nearby genes (BERGER 2002; BHAUMIK *et al.* 2007; SHILATIFARD 2006). There are two non-exclusive theories of how histone modifications change the functional properties of chromatin. One theory is that modifications change the physical properties of nucleosomes themselves in the context

of multinucleosomal arrays and thereby change their interactions with each other (HANSEN *et al.* 1998; WOLFFE and HAYES 1999). The other and more widely accepted theory is that histone modifications provide marks that recruit the activities of other factors that further modify chromatin and can control transcriptional activity.

This latter theory is a central idea of the "histone code hypothesis," which holds that the transcriptional state of genes can be encoded in the modification state of the surrounding chromatin (JENUWEIN and ALLIS 2001; STRAHL and ALLIS 2000). This hypothesis suggests that the modifications on histone tails are molecular marks that are read by functional binding modules that are present on many chromatin-modifying factors. In this view, covalent marks recruit the binding of enzymes or other effectors that then execute the molecular program. A central tenet of the histone code is the possibility of cross-regulatory interactions among multiple modifications and also among the factors that respond to them. These putative higher-order interactions provide a platform for combinatorial regulation of the chromatin state by reading the entire pattern of histone marks rather than responding to each mark independently (BERGER 2007; BUSZCZAK *et al.* 2009; FISCHLE 2008; FISCHLE *et al.* 2003b). As elaborated below, since specific histone modifications play a central role in Polycomb silencing, the PcG can be considered to supply a subset of the information held in the histone code.

D. Recruitment of Polycomb Complexes

Polycomb Response Elements allow genes to be recognized by the Polycomb system. If these genes are not being actively transcribed at the time PcG proteins become engaged (and thus not protected by the Trithorax system), then gene silencing will be maintained by the PcG. However, it is not clear how all three known complexes (plus additional unknown complexes and factors) are targeted to their proper sites of activity. Several studies have assessed the presence of specific PcG proteins on chromatin when certain other factors are removed. The removal of Pleiohomeotic (PHO) from cultured *Drosophila* cells leads to the loss of PRC2 and PRC1 from the PRE upstream of the *Hox* gene *Ultrabithorax* (*Ubx*) as detected by chromatin immunoprecipitation (WANG *et al.* 2004b). The same study found that PHO was still detected on the *Ubx* PRE after removing PRC2 or PRC1 components from the system. This suggests that the recruitment of PhoRC to the PRE is an early event in Polycomb repression and that the other complexes are recruited subsequently. Presumably, this early recognition involves DNA-binding by PHO to its target sites in the PRE (see below; MULLER and KASSIS 2006). Several studies using similar approaches have reported the loss of PRC1 targeting consequent to PRC2 knockdown and conversely, retention of PRC2 despite PRC1 knockdown. (CAO *et al.* 2002; RASTELLI *et al.* 1993; WANG *et al.* 2004b). Mammalian cells also show this relationship between the targeting of PRC2 and PRC1 (BOYER *et al.* 2006; CAO *et al.* 2005).

Taken together, these studies suggest a model where recruitment of Polycomb complexes proceeds hierarchically, beginning with PhoRC, which promotes the recruitment of PRC2, which in turn promotes the recruitment of PRC1. However, the case for this step-by-step model is by no means conclusive. All Polycomb complexes persist at their target sites as silencing is established, possibly participating in protein-protein interactions with each other. Additionally, many models of Polycomb repression envision the creation of intra-locus looping structures that require the various Polycomb complexes to interact with the PRE, modified nucleosomes in the coding region of the silenced gene, and with each other (MULLER and KASSIS 2006; SCHWARTZ and PIRROTTA 2007; WANG *et al.* 2004b). Thus, some researchers hypothesize that all PcG complexes may arrive at chromatin simultaneously and independently, even if they then interact with each other at chromatin sites to establish Polycomb repression (SCHWARTZ and PIRROTTA 2007).

E. Pleiohomeotic Repressive Complex

Pleiohomeotic (PHO) and the closely-related protein PHO-Like (PHOL) are the only known Polycomb group proteins with the ability to recognize specific DNA sequence (BROWN *et al.* 2003; BROWN *et al.* 1998). When PHO was purified from *Drosophila* nuclear extracts using an epitope tag, it was found to be in complex with a second partner subunit, dSFMBT (KLYMENKO *et al.* 2006). Knockout of the *dsfmbt* gene produced the canonical Polycomb phenotype, verifying that *dsfmbt* satisfies the genetic criteria for membership in the PcG. Notable features of dSFMBT include a subtype of

Cys_2-Cys_2 zinc finger, four MBT repeats, and a SAM domain close to the C-terminus. These features are elaborated in the subsequent section on the SCM repressor, which also shares these three functional domains.

The experimental studies suggest that target recognition and the recruitment of other PcG complexes are the major functional roles for PhoRC in Polycomb repression. The role of PREs in mediating Polycomb repression and the critical role of PHO binding to PREs have been discussed above. Thus, PhoRC would seem to be a central element in organizing the locus for subsequent chromatin modifications in Polycomb repression. Recruitment is usually hypothesized to take place via protein-protein interactions between PhoRC and other PcG complexes. Consistent with this idea, PHO interacts with both the Extra Sex Combs (ESC) and Enhancer-of-Zeste (E(Z)) components of PRC2 *in vivo* and is required for E(Z) binding to the PRE in cultured *Drosophila* cells (WANG *et al.* 2004b). Interactions between PHO and PRC1 components have also been reported (MOHD-SARIP *et al.* 2002), including an interaction where PHO and PRC1 bind synergistically to a PRE element (MOHD-SARIP *et al.* 2005; MOHD-SARIP *et al.* 2006). Although dSFMBT is also required for Polycomb repression (KLYMENKO *et al.* 2006), so far research has not shed light on whether it makes any contribution to this recruitment function.

F. Polycomb Repressive Complex 2 and Histone Methylation

As mentioned previously, PRC2 was discovered after affinity-purification from nuclear extracts derived from transgenic *Drosophila* embryos expressing FLAG-tagged Extra Sex Combs (ESC; MULLER *et al.* 2002). In addition to the protein Enhancer of Zeste (E(Z)), which was already known to associate with ESC (JONES *et al.* 1998; NG *et al.* 2000), the other components of this complex were identified as the PcG protein Suppressor of Zeste-12 (SU(Z)12) and Nucleosome Remodeling Factor-55 (NURF55). While the E(Z), ESC, and SU(Z)12 subunits are all three dedicated PcG proteins, NURF55 is more functionally diverse, being involved in chromatin assembly and remodeling, and deacetylation (MARTINEZ-BALBAS *et al.* 1998; TYLER *et al.* 1996; VERREAULT *et al.* 1996). PRC2 reconstitution experiments (KETEL *et al.* 2005), using a baculovirus expression system, established that E(Z) is located in the center of the complex, with contacts to both ESC and SU(Z)12, as illustrated in Figure 1. NURF55 is primarily in contact with SU(Z)12. Several labs independently confirmed the identities of these PRC2 subunits, with some of them purifying the corresponding complex from mammalian cells (CAO *et al.* 2002; CZERMIN *et al.* 2002; KUZMICHEV *et al.* 2002). The complexes purified from *Drosophila* and from mammalian cells are remarkably similar in subunit composition.

E(Z) contains a SET domain, which was previously shown to be the catalytic domain in a class of chromatin-modifying enzymes called histone methyltransferases (DILLON *et al.* 2005; REA *et al.* 2000). Thus, there was interest in determining whether PRC2

would also show histone methyltransferase (HMTase) activity. This activity can be assayed *in vitro* by combining the putative enzyme with a histone-containing substrate and radiolabeled S-adenosyl-methionine (SAM) which serves as a methyl group donor. When this purified PRC2 complex was analyzed by HMTase assay, it was able to methylate histone H3 *in vitro* (CZERMIN *et al.* 2002; MULLER *et al.* 2002).

Further investigation demonstrated that the primary specificity of the PRC2 HMTase is for lysine-27 of histone H3 (H3-K27; CAO *et al.* 2002; MULLER *et al.* 2002). Edman degradation of methylated histone H3 substrates revealed a strong preference for the H3-K27 (MULLER *et al.* 2002). This specificity was further confirmed using *in vitro* HMTase assays with recombinant histones bearing point mutations as a substrate. When H3-K27 was mutated to an alanine, PRC2 was no longer able to methylate histone substrates, whereas mutating lysine 9 to alanine had no effect on the HMTase activity of PRC2 (MULLER *et al.* 2002). Lysine residues can accept one, two, or three methyl groups (mono- di- and tri-methylation, respectively), and PRC2 has been implicated in attaching all three methyl groups (JOSHI *et al.* 2008; NEKRASOV *et al.* 2007). While mono- and di-methylation of H3-K27 are widespread throughout the *Drosophila* genome, trimethyl-H3-K27 accumulates in limited regions that coincide with PcG target sites (EBERT *et al.* 2004). PRC2 is the only known H3-K27 HMTase in *Drosophila*. It is presumed to be responsible for all methylation states on H3-K27, but the trimethyl form is most strongly associated with, and functionally implicated in, Polycomb repression.

PRC2 can be reconstituted in a baculovirus expression system by co-expressing the four core subunits, and the resulting complex is functional in the HMTase assay (MULLER *et al.* 2002). This tool allowed researchers to establish a link between methylation and Polycomb silencing. Site directed mutations to the E(Z) SET domain that abolished enzymatic activity were also unable to rescue Polycomb silencing when tested by transgenesis in *Drosophila* (MULLER *et al.* 2002). More recently, SET domain mutations recovered in screens for loss of Polycomb silencing have been found methylation deficient when recombinant versions were tested in the HMTase assay (JOSHI *et al.* 2008) despite normal assembly into PRC2 complex. These results suggest that the methylation function of PRC2 is a required component of the Polycomb silencing mechanism. However, the precise role that methylation plays in that mechanism remains unclear, as will be discussed below.

A remarkable feature of E(Z) is that, unlike some other SET-domain proteins, it is virtually catalytically inactive on its own. In order to achieve robust methyltransferase activity, E(Z) requires assembly with both ESC and SU(Z)12. The requirements for ESC and SU(Z)12 were defined *in vitro* using recombinant PRC2 (CAO *et al.* 2002; KETEL *et al.* 2005; PASINI *et al.* 2004). In contrast, NURF55 contributes little to *in vitro* PRC2 HMTase. Furthermore, mutant *Drosophila* embryos that are null for ESC also lack H3-K27 methylation (KETEL *et al.* 2005). It is not clear how the non-catalytic subunits contribute mechanistically to PRC2 function. There are at least three different possible roles for the non-catalytic subunits in potentiating the methyltransferase activity of PRC2. First, the SET domain of E(Z) might be in the proper physical

conformation for catalytic activity only when E(Z) is bound to ESC and SU(Z)12. Second, the non-catalytic subunits might act as adaptor proteins or scaffolds that facilitate interactions with the histone substrate. Both the ESC and SU(Z)12 subunits have been implicated in histone or nucleosome binding (NEKRASOV *et al.* 2005; TIE *et al.* 2007). However, there exist mutations in ESC that do not interfere with nucleosome binding but still eliminate histone methylation (KETEL *et al.* 2005), which suggests that ESC contributes somehow more directly to the enzymatic activity of PRC2. Third, non-catalytic subunits could be involved with SAM binding to facilitate methyl group donation to the catalytic core. Furthermore, whatever role these non-catalytic subunits might play in histone methylation, they may also perform additional roles in PRC2 function. For example, one active area of research concerns whether and how PRC2 partners with histone deacetylases or DNA methyltransferases to accomplish or enhance gene silencing (reviewed in SIMON and LANGE 2008).

An interesting source of variability in composition of the PRC2 complex that may occur in both *Drosophila* and mammals is the incorporation of alternate versions of the ESC subunit into PRC2. In *Drosophila*, the *esc* gene has been duplicated. The second gene copy has been called *esc-like*. Both the ESC and ESC-Like (ESCL) proteins contain seven WD40 repeats which together form a "beta propeller" structure (see chapter 2). The WD40 repeats are joined by loops which protrude from the plane of the propeller on both sides. There are several surface loops that are highly conserved among the ESC homologues in different species, and these loops correspond to the most similar regions between ESC and ESCL. Both proteins also have an unstructured N-terminal tail and

this tail region is quite divergent. In addition to having low sequence homology between the tails, they have strikingly different lengths. The ESC tail consists of approximately 70 residues, while ESCL has just over 100. Because ESC expression drops off dramatically during post-embryonic stages in *Drosophila* (NG *et al.* 2000; SIMON 1995), it is interesting to know whether ESCL can substitute for ESC in fly PRC2 and what if any functional differences result. The role of ESCL will be discussed more fully in Chapter Two, which will present data addressing its participation in Polycomb repression.

Intriguingly, chromatographic separations of nuclear extracts have found different isoforms of the ESC homologue EED within the human PRC2 complex (KUZMICHEV *et al.* 2004; KUZMICHEV *et al.* 2005). These isoforms differ in the lengths of the N-terminal tails of the different EED species. These isoforms also can be obtained when EED message is translated *in vitro* because the ribosomes begin translating at alternative start codons. There is evidence that the inclusion of different EED isoforms in human PRC2 can shift the substrate preference from histone H3 to histone H1 (KUZMICHEV *et al.* 2004; KUZMICHEV *et al.* 2005). However, there is little information on possible functional roles for histone H1 methylation in Polycomb repression.

The molecular role that histone methylation plays in the mechanism of Polycomb repression is largely unsettled. The first theories focused on the chromodomain of the PRC1 subunit Polycomb (PC) because the related chromodomain of Heterochromatin Protein 1 (HP1) binds to methylated H3-K9 produced by the SET domain HMTase

SU(VAR)3-9 (BANNISTER *et al.* 2001; LACHNER *et al.* 2001). By analogy, it was hypothesized that H3-K27 methylation would produce binding sites for the chromodomain of PC and thus provide the mechanism for the recruitment of PRC1 (CAO *et al.* 2002; SIMON 2003). Aside from the evidence that PC does, in fact, bind specifically to trimethyl-H3-K27 *in vitro* (discussed below in the context of PRC1), this model receives support from many demonstrations that the removal of PRC2 from Polycomb target sites leads to the loss of PRC1 (CAO *et al.* 2002; RASTELLI *et al.* 1993; WANG *et al.* 2004b). Furthermore, PRC1 can be competed off of chromosomes by incubation with H3 tail peptides containing trimethyl-K27 (RINGROSE *et al.* 2004).

Nevertheless, there are problems that prevent acceptance of this PRC1 recruitment model in such a simple form. Polycomb proteins tend to be found at discrete locations within target loci, but trimethyl-H3-K27 is found broadly distributed across silenced loci (PAPP and MULLER 2006; SCHWARTZ *et al.* 2006). Moreover, the PRE is generally depleted of nucleosomes (KAHN *et al.* 2006; MULLER and KASSIS 2006), so methyl-H3-K27 cannot be solely responsible for recruiting PRC1 directly to the PRE. There is also at least one example in mammalian stem cells where researchers report PRC1 bound to a Polycomb target site in the absence of PRC2 expression or H3-K27 methylation (PASINI *et al.* 2007).

Perhaps the analogy with heterochromatin maintenance by SU(VAR)3-9 and HP1 led researchers to premature views about H3-K27 methylation and PRC1 recruitment before other alternatives had been fully investigated. The experiments that remove

PRC2 from PcG targets generally disrupt the entire complex, so it is difficult to draw definitive conclusions about the role of methylation *per se* versus possible contributions from protein-protein interactions between PRC2 and PRC1. Adding to the complexity, PRC2 also acts in a context where PhoRC and possibly other unknown factors are present on chromatin. Protein-protein interactions have, in fact, been observed among all three of the characterized PcG complexes (MULLER and KASSIS 2006), including interactions between PHO and PRC1 (MOHD-SARIP *et al.* 2005; MOHD-SARIP *et al.* 2006). Additionally, a self-binding interaction domain found in the Sex Combs on Midleg repressor (see below) is also found on members of PRC1 and PhoRC, raising the possibilities of additional cross interactions between PcG complexes. Finally, the unresolved questions about additional PRE binding proteins raises the possibility that any number of unknown factors might help recruit PRC1. Ultimately, PRC1 may arrive at chromatin through a combination of methylation-dependent and methylation-independent mechanisms. One way to address the role of H3-K27 methylation empirically would be to create ectopic methylation sites in the absence of a PRE, and thus in absence of PhoRC and any other factors that bind to PREs. A system that targets PRC2 to ectopic sites and then observes the resultant effects on *Drosophila* chromosomes is presented in Chapter Three.

Instead of initial targeting of PRC1, recent theories have suggested that PC binding to trimethyl-H3-K27 may help stabilize intra-locus looping that promotes Polycomb silencing (PAPP and MULLER 2006; SCHWARTZ and PIRROTTA 2007). Looped structures might bring a distal PRE into contact with the promoter, or facilitate the spread of

methyl-H3-K27 over the length of the silenced gene (SCHWARTZ and PIRROTTA 2007; SIMON 2009). In high-resolution chromatin immunoprecipitation experiments, (SCHWARTZ *et al.* 2006) detect PRC1 signals spread out over a broad area within PcG loci in addition to a peak at the PRE. They interpret this pattern as representing PRC1 anchored to the PRE by DNA-binding factors and then bridging to other points throughout the locus by binding to the widely-distributed methyl-H3-K27 via the chromodomain of PC.

G. Polycomb Repressive Complex 1 and the Mechanism of Repression

The PRC1 complex was the first functional PcG complex to be isolated (SHAO *et al.* 1999). It also contains four core subunits. The Polycomb (PC) protein itself is a member of this complex along with the PcG proteins Polyhomeotic (PH) and Posterior Sex Combs (PSC), and Sex Combs Extra (SCE, also known as dRING1).

As mentioned above, a biochemical activity that has been described for PRC1 is binding to trimethyl-H3-K27 via the chromodomain of PC. The chromodomain is shared among proteins that bind to methyl-lysine residues on histones, including HP1 (FISCHLE *et al.* 2003c). This binding activity was demonstrated with *in vitro* binding and structural studies (FISCHLE *et al.* 2003c; MIN *et al.* 2003) and the role of the PC chromodomain in chromatin targeting has been demonstrated in *Drosophila* cell culture experiments (MESSMER *et al.* 1992).

PRC1 is thought to be the PcG complex most directly responsible for actually silencing the target gene, but researchers have not established the actual mechanism that prevents transcription. One theory is that PRC1 promotes a change in chromatin structure that simply denies access to activating factors (FITZGERALD and BENDER 2001; MCCALL and BENDER 1996). PRC1 has been observed to produce chromatin compaction *in vitro* (FRANCIS *et al.* 2004), but its applicability to the *in vivo* situation has not been demonstrated. A related PRC1 function might be to inhibit nucleosome remodeling (KING *et al.* 2002; SHAO *et al.* 1999), a process in which nucleosomes are physically rearranged on chromatin so as to make activation sites accessible. In contrast to an accessibility mechanism, (DELLINO *et al.* 2004) have observed RNA polymerase bound to the 5' end of *Hox* genes under Polycomb repression, suggesting that genes under Polycomb repression are, in fact, still accessible to the transcription machinery. Since the promoter region has not melted, however, they conclude that Polycomb repression is interfering with the initiation of transcription (DELLINO *et al.* 2004; PAPP and MULLER 2006). Another mechanism under consideration reflects the fact that the dRING1 and PSC components of PRC1 have ubiquitin ligase activity *in vivo* (CAO *et al.* 2005; WANG *et al.* 2004a). This activity modifies lysine-119 of histone H2A. Since unbiquitylation is an extraordinarily bulky modification, it is conceivable that histone ubiquitylation would present an obstacle to the procession of RNA polymerase, and so this might provide a mechanism for preventing transcriptional elongation (STOCK *et al.* 2007; ZHOU *et al.* 2008). It is also conceivable that ubiquitylation might repress genes by another, as-yet undescribed mechanism.

H. The SCM Polycomb Repressor

Sex Combs on Midleg (SCM) is one of the PcG proteins that has not been incorporated yet into the emerging framework of PcG complexes. Loss of the SCM protein causes a severe homeotic phenotype equivalent to the loss of PRC2 or PRC1 components (BORNEMANN *et al.* 1998; BREEN and DUNCAN 1986), indicating that SCM is also an important contributor to Polycomb silencing. Immunostainings of polytene chromosomes (BORNEMANN *et al.* 1998; PETERSON *et al.* 1997) and chromatin immunoprecipitations (see Chapter Four) place SCM at PcG target sites under repression. SCM is a 98kD protein, but fractionates at approximately 500kD in gel filtration experiments on *Drosophila* nuclear extracts, suggesting that it acts in a complex or perhaps a self-multimer (PETERSON *et al.* 2004). However, SCM has not been found in stable association with any of the previously characterized PcG complexes.

The domain structure of SCM has motivated speculation about what role it might play in Polycomb repression. SCM contains several domains of interest (PETERSON *et al.* 2004; see Chapter Four, Figure 1) including two putative zinc fingers, two MBT repeats, and an SPM domain near its C-terminal end. The zinc fingers do not provide sequence-specific DNA-binding, and no other function has been found for them. The SPM domain is a protein-protein interaction motif, and *in vitro* experiments have suggested an interaction between SCM and a similar domain in PH (BORNEMANN *et al.* 1996; PETERSON *et al.* 1997; PETERSON *et al.* 2004). SFMBT is even more similar in

domain organization to SCM (see Chapter Four, Figure 1) and also includes a SPM domain, prompting speculation that SCM may physically partner with SFMBT. Although biochemical tests demonstrate that the SPM domain of SCM has the capacity to bind to either PH or SFMBT *in vitro*, there is not evidence for either stable association *in vivo*. Purifications of PRC1 have detected a substoichiometric enrichment of SCM (SAURIN *et al.* 2001; SHAO *et al.* 1999), which may reflect SCM interaction with PRC1, possibly stabilized when both are present on the chromatin template.

More recent work with SCM has revealed that its MBT repeats have an affinity for mono- and di-methyl-lysine (GRIMM *et al.* 2007; SANTIVERI *et al.* 2008), albeit at a much lower affinity than PC has for trimethyl-H3-K27. This may suggest other possible binding roles for both SCM and SFMBT, but it is not clear if the potential binding partners would be methylated histones or methylated non-histone proteins. Additionally SCM might be in complex with as-yet undescribed proteins that bind to DNA or to nucleosomes. Chapter Four presents *in vivo* studies to further address the role of SCM in PcG repression. Specifically, a series of molecular epistasis experiments determine the stage at which SCM is recruited to chromatin during Polycomb repression.

III. Biological and Biomedical Significance of Polycomb Proteins

While the foregoing discussion has focused on PcG research in *Drosophila*, it is clear that there are parallel mechanisms in mammalian systems. In fact, it is remarkable that the core components of the PRC1 and PRC2 complexes, and their biochemical activities, are conserved from flies to humans (BOYER *et al.* 2005; CAO *et al.* 2002; KUZMICHEV *et al.* 2002; LEVINE *et al.* 2002). Furthermore, the developmental role of PcG proteins in repressing Hox genes is also conserved in mammals (SCHUMACHER *et al.* 1996; TAKIHARA *et al.* 1997; VAN DER LUGT *et al.* 1994). PhoRC components are also conserved between flies and mammals, but it is less clear whether these components conserve function in mammals (SIMON 2009) and purification of a PhoRC from mammalian cells has yet to be reported. Individual Polycomb proteins are even further conserved from plants to humans (PIEN and GROSSNIKLAUS 2007; SCHUETTENGRUBER *et al.* 2007), and Polycomb proteins repress *Hox* genes in *C elegans* (ROSS and ZARKOWER 2003). Overall conservation of the PcG machinery does decline in more distant species, however, with *C elegans* and *Arabadopsis* lacking recognizable PRC1 subunits. It is interesting to note that the most widely conserved components of the PcG are members of PRC2, yet the Polycomb system has retained its silencing function in all of the phylogenetic branches where it appears. It is interesting to consider whether PRC2 has some silencing ability of its own even in the absence of PRC1, or whether distant organisms have evolved parallel systems to perform the silencing functions supplied by PRC1.

As suggested by the wide conservation of PcG proteins, they participate in several critical biological processes that motivate much of the research in the field. Transcriptional regulation is a basic cellular process that underlies many phenomena of interest to medical researchers. The overarching medical justification of most basic research is to understand biological processes when they are functioning normally so as to better understand what has gone wrong in a disease state. The Polycomb system has, in fact, been implicated in cancer progression and metastasis (see reviews in LESSARD and SAUVAGEAU 2003; SIMON and LANGE 2008; SPARMANN and VAN LOHUIZEN 2006; VALK-LINGBEEK *et al.* 2004) For example, the mammalian E(Z) homologue EZH2 is commonly found overexpressed in tumor samples from patients, and expression levels have a positive correlation with tumor growth and invasiveness (CROONQUIST and VAN NESS 2005; KLEER *et al.* 2003; VARAMBALLY *et al.* 2002). This suggests that analysis of PcG function in cancer might yield diagnostic or even therapeutic advances.

Another area of great interest for medical researchers that involves PcG proteins is stem cell maintenance (reviewed in SIMON 2009). The Polycomb system seems to maintain the stem cell state by repressing cell differentiation factors, since the loss of PcG components often leads to premature differentiation (BOYER *et al.* 2006; LEE *et al.* 2006; PASINI *et al.* 2007). Some of this work suggests that the PRC2 complex may participate in silencing these differentiation factors by acting as co-repressors with the Oct4, Sox2, and Nanog repressors (BOYER *et al.* 2005; BOYER *et al.* 2006; LEE *et al.* 2006). Recent investigation into the chromatin state of embryonic stem cells has

revealed that differentiation genes are simultaneously marked with methylation on both H3-K27 and H3-K4 (BERNSTEIN *et al.* 2006). This marking is surprising because H3-K4 is generally considered an activating mark applied by Trithorax Group proteins (SCHUETTENGRUBER *et al.* 2007). This doubly-marked chromatin is sometimes interpreted as a "sensitized" state in which silenced differentiation factors can be quickly activated in response to differentiation cues (BERNSTEIN *et al.* 2006).

Other biological processes that depend on PcG components include mammalian X inactivation and vernalization in plants. X inactivation compensates for the presence of two X chromosomes in female mammals by silencing one entire chromosome. During this process, PcG proteins mark the entire X chromosome with methyl-H3-K27 (HEARD 2005). In vernalization, plants avoid flowering before the end of the winter season by requiring a prolonged exposure to cold temperatures before they will produce flowers (SCHMITZ and AMASINO 2007). Prior to winter, flowering is inhibited by expression of the FLOWERING LOCUS C (FLC) gene. A prolonged cold period triggers silencing of FLC by plant PcG proteins so that flowering can proceed once warmer temperatures arrive in the spring (GENDALL *et al.* 2001).

Additionally, we consider the importance of Polycomb research to the field of epigenetics. Epigenetics is defined as heritable information not transmitted by the genetic sequence. Current interest in the field centers on information about the transcriptional state of genes being passed between generations of cellular progeny in proliferating tissue as well as between generations of organisms in the reproductive

cycle. Histone modifications are envisioned as an important mechanism for encoding and transmitting epigenetic information (FELSENFELD and GROUDINE 2003). Because the chromatin modifications and persistent repressive state conferred by the Polycomb system survives through the cell cycle to daughter cells, research into Polycomb repression is expected to enlighten mechanisms of epigenetic inheritance. The underlying mechanism for transmitting the transcriptional state through the cell cycle is obscure because chromatin proteins are frequently stripped from DNA during replication or mitosis, and research has not defined mechanisms whereby the histone modification state would be reliably reapplied as chromatin states are reassembled. Greater understanding of the mechanism of Polycomb repression is likely to promote our understanding of epigenetic inheritance.

IV. Summary of Dissertation

The following chapters present work on the recruitment and molecular functions of PcG proteins during Polycomb repression in *Drosophila*. Chapter Two presents a study of the ESCL Polycomb repressor (see above). This study includes genetic and biochemical investigations into whether ESCL participates in Polycomb repression as an alternative subunit of the PRC2 complex in place of ESC.

Chapter Three documents a system intended to study H3-K27 methylation by producing ectopic methylation sites in the genome of living *Drosophila*. The strategy for

producing ectopic methylation is to tether the PRC2 HMTase complex to transgenic sites by using a heterologous DNA-binding domain. This chapter presents the development of tools and the preliminary results obtained from testing this system *in vivo*.

Chapter Four presents an investigation into the recruitment of the SCM Polycomb repressor (see above) to chromatin during Polycomb repression. Molecular epistasis experiments are performed to help integrate SCM into the emerging framework of PcG complexes. By removing defined components of the PcG and then observing which other components are still able to bind to the *Ubx* PRE, these experiments address the relationship between SCM and the known Polycomb complexes.

Finally, an appendix presents results from preliminary investigations into the occurrence of serine-28 phosphorylation on histone H3 in *Drosophila*. Serine-28 (H3-S28) is adjacent to H3-K27, and there is interest in whether H3-S28 phosphorylation can impact Polycomb repression.

Chapter Two

Alternative ESC and ESC-Like subunits of a Polycomb group histone methyltransferase complex are differentially deployed during *Drosophila* development

Liangjun Wang[1], Neal Jahren[2], Marcus L. Vargas[2], Erica F. Andersen[2], Judith Benes[1], Junyu Zhang[1], Ellen L. Miller[2], Richard S. Jones[1,*], and Jeffrey A. Simon[2,*]

[1]Department of Biological Sciences, Southern Methodist University, Dallas, Texas.

[2]Department of Genetics, Cell Biology and Development, University of Minnesota.

[*]Corresponding authors: simon004@umn.edu, rjones@mail.smu.edu

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I contributed the developmental western blot in Figure 4 and the genetic analysis presented in Figure 3 and table 1. The text is presented unchanged from the accepted version of the manuscript except that the abstract has been removed. All figure content is present, but has been rearranged to comply with dissertation formatting requirements.

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v1.3

I. Introduction

The Polycomb group (PcG) proteins of *Drosophila* are chromatin components that maintain transcriptional off states during development (FRANCIS and KINGSTON 2001; RINGROSE and PARO 2004; SIMON and TAMKUN 2002). PcG repressors are implicated in silencing many target genes in the fly genome (FRANKE *et al.* 1992; MOAZED and O'FARRELL 1992; RINGROSE *et al.* 2003). The most well-characterized targets are the fly *Hox* genes, such as *Ultrabithorax (Ubx*) (BEUCHLE *et al.* 2001; MCKEON and BROCK 1991; SIMON *et al.* 1992; STRUHL and AKAM 1985). Genetic studies reveal that most PcG repressors are needed continuously to repress *Hox* genes throughout development. The PcG requirement begins at about 3-4 hours of embryonic development, when the initial *Hox* repressors such as Hunchback and Kruppel decay. PcG repressors then maintain *Hox* repression during the remainder of embryogenesis and through subsequent larval and pupal stages. Polycomb response elements (PREs), located in *Hox* regulatory regions, are also needed during post-embryonic stages to ensure maintenance of repression (BUSTURIA *et al.* 1997). Thus, the PcG repression system provides stable gene silencing for long periods of developmental time and through many rounds of cell division in proliferating tissues.

There are approximately fifteen fly PcG proteins with roles in *Hox* gene repression established by genetic studies. Biochemical studies have defined compositions of complexes built from PcG proteins and have revealed some of their molecular functions in chromatin. These studies have generally employed purification of epitope-tagged

PcG complexes from fly embryo extracts, subunit identification, and analyses of *in vitro* functions using either native or recombinant PcG complexes. The two best-characterized fly PcG complexes are the ESC-E(Z) complex and Polycomb repressive complex 1 (PRC1) (CZERMIN *et al.* 2002; FRANCIS *et al.* 2001; MULLER *et al.* 2002; NG *et al.* 2000; SAURIN *et al.* 2001; SHAO *et al.* 1999; TIE *et al.* 2001). These two protein complexes are biochemically separable and contain non-overlapping sets of PcG subunits. In addition, the DNA-binding PcG proteins PHO and PHOL are required to target PcG complexes to *Hox* genes *in vivo* (BROWN *et al.* 2003; WANG *et al.* 2004b).

The fly ESC-E(Z) complex contains four core subunits: Extra sex combs (ESC), Enhancer of zeste [E(Z)], Suppressor of zeste-12 [SU(Z)12] and NURF-55 (CZERMIN *et al.* 2002; MULLER *et al.* 2002). This complex possesses enzyme activity that methylates histone H3 with primary specificity for lysine-27 (K27) in the N-terminal tail (CZERMIN *et al.* 2002; MULLER *et al.* 2002). Chromatin complexes containing conserved human homologs have very similar subunit compositions and share histone methyltransferase activity for H3-K27 (CAO *et al.* 2002; KUZMICHEV *et al.* 2002). Since the PC subunit of PRC1 binds specifically to methylated H3-K27, this chromatin modification is thought to help recruit PRC1 to target genes (CAO *et al.* 2002; CZERMIN *et al.* 2002; FISCHLE *et al.* 2003c; MIN *et al.* 2003). Indeed, genetic loss of methylated H3-K27 correlates with PC dissociation from a *Ubx* PRE *in vivo* (CAO *et al.* 2002). Thus, an ordered series of events has been hypothesized whereby the ESC-E(Z) complex marks the local chromatin for PcG repression and this in turn attracts PRC1, which is most directly

responsible for keeping target genes transcriptionally silent (CAO *et al.* 2002; SIMON 2003; WANG *et al.* 2004b).

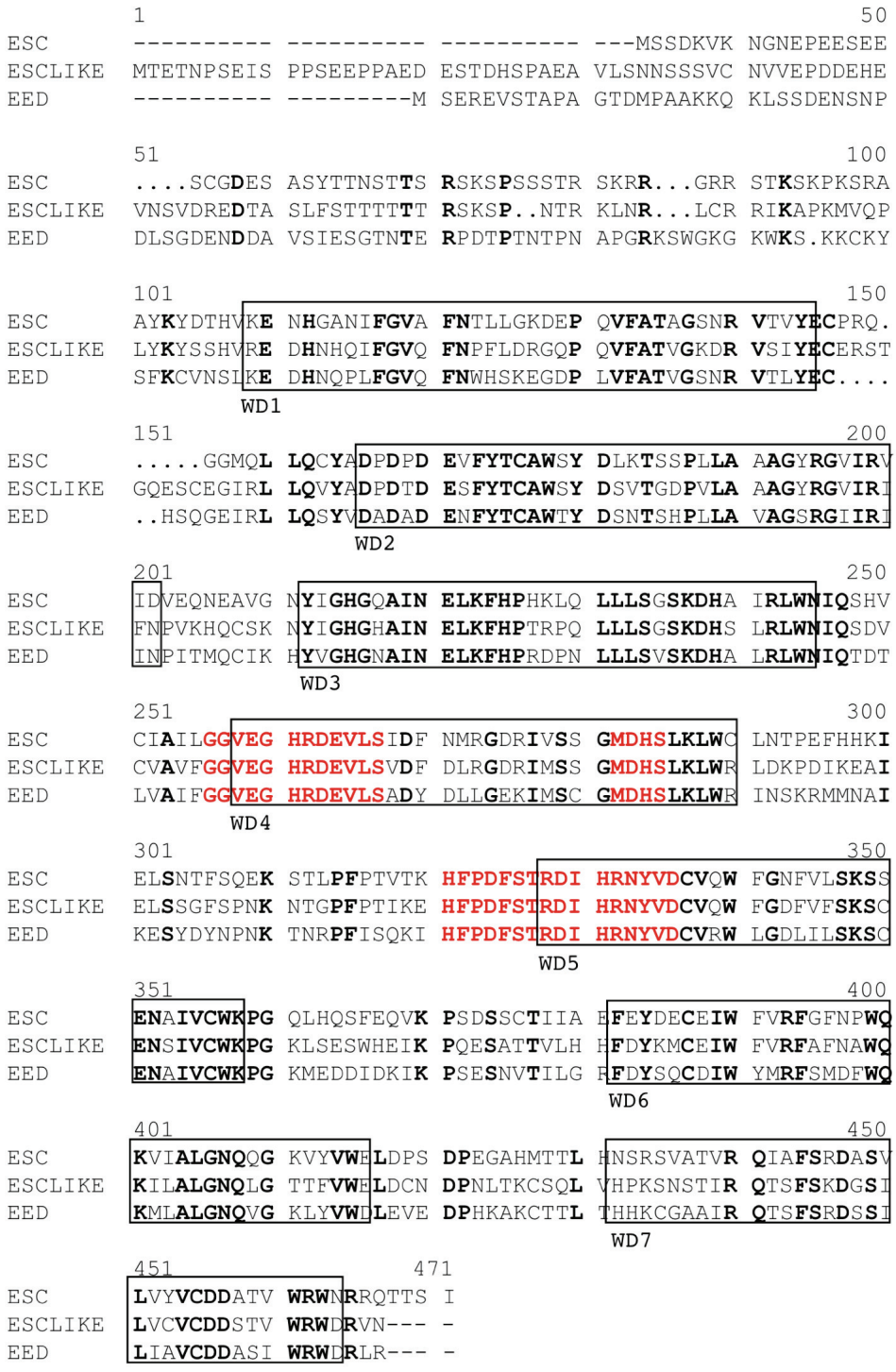
The SET domain of E(Z) provides the histone methyltransferase (HMTase) catalytic function (MULLER *et al.* 2002). In agreement with this central role, E(Z) is required throughout fly development to maintain *Hox* gene repression. The tight correlation between E(Z) function, K27 methylation, and *Hox* silencing *in vivo*, as demonstrated with conditional *E(z)* alleles and site-directed E(Z) SET domain mutants (CAO *et al.* 2002; CARRINGTON and JONES 1996; MULLER *et al.* 2002), implies that the HMTase activity is itself needed continuously as development proceeds. In addition to E(Z), the noncatalytic ESC and SU(Z)12 subunits are required for HMTase activity *in vitro* and ESC is required for global H3-K27 methylation in embryos (CAO and ZHANG 2004a; KETEL *et al.* 2005; NEKRASOV *et al.* 2005). Since E(Z) complexes have only been purified so far from embryos, much remains to be determined about the nature and composition of E(Z) complexes that maintain K27 methylation during post-embryonic stages.

Genetic and expression studies on fly ESC suggest that subunit contributions to the HMTase complex may change during development. E(Z) and SU(Z)12 are critical for *Hox* repression during both embryonic and post-embryonic stages. In contrast, ESC is critically required during embryogenesis, but its functional role is greatly diminished during post-embryonic stages (GLICKSMAN and BROWER 1988; SIMON *et al.* 1995; STRUHL and BROWER 1982). Indeed, ESC is the only PcG member whose maternal

supply to the embryo is sufficient for survival to adult stages in flies lacking zygotic product (STRUHL 1981). In agreement with the genetic studies, ESC mRNA and protein levels are most abundant during embryonic stages but then are dramatically reduced during larval and pupal development (FURUYAMA *et al.* 2003; GUTJAHR *et al.* 1995; NG *et al.* 2000; SATHE and HARTE 1995; SIMON *et al.* 1995). These studies have defined a long-standing paradox: how can PcG repression be maintained stably throughout development despite changes in the requirement for ESC? In molecular terms, how can an E(Z) HMTase complex continue to function despite changes in availability of a vital core subunit? A potential solution to this ESC paradox is provided by the discovery, through the fly genome project, of a second ESC-related gene product in flies. This gene, called *esc-like*, produces a protein which is strikingly similar to ESC (Fig. 1). We show here that ESC-Like (ESCL) is expressed at peak levels during post-embryonic stages, which corresponds to times of diminished ESC. We find overlapping requirements for ESC and ESCL in PcG repression via genetic studies *in vivo* and as assessed by RNA interference studies in post-embryonic cultured fly cells. Chromatin immuneprecipitations show that ESCL associates and tracks with E(Z) in wing discs and *in vitro* tests show that ESCL can replace ESC in catalytically active HMTase complexes. Our results suggest that the composition of E(Z) HMTase complexes changes during fly development, with the ESC subunit predominating in embryos and ESCL playing a major role during post-embryonic stages.

Figure 1: Amino acid sequence and conservation of the *Drosophila* ESC-Like protein. A) Sequence alignment of fly ESC, fly ESC-LIKE, and mouse EED proteins. The seven WD repeats are enclosed in boxes. Residues in red correspond to predicted loops shown in red in Figure 2 and are absolutely conserved in all three proteins. Other residues that are identical in all three proteins are shown in bold. In pairwise comparisons, ESC is 55% identical to EED and ESCL is 60% identical to EED.

Figure 1


1 50
ESC ---------- ---------- ---------- ---MSSDKVK NGNEPEESEE
ESCLIKE MTETNPSEIS PPSEEPPAED ESTDHSPAEA VLSNNSSSVC NVVEPDDEHE
EED ---------- ---------M SEREVSTAPA GTDMPAAKKQ KLSSDENSNP
51 100
ESC ....SCGDES ASYTTNSTTS RSKSPSSSTR SKRR...GRR STKSKPKSRA
ESCLIKE VNSVDREDTA SLFSTTTTTT RSKSP..NTR KLNR...LCR RIKAPKMVQP
EED DLSGDENDDA VSIESGTNTE RPDTPTNTPN APGRKSWGKG KWKS.KKCKY
101 150
ESC AYKYDTHVKE NHGANIFGVA FNTLLGKDEP QVFATAGSNR VTVYECPRQ.
ESCLIKE LYKYSSHVRE DHNHQIFGVQ FNPFLDRGQP QVFATVGKDR VSIYECERST
EED SFKCVNSLKE DHNQPLFGVQ FNWHSKEGDP LVFATVGSNR VTLYEC....
WD1
151 200
ESC .....GGMQL LQCYADPDPD EVFYTCAWSY DLKTSSPLLA AAGYRGVIRV
ESCLIKE GQESCEGIRL LQVYADPDTD ESFYTCAWSY DSVTGDPVLA AAGYRGVIRI
EED ..HSQGEIRL LQSYVDADAD ENFYTCAWTY DSNTSHPLLA VAGSRGIIRI
WD2
201 250
ESC IDVEQNEAVG NYIGHGQAIN ELKFHPHKLQ LLLSGSKDHA IRLWNIQSHV
ESCLIKE FNPVKHQCSK NYIGHGHAIN ELKFHPTRPQ LLLSGSKDHS LRLWNIQSDV
EED INPITMQCIK HYVGHGNAIN ELKFHPRDPN LLLSVSKDHA LRLWNIQTDT
WD3
251 300
ESC CIAILGGVEG HRDEVLSIDF NMRGDRIVSS GMDHSLKLWC LNTPEFHHKI
ESCLIKE CVAVFGGVEG HRDEVLSVDF DLRGDRIMSS GMDHSLKLWR LDKPDIKEAI
EED LVAIFGGVEG HRDEVLSADY DLLGEKIMSC GMDHSLKLWR INSKRMMNAI
WD4
301 350
ESC ELSNTFSQEK STLPFPTVTK HFPDFSTRDI HRNYVDCVQW FGNFVLSKSS
ESCLIKE ELSSGFSPNK NTGPFPTIKE HFPDFSTRDI HRNYVDCVQW FGDFVFSKSC
EED KESYDYNPNK TNRPFISQKI HFPDFSTRDI HRNYVDCVRW LGDLILSKSC
WD5
351 400
ESC ENAIVCWKPG QLHQSFEQVK PSDSSCTIIA EFEYDECEIW FVRFGFNPWQ
ESCLIKE ENSIVCWKPG KLSESWHEIK PQESATTVLH HFDYKMCEIW FVRFAFNAWQ
EED ENAIVCWKPG KMEDDIDKIK PSESNVTILG RFDYSQCDIW YMRFSMDFWQ
WD6
401 450
ESC KVIALGNQQG KVYVWELDPS DPEGAHMTTL HNSRSVATVR QIAFSRDASV
ESCLIKE KILALGNQLG TTFVWELDCN DPNLTKCSQL VHPKSNSTIR QTSFSKDGSI
EED KMLALGNQVG KLYVWDLEVE DPHKAKCTTL THHKCGAAIR QTSFSRDSSI
WD7
451 471
ESC LVYVCDDATV WRWNRRQTTS I
ESCLIKE LVCVCDDSTV WRWDRVN--- -
EED LIAVCDDASI WRWDRLR--- -

II. Materials and Methods

***Drosophila* strains.** All standard fly stocks were obtained from the Bloomington stock center. *esc* is located at polytene cytological position 33A2, which corresponds to coordinates 2839 to 5061 of genomic scaffold sequence AE003634. *escl* is located at cytological position 33B5 and coordinates 166586 to 168478 of AE003634. *Df(2L)esc*^{10} [Df(2L)33A1; 33B2] deletes *esc*, but not *escl*. The proximal breakpoint of *Df(2L)esc*^{10} is ~140 kb proximal to *esc* and ~20 kb distal to *escl* (FREI *et al.* 1985). *Df(2L)Prl* [Df(2L)32F1-3; 33F1-2] deletes both *esc* and *escl* (FREI *et al.* 1988). *esc*^{6} is an apparent null allele resulting from a splice site mutation that truncates the protein at residue G20 and *esc*^{2} is an apparent null resulting from a frameshift at residue V404 (GUTJAHR *et al.* 1995; SATHE and HARTE 1995; STRUHL 1981). *esc*^{5} is a nonsense allele, Q171stop (SATHE and HARTE 1995).

Generation of *escl* transgenes and germline transformants. A 2.3 kb genomic *escl* fragment was obtained by PCR using wild-type adult fly DNA as template and tailed primers positioned 0.3 kb upstream of the start codon and 0.5 kb downstream of the stop codon. These primers lie within the coding regions of the closely flanking genes CG16969 and CG31866, respectively. The 2.3 kb fragment was inserted into pCasper4 to generate the transformation construct pCas-ESCL2.3. A FLAG-tagged version was also constructed in pCasper4, which is identical except for insertion of a FLAG-encoding oligonucleotide just upstream of the ESCL start codon. Germline transformants were generated in a *y Df(1)w*^{67c23} genetic background.

Antibodies, Western blots, and co-immunoprecipitations. Rabbit anti-ESCL and anti-ESC antisera were raised against His_6-fusion proteins containing ESCL residues 2-96 and ESC residues 2-64, respectively. Anti-ESCL and anti-ESC antibodies were affinity purified, using the same respective fusion proteins, as previously described (O'CONNELL *et al.* 2001) except that antisera were first preadsorbed against His_6-dMes4-420-633. Protein extracts from embryos, larvae, and pupae for use in Western blots were prepared as described (NG *et al.* 2000). Relative protein concentrations were determined by Coomassie blue staining of proteins after SDS-PAGE. Western blots were performed using affinity-purified anti-ESCL at 1:1000 and goat anti-rabbit-HRP at 1:10,000 (BioRad) and signals were developed using an ECL chemiluminescence detection kit (Amersham). Protein extracts for use in co-immunoprecipitations were prepared from MCW12 cells as described for SL2 cells (WANG *et al.* 2004b). Immunoprecipitations were performed as previously described (JONES *et al.* 1998) using 10 μl of affinity-purified anti-ESCL or anti-ESC antibodies.

Cell culture and RNA interference. MCW12 cells, which were derived from wing imaginal discs, were a generous gift from Deborah Cottam and Martin Milner (University of St. Andrew). Aliquots were thawed and ~1.5 x 10^6 cells were cultured in 6-well plates in SS3 medium (Sigma) supplemented with 2% heat-inactivated fetal calf serum, 2.5% fly extract and 12.5 IU/100 ml insulin. Fly extract was prepared as described (CURRIE *et al.* 1988). Because the MCW12 cells grew for only approximately two weeks before they ceased dividing, fresh aliquots of cells were thawed for each

experiment. RT-PCR analysis of transcript levels was performed as previously described for SL2 cells (WANG *et al.* 2004b) using the following gene-specific primers: *Antp*, 5'-AGC AAC AGC CCT CGC AGA AC-3' and 5'-AAC TCC CGA CTG CTG CTG GT-3'; *Ubx*, 5'-CGA GGA AAT CCG TCA GCA GAC-3' and 5'-CAG AGT AAC CAA TTT GTT TTT CAC-3'; *Abd-B*, 5'-CTC CCC TCG CAA TTA CCA AAG G-3' and 5'-TGC CGT GTG CCG CTT GAC CG-3'; *RpII140*, 5'-CCT GCT GGA TCG TGA TTA ACG C-3' and 5'-GTT GAT GAT GAA GTA GCC ACC G-3'. dsRNA was prepared as previously described (WANG *et al.* 2004b) and included the following sequences: *Pc*, 935 bp extending from 120 to 955 bp downstream of the ATG; *esc*, 598 bp extending from 50 bp upstream of the ATG to 545 bp downstream; *escl*, 915 bp extending from 700 to 1615 bp downstream of the ATG; GFP, 662 bp extending from 25 to 687 bp downstream of the ATG. Freshly thawed aliquots of MCW12 cells were cultured for one week, then transfected with dsRNA as previously described (CAO *et al.* 2002; WANG *et al.* 2004b) except that the second transfection was performed 4 days after the first transfection due to the slower growth rate of MCW12 cells relative to SL2 cells.

ChIP Assays. Chromatin immunoprecipitations of hand dissected wing imaginal discs were performed essentially as previously described (CAO *et al*. 2002; WANG *et al*. 2004b). Wing disc ChIP input control PCR reactions contained total DNA extracted from fixed and sonicated chromatin equivalent to DNA from 10%, 1%, or 0.1% of an imaginal disc, respectively. Each PCR reaction performed on DNA from immunoprecipitated chromatin contained the equivalent of one wing imaginal disc.

Wing discs were isolated from either normal [$E(z)^{61}$ larvae reared at 18°C] or *esc* mutant larvae. In order to obtain *esc* mutant wing discs, esc^5/*In(2LR), Gla Bc Elp* adults were crossed to esc^6/*In(2LR), Gla Bc Elp* adults and wing discs were dissected from Bc^+ late third instar larval progeny. ChIP assays of MCW12 cells were performed essentially as previously described for SL2 cells (WANG *et al.* 2004b). Input control PCR reactions contained total DNA from fixed and sonicated chromatin equivalent to 10% of the immunoprecipitated chromatin used in each ChIP PCR reaction.

Generation and analysis of recombinant ESC-E(Z) and ESCL-E(Z) complexes. Baculovirus expression of recombinant complexes was performed using the Bac-to-Bac system (Invitrogen). Full-length cDNAs encoding FLAG-ESC, E(Z), SU(Z)12 and NURF-55 inserted into pFastBac1 were described previously (MULLER *et al.* 2002). A FLAG-ESCL construct was produced by inserting a 1.8 kb *escl* cDNA fragment into pFastBac1, followed by insertion of a FLAG oligonucleotide at the N-terminus. Anti-FLAG immunoaffinity purification of complexes was essentially as described (MULLER *et al.* 2002) except washes in BC buffer were performed up to 1.2 M KCl and elutions were performed in batch for 1 hour with 0.8 mg/ml FLAG peptide. The ESCL-E(Z) complex was purified twice independently and in parallel with an ESC-E(Z) complex used for comparative HMTase tests. HMTase assays were performed as described (MULLER *et al.* 2002), with duplicate assays performed on independently prepared complexes. Polynucleosome substrate, consisting of 8-12mers purified from HeLa cells, was used for the experiment in Fig. 7B and was prepared as described (KETEL *et al.* 2005). The histone substrates used in Fig. 7C were H3/H4 tetramers containing

wild-type or K9A or K27A mutant forms of H3 and were kindly provided by Nicole Francis (Harvard University).

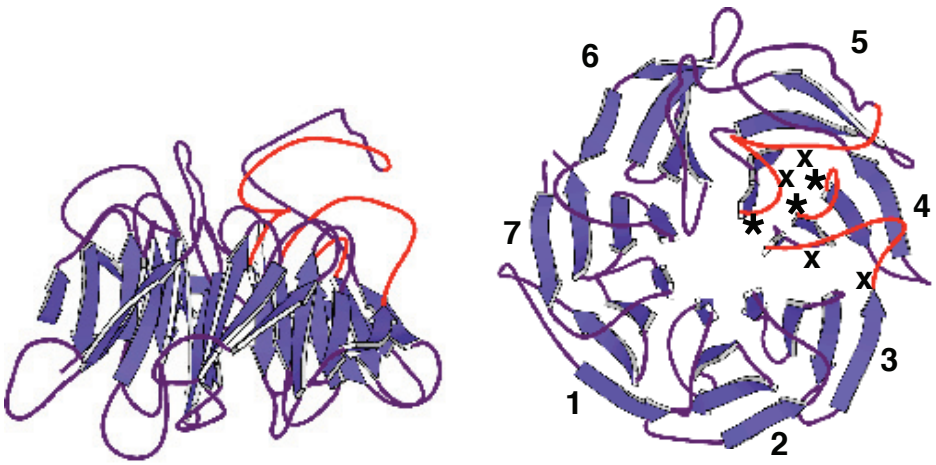
III. Results

The *Drosophila esc-like* gene encodes an ESC-related protein

One of the unexpected findings from the *Drosophila* genome project was the discovery of a second *esc*-related gene, designated CG5202. This gene, *esc-like (escl)*, encodes a protein that shares about 60% overall identity with fly ESC and with the mammalian homolog, EED (Fig. 1). The bulk of ESC and ESCL is comprised of a domain with seven WD repeats, which is 63% identical between the two proteins. Each protein also possesses a smaller and less conserved (37% identical) N-terminal tail. The ESCL tail is 35 amino acids longer than the ESC tail, which contributes to a slightly larger ESCL protein of 462 residues and an expected mass of 52 kD. A predicted three-dimensional structure for ESC was derived previously (NG *et al.* 1997) based upon the folding of multiple WD repeats into a ß-propeller (WALL *et al.* 1995). This circular structure serves as a scaffold for protein interactions and features variable surface loops that emanate above and below the plane of the propeller. The surface loops highlighted in red (Fig. 2) correspond to regions that are absolutely identical in ESC, ESCL and EED (Fig. 1) and are thus likely to be functionally important. Indeed, analysis of *esc* alleles and site-directed *esc* mutations in these loops (indicated in Fig. 2), have implicated these ESC regions in repressor function *in vivo* (GUTJAHR *et al.* 1995; NG *et al.* 2000; NG *et al.* 1997; SATHE and HARTE 1995) and in E(Z) binding and HMTase function of

Figure 2: Predicted structure of ESC protein. Side and top views showing predicted structure (NG *et al.* 1997) of the ESC ß-propeller formed from the WD repeats. Loops in red are identical among ESC, ESCL and EED. Numbers correspond to the WD repeats marked in boxes in Figure 1. "*" indicates positions of loss-of-function *esc* missense alleles (GUTJAHR *et al.* 1995; SATHE and HARTE 1995) and "X" indicates positions of site-directed *esc* mutations that cause loss-of-function in vivo (NG *et al.* 2000; NG *et al.* 1997).

Figure 2



the complex *in vitro* (JONES *et al.* 1998; KETEL *et al.* 2005; TIE *et al.* 1998). This pattern of conservation suggests that the molecular function of ESCL is similar to the function of ESC. Thus, we pursued genetic and molecular tests to address the roles of ESCL *in vivo* and *in vitro*.

Evidence for overlap of ESC and ESC-Like functions *in vivo*

There are no pre-existing mutant alleles of the *escl* gene. However, since *escl* is closely linked to *esc,* about 160 kb proximal within cytogenetic region 33B, deficiencies are available that remove just *esc* or both *esc* and *escl*. We used such deficiencies in genetic interaction tests to address if heterozygosity for *escl* enhances the severity of phenotypes observed with loss of *es*c function. Our tests employed $Df(2L)esc^{10}$, which removes *esc* but leaves *escl* intact, *Df(2L)Prl*, which deletes both *esc* and *escl,* and two apparent *esc* null alleles, esc^{6} and esc^{2}. esc^{6} disrupts the intron 1 splice donor, leading to a prematurely truncated protein of only 24 amino acids (GUTJAHR *et al.* 1995). esc^{2} creates a frameshift within the seventh WD repeat that replaces the normal 22 C-terminal amino acids with 44 novel residues (GUTJAHR *et al.* 1995; SATHE and HARTE 1995).

As described previously (STRUHL 1981), we observed that animals with the genotype $esc^{6}/Df(2L)esc^{10}$ survive to adulthood and display an extra sex combs phenotype, a hallmark of partial PcG loss-of-function. Although complete loss of esc^{+} product is embryonic lethal, these $esc^{6}/Df(2L)esc^{10}$ adults survive due to maternal esc^{+} product which supplies sufficient function for viability. In contrast, we found that

esc^{6}*/Df(2L)Prl* is a lethal genotype. This result is consistent with heterozygosity for *escl* enhancing the zygotic loss of *esc* to create synthetic lethality. However, the lethality could also be due to other lethal mutations on the *esc*^{6} chromosome located within the *Df(Prl)* interval. To directly address whether *escl*$^{+}$ dosage is responsible, a transgenic copy of *escl* was tested for its ability to restore viability to *esc*^{6}*/Df(2L)Prl* animals. A 2.3 kb genomic fragment, which extends into the predicted flanking genes on either side of *escl*, was generated by PCR using wild-type adult DNA as template. This fragment was inserted into the P element vector pCasper4 and transformant lines carrying a single copy of this *escl*$^{+}$ transgene were produced. Crosses were performed to produce animals with the genotype *esc*^{6}*/Df(2L)Prl; P[escl*$^{+}$*]/+*. These individuals survive to adulthood with an extra sex combs phenotype similar to *esc*^{6}*/Df(2L)esc*^{10}. Thus, the lethality is rescued by increasing *escl*$^{+}$ dosage from one to two copies. These results suggest that there is overlap between the functions of *esc* and *escl in vivo*.

A similar set of genetic tests using *esc*^{2} instead of *esc*^{6} was also performed. Although genetic enhancement of *esc* by *escl* was again observed, the severity of the phenotypes was less extreme, involving visible homeotic transformations rather than synthetic lethality. Specifically, *esc*^{2}*/Df(2L)esc*^{10} males display an intermediate extra sex combs phenotype (Fig. 3A, Table 1) that is less severe than that seen with *esc*^{6}*/Df(2L)esc*^{10}. The *esc*^{2}*/Df(2L)esc*^{10} animals typically showed sex combs on only 3 or 4 out of 6 legs and, when present, the ectopic sex combs typically contained only 1-4 teeth (Fig. 3A, second row). In addition, they appeared wild-type with respect to antennal development. In contrast, *esc*^{2}*/Df(2L)Prl* animals show nearly full sex combs on all six

Figure 3: Genetic enhancement of *esc* loss by reduced *escl* dosage. Extra sex combs phenotypes (A) and antenna-to-leg transformations (B) are shown for the indicated genotypes. T1, T2 and T3 indicate the first, second, and third thoracic legs, respectively. Arrow under T2 indicates example of a partial extra sex comb. Bold arrows under (B) indicate partial transformations of antennae towards legs. Genetic enhancement is evident by comparing phenotype of $esc^{2}/Dfesc^{10}$ (second row) to phenotype of $esc^{2}/Df(Prl)$ (third row). The enhanced $esc^{2}/Df(Prl)$ phenotype is rescued by a single copy of an *escl* transgene (fourth row).

Figure 3

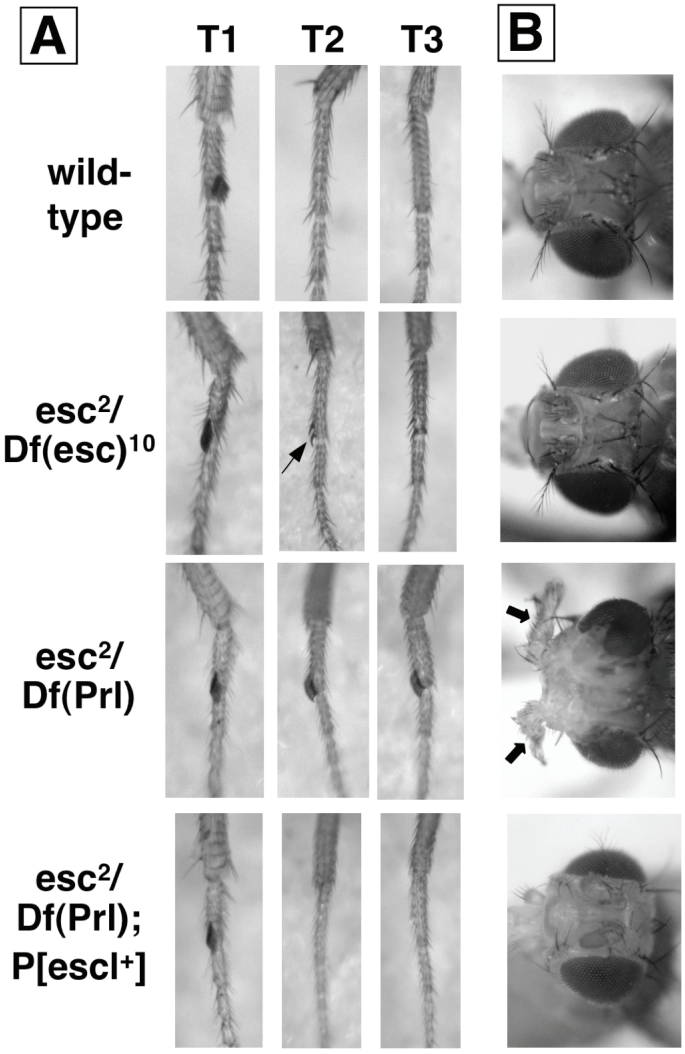

A
T1
T2
T3
B
wild-type
esc2/ Df(esc)10
esc2/ Df(Prl)
esc2/ Df(Prl); P[escl+]

Table 1: Rescue of extra sex combs phenotype by ESCL transgenes.

Genotype	Ave. number of legs with sex comb teeth
esc^{2}*/ Df(esc)*^{10}	3.7 (38)
esc^{2}*/ Df(Prl)*	5.9 (9)
esc^{2}*/ Df(Prl); P[ESCL]/ +*[a]	2.4 (18)
esc^{2}*/ Df(Prl)*	5.5 (8)
esc^{2}*/ Df(Prl); P[FL-ESCL]/ +*[a]	2.0 (8)

[a]Two independent tests for rescue of the extra sex combs phenotype in *esc*^{2}*/ Df(Prl)* adults were performed using either an untagged ESCL transgene or a FLAG-tagged transgene. The number of legs with sex comb teeth ranges from two (wild-type) to six (extreme mutant phenotype). Numbers in parentheses are number of adult males of indicated genotypes recovered and scored from among approximately 300 *esc*^{2}-containing male progeny each in the *P[ESCL]* or *P[FL-ESCL]* rescue crosses.

legs (Fig. 3A, Table 1) as well as partial antenna-to-leg transformations (Fig. 3B). Thus, the severity of homeotic transformations is enhanced here by heterozygosity for *escl*. Once again, the enhancement is specifically due to *escl* dosage because addition of an $escl^{+}$ transgene rescues these defects (Fig. 3 bottom, Table 1). These phenotypes further support *esc/escl* overlap and also indicate that *escl* function *in vivo* includes a role in *Hox* gene repression.

ESC-Like expression peaks during post-embryonic development

In order to examine ESCL expression during development, and its associations with other PcG proteins, we generated polyclonal antibodies against the ESCL N-terminal tail. The ESCL immunogen spanned residues 2-96, a region which lacks significant sequence similarity to ESC. Figure 4A shows that this antibody detects a species of approximately the correct size, ~60 kD, on a Western blot of total extract from wild-type pupae. This same species, plus a slightly larger one, are detected in pupal extract from a transformant line that contains a FLAG-ESCL transgene (lane 2). This transgene produces functional ESCL product since, like the untagged transgene, it rescues synthetic lethality of esc^{6}*/Df(2L)Prl* animals as well as the homeotic phenotypes of esc^{2}*/Df(2L)Prl* animals (Table 1). The detection of this doublet identifies the lower species as endogenous ESCL and indicates that FLAG-ESCL is expressed at a comparable level. Western blot analysis of purified recombinant FLAG-ESCL versus recombinant FLAG-ESC (lanes 3 and 4) confirms antibody specificity for ESCL and lack of cross-reactivity with ESC.

Figure 4: Western blot analysis of ESCL expression during development. A) Western blot demonstrating reactivity of affinity-purified anti-ESCL antibody. Leftmost lanes contain mid-pupal extracts from wild-type (lane 1) and FLAG-ESCL germline transformant (lane 2). Rightmost lanes contain approximately 25 ng of purified recombinant FLAG-ESCL-E(Z) complex (lane 3) and purified recombinant FLAG-ESC-E(Z) complex (lane 4). B) Developmental Western blot using anti-ESCL antibody and extracts from the indicated stages. Embryonic stages are in hours and L3 indicates crawling third-instar larvae. Pupal stages are white prepupae, tan mid-stage pupae, and late pupae with eye and body pigmentation. Protein loads were balanced by Coomassie blue staining of extracts after SDS-PAGE.

Figure 4

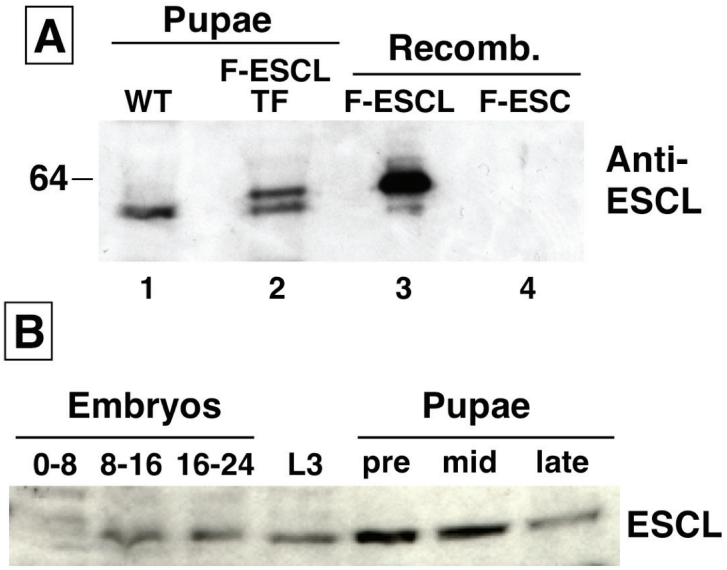

A
Pupae
Recomb.
F-ESCL
WT
TF
F-ESCL
F-ESC
64
Anti-
ESCL
1
2
3
4
B
Embryos
Pupae
0-8
8-16
16-24
L3
pre
mid
late
ESCL

Figure 4B shows a developmental Western blot performed on extracts from wild-type embryos, larvae, and pupae of the indicated stages. In contrast to ESC, which peaks during mid-embryogenesis and then is dramatically reduced (FURUYAMA *et al.* 2003; NG *et al.* 2000), ESCL accumulation persists through late embryonic and larval stages and then peaks during early pupal development. These differential ESC and ESCL expression profiles are also apparent at the mRNA level (*Drosophila* Developmental Gene Expression Timecourse, http://genome.med.yale.edu/Lifecycle/). Thus, ESCL is most abundant at developmental times that correspond to times of diminished ESC.

ESC-Like associates with E(Z) on target gene chromatin

ESC functions *in vivo* as a noncatalytic subunit in E(Z) HMTase complexes (CZERMIN *et al.* 2002; KETEL *et al.* 2005; MULLER *et al.* 2002; NEKRASOV *et al.* 2005). The high degree of ESC/ESCL similarity (Fig. 1), including regions required for E(Z) binding, suggests that ESCL also functions together with E(Z). To test this, we performed chromatin immunoprecipitation (ChIP) assays to determine if ESCL colocalizes with E(Z) on *Hox* gene DNA *in vivo*. Previous ChIP analyses using wing imaginal discs have shown that E(Z) associates with the transcription start region and with a Polycomb response element (PRE) of the *Ubx* gene (CAO *et al.* 2002; WANG *et al.* 2004b). Within these regulatory regions, E(Z) binding is detected primarily on fragment p2, which includes the start site, and on fragments b4 and b5 of the upstream PRE (Fig. 5A-C). We find that ESCL tracks precisely with E(Z) on these same chromatin fragments in wing discs (Fig. 5B, C). Based upon co-immuneprecipitations from soluble extracts

Figure 5: Associations of E(Z), ESCL and ESC with *Ubx* regulatory DNA in wing imaginal discs. Chromatin immunoprecipitations (ChIPs) were performed to compare distributions of E(Z), ESCL and ESC on fragments (shown in A) from the *Ubx* promoter region and an upstream PRE. Fragment numbering is as described (WANG *et al.* 2004b). B) Distributions on the *Ubx* promoter in wild-type wing discs. C) Distributions on the *Ubx* PRE in wild-type wing discs. D) Distributions on the *Ubx* promoter and PRE in *esc* mutant wing discs. In each panel, antibodies used in the immunoprecipitations are indicated at the top and PCR-amplified regions are indicated to the left. "Mock" indicates control IP with crude rabbit preimmune antiserum and "Rp" indicates a control fragment from the *RpII140* promoter.

Figure 5

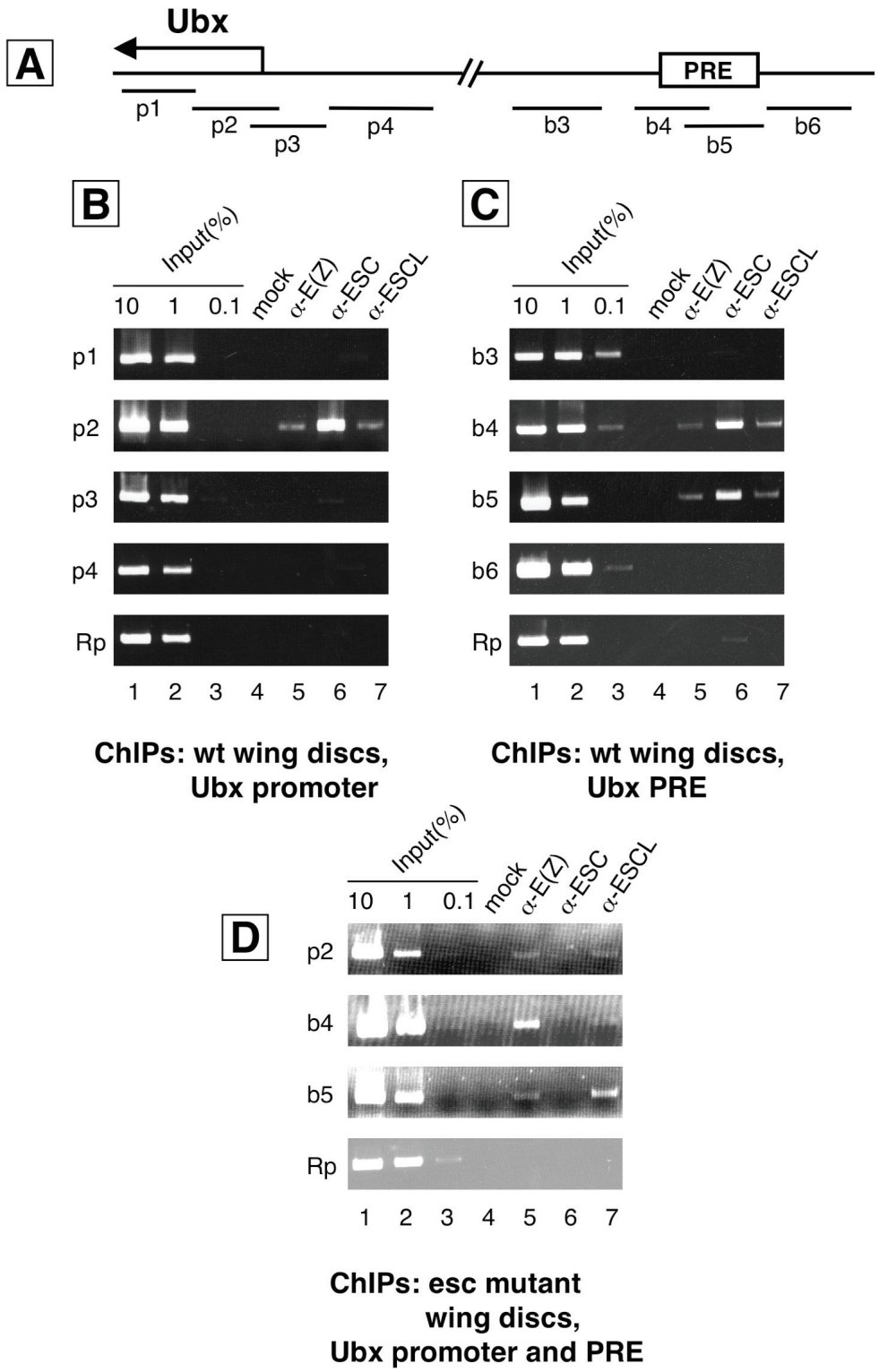
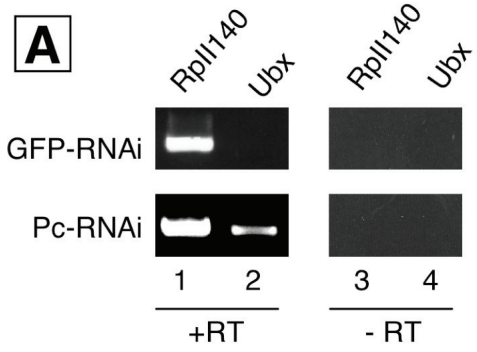
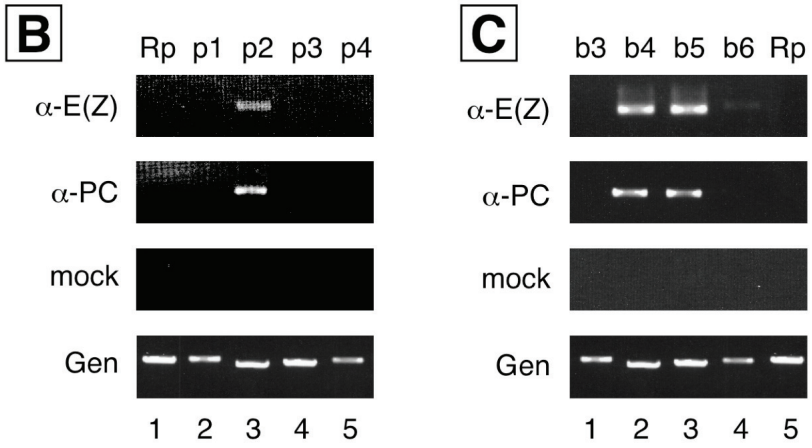

A
Ubx
PRE
p1
p2
p3
p4
b3
b4
b5
b6
B
Input(%)
10
1
0.1
mock
α-E(Z)
α-ESC
α-ESCL
p1
p2
p3
p4
Rp
1 2 3 4 5 6 7
ChIPs: wt wing discs,
Ubx promoter
C
Input(%)
10
1
0.1
mock
α-E(Z)
α-ESC
α-ESCL
b3
b4
b5
b6
Rp
1 2 3 4 5 6 7
ChIPs: wt wing discs,
Ubx PRE
D
Input(%)
10
1
0.1
mock
α-E(Z)
α-ESC
α-ESCL
p2
b4
b5
Rp
1 2 3 4 5 6 7
ChIPs: esc mutant
wing discs,
Ubx promoter and PRE

Figure 6: Analysis of PcG functions in wing disc-derived MCW12 cells. A) RNA interference assay using MCW12 cells. RT-PCR analysis of *Ubx* and *RpII140* expression in MCW12 cells transfected with *Pc* or *GFP* dsRNAs. Panels (B) and (C) show ChIP analysis of the distributions of E(Z) and PC on the *Ubx* promoter (B) and *Ubx* PRE (C) in MCW12 cells. Fragments are shown in Fig. 5A. "Rp" indicates control fragment from the *RpII140* promoter, "mock" indicates control IP with crude rabbit preimmune antiserum, and "Gen" indicates input control PCR reactions on genomic DNA. D) Co-immuneprecipitations from MCW12 cell extracts showing association of E(Z) with ESC or ESCL. Proteins were immuneprecipitated with anti-ESC or anti-ESCL antibodies or Protein A alone, as indicated above the panels, and Western blots were probed with anti-E(Z) antibodies. Input lanes contain crude extract equivalent to 10% or 20% of the extract, respectively, used in anti-ESC (left panel) or anti-ESCL (right panel) immuneprecipitations. E) RNA interference assay using MCW12 cells. RT-PCR analysis of *Ubx* and *RpII140* expression in MCW12 cells transfected with *esc* dsRNA (left panel), *escl* dsRNA (middle panel) or a mixture of both *esc* and *escl* dsRNAs (right panel). In (A) and (E), "+RT" indicates reverse transcriptase added and "-RT" indicates reverse transcriptase omitted from reverse transcription reaction.

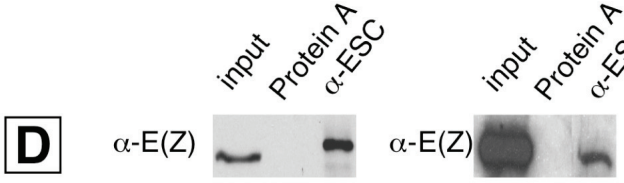
Figure 6



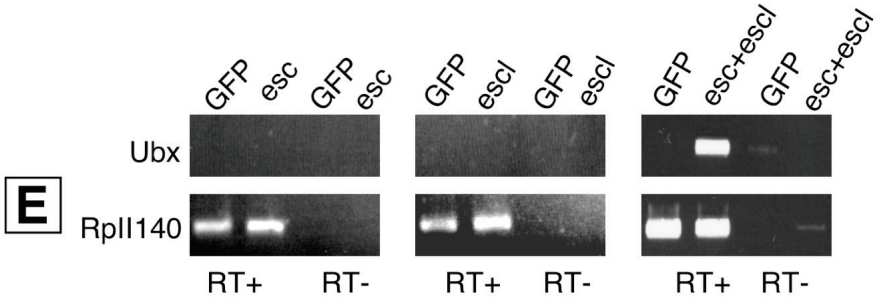
RNAi: Pc in MCW12 cells



ChIPs: MCW12 cells



Co-IPs: MCW12 cells



RNAi: esc, escl in MCW12 cells

(Fig. 6D) and analysis of a recombinant ESCL-E(Z) complex (see below), this close tracking likely reflects a complex containing both ESCL and E(Z).

In addition, we performed ChIP assays to determine if ESC is also present in *Hox* gene chromatin from wing discs. Somewhat surprisingly, despite its diminished expression levels in larvae, ESC is readily detected on the same chromatin fragments as E(Z) and ESCL (Fig. 5B, C). When these ChIP experiments were repeated using wing discs from *esc* null mutant larvae, ESC is no longer detected but E(Z) and ESCL remain associated with the *Ubx* promoter and PRE (Fig. 5D). Thus, E(Z) and ESCL can bind to target sites in wing disc chromatin in the absence of ESC. These results are consistent with E(Z) complexes in this tissue containing either ESC or ESCL, or possibly a combination of both, reinforcing the possibility that these proteins have overlapping functions in larvae.

Requirement for ESCL and ESC in *Ubx* repression in post-embryonic cells

The respective contributions of ESCL and ESC to *Ubx* repression in wing imaginal discs was tested using a cell line, MCW12, that was derived from wing imaginal discs (D. Cottam and M. Milner, unpublished). These cells have retained wing imaginal disc-like patterns of *Hox* gene expression; RT-PCR analysis indicated expression of *Antp*, but lack of *Ubx*, *abdA*, or *AbdB* mRNA in control MCW12 cells (Fig. 6A and data not shown). However, RNAi-mediated knock-down of PC resulted in dramatic *Ubx* derepression (Fig. 6A), reminiscent of *Ubx* derepression observed in wing disc somatic clones of PcG mutant alleles *in vivo* (BEUCHLE *et al.* 2001). In addition, ChIP assays

revealed the same distributions of PC and E(Z) at the *Ubx* PRE and promoter regions in MCW12 cells as in wing imaginal discs (Fig. 6B, C; CAO *et al.* 2002; WANG *et al.* 2004b). Thus, we conclude that MCW12 cells have retained PcG-mediated silencing of *Ubx*, as in wing imaginal discs *in vivo*.

Co-immunoprecipitations demonstrated that E(Z) is physically associated with both ESC and ESCL in MCW12 cell extracts (Fig. 6D). To determine whether ESC and/or ESCL are functionally required for PcG-mediated silencing of *Ubx*, we performed RNAi-mediated knock-down experiments. MCW12 cells were transfected with *esc* dsRNA, *escl* dsRNA, or an equal mixture of both dsRNAs and the effects upon *Ubx* expression were monitored by RT-PCR (Fig. 6E). Knock-down of either ESC or ESCL alone had no effect on levels of *Ubx* RNA. However, simultaneous knock-down of both ESC and ESCL resulted in *Ubx* derepression (Fig. 6E, right panel) similar to that observed following PC knock-down (Fig. 6A). These results indicate that ESC and ESCL are both functionally required for *Ubx* repression in this wing disc-derived cell line. Taken together with the coincident distributions of E(Z), ESC and ESCL in *Ubx* wing disc chromatin (Fig. 5B, C), these results provide further evidence that *in vivo* functions of ESC and ESCL overlap, at least in this imaginal tissue.

Histone methyltransferase activity of complexes containing ESCL in place of ESC

A recombinant fly ESC-E(Z) complex containing ESC, E(Z), SU(Z)12 and NURF-55 has robust HMTase activity that methylates K27 of histone H3 (MULLER *et al.* 2002). However, if the ESC subunit is singly removed, or impaired by mutation, then the

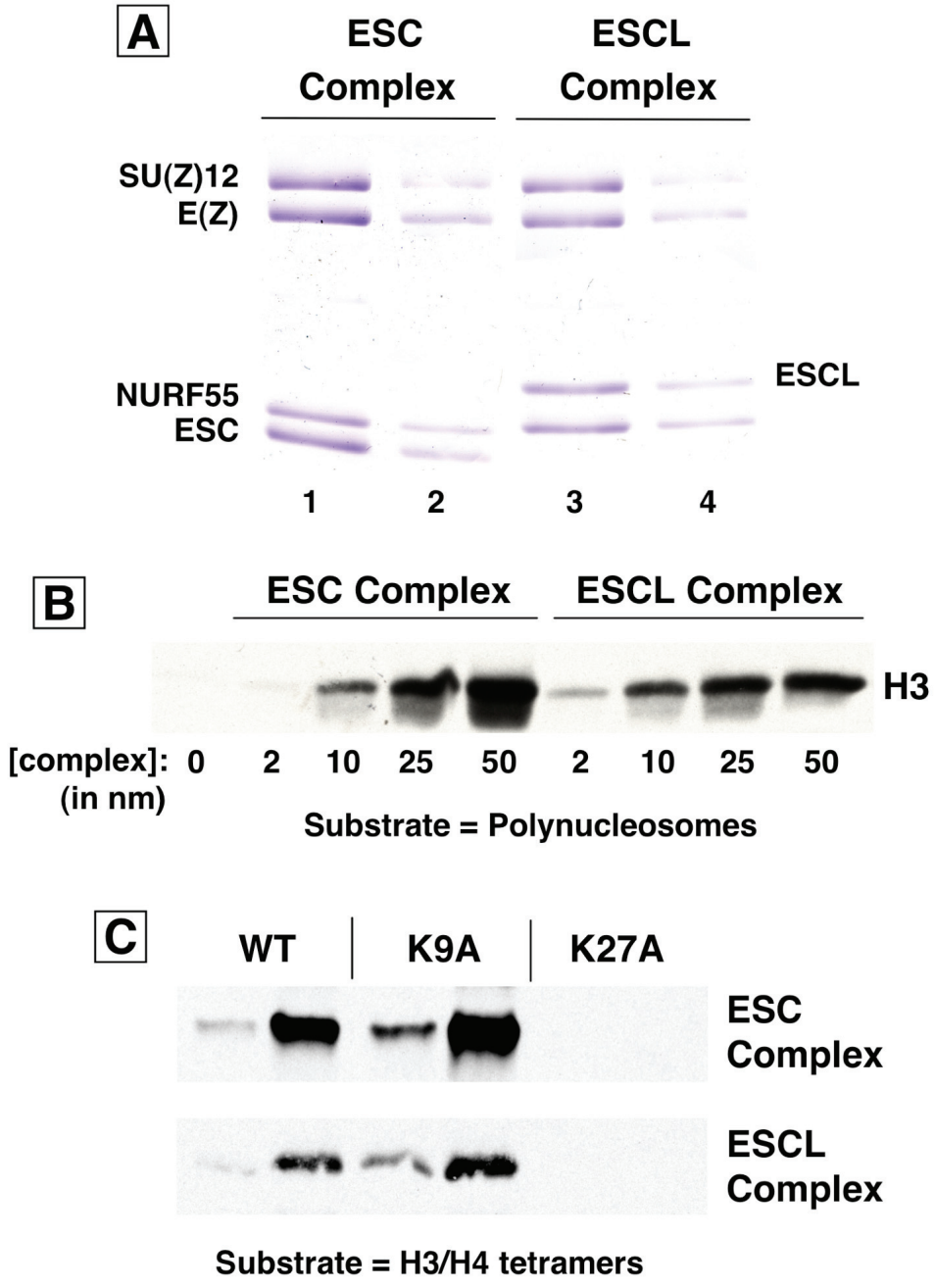
resulting mutant complexes have dramatically reduced HMTase (KETEL *et al.* 2005; NEKRASOV *et al.* 2005). In agreement with this, genetic disruption of ESC yields fly embryos with little or no detectable H3-K27 methylation (KETEL *et al.* 2005). Thus, although its biochemical mechanism is not yet clear, ESC plays a vital role in potentiating the HMTase activity of the ESC-E(Z) complex.

We reasoned that if ESCL functions molecularly in a manner similar to ESC, then it might also assemble stably into a complex with E(Z) and make key contributions to HMTase activity. To test these possibilities, we used a baculovirus system to co-express ESCL along with E(Z), SU(Z)12 and NURF-55 and determined if these four components could be purified together as a stable complex. Purification was achieved using anti-FLAG immunoaffinity using a FLAG tag placed at the extreme N-terminus of ESCL. Figure 7A shows that a four-subunit ESCL-E(Z) complex is obtained (lanes 3 and 4). This ESCL-E(Z) complex appears identical in subunit composition to an ESC-E(Z) complex purified in parallel (lanes 1 and 2) except for the replacement of ESC with ESCL.

The HMTase levels of these ESC-E(Z) and ESCL-E(Z) complexes were then compared using a polynucleosome substrate. Figure 7B demonstrates that the two recombinant complexes display similar levels of H3 methylation activity. Since single loss of ESC from its complex leads to at least a 25-50 fold reduction in HMTase *in vitro* (KETEL *et al.* 2005; NEKRASOV *et al.* 2005), this result indicates that ESCL can potentiate the enzyme activity in a manner similar to ESC.

Figure 7: Histone methyltransferase activities of recombinant E(Z) complexes containing either ESC or ESCL. A) Subunit compositions of complexes purified after co-expression of E(Z), SU(Z)12 and NURF-55 with either ESC (leftmost lanes) or ESCL (rightmost lanes). Complexes were purified using FLAG tags on either ESC or ESCL. The second lane of each pair shows 40% as much material loaded. B) Comparison of HMTase activities of four-subunit ESC-E(Z) and ESCL-E(Z) complexes. Numbers indicate concentrations of complexes (in nM) in HMTase reactions using 1 ug HeLa polynucleosomes as substrate. C) Comparison of lysine specificities of four-subunit ESC-E(Z) and ESCL-E(Z) complexes. HMTase reactions were performed using each complex at a concentration of 125 nM and H3/H4 tetramers containing either wild-type H3 or the indicated H3 mutant. Pairs of lanes show reactions using either 50 ng or 200 ng of the indicated histone substrate.

Figure 7


A
ESC Complex
ESCL Complex
SU(Z)12
E(Z)
NURF55
ESC
ESCL
1
2
3
4
B
ESC Complex
ESCL Complex
H3
[complex]: (in nm)
0
2
10
25
50
2
10
25
50
Substrate = Polynucleosomes
C
WT
K9A
K27A
ESC Complex
ESCL Complex
Substrate = H3/H4 tetramers

Edman degradation analysis of nucleosomes methylated *in vitro*, and studies using *E(z)* and *esc* mutants *in vivo*, indicate that the primary specificity of the fly ESC-E(Z) HMTase is for K27 of histone H3 (CAO *et al.* 2002; KETEL *et al.* 2005; MULLER *et al.* 2002). In addition, methylation of histone substrates bearing lysine substitutions confirmed that the recombinant four-subunit ESC-E(Z) complex displays this K27 specificity (MULLER *et al.* 2002). We used this latter assay here to compare the lysine specificity of the ESCL-E(Z) complex to the ESC-E(Z) complex (Fig. 7C). Histone H3/H4 tetramers containing wild-type H3, or mutant forms of H3 bearing substitutions at K9 or K27, were used as substrate in HMTase assays. Figure 7C shows that the ESCL-E(Z) complex displays a similar marked preference for methylation of K27. Taken together, these *in vitro* assays show that ESCL can functionally substitute for ESC in the four-subunit HMTase complex.

IV. Discussion

ESC and E(Z), and their homologs, are functional partners in the chromatin of plants, invertebrates, and mammals. Working together, they control a diverse array of developmental processes including flower and seed differentiation in *Arabidopsis* (GOODRICH *et al*. 1997; SPILLANE *et al*. 2000; YOSHIDA *et al*. 2001), germline development in *C. elegans* (HOLDEMAN *et al*. 1998; KORF *et al*. 1998), X chromosome inactivation in mice (PLATH *et al*. 2003; SILVA *et al*. 2003; WANG *et al*. 2001a), and *Hox* gene repression in flies and mammals (JONES and GELBART 1990; SCHUMACHER *et al*. 1996; SIMON *et al*. 1992; STRUHL and AKAM 1985). Recent studies show that this

partnership reflects a requirement for ESC in potentiating histone methyltransferase (HMTase) activity of E(Z) (KETEL *et al*. 2005; MONTGOMERY *et al*. 2005; NEKRASOV *et al*. 2005). In light of this functional interdependence, a paradox is presented by developmental studies in *Drosophila*, which show that ESC is primarily needed during early embryogenesis, whereas E(Z) is required throughout embryonic, larval and pupal development. Our analysis of ESCL, which can replace ESC in E(Z) HMTase complexes *in vitro*, provides a plausible solution to this puzzle. ESCL expression is largely complementary to that of ESC, peaking during later developmental stages, and our functional studies show that ESCL is partially redundant with ESC in imaginal tissues. These results, together with prior genetic data that address *esc* time of action (see below), indicate that ESC predominates in embryos whereas both ESCL and ESC make functional contributions during post-embryonic development.

Developmental times of ESC and ESCL function in PcG repression

Phentoypic analyses of *esc* loss-of-function mutants provided the original evidence that the primary time of ESC action is during embryogenesis. Although complete loss of esc^{+} product is embryonic lethal and yields wholesale mis-expression of *Hox* genes (SIMON *et al.* 1992; STRUHL and AKAM 1985), it was shown that maternally provided esc^{+} product provides sufficient function during embryogenesis to enable zygotically null esc^{-} animals to survive to adulthood (STRUHL 1981; STRUHL 1983). These esc^{-} adults are fertile, healthy, and phenotypically normal except for minor homeotic transformations such as extra sex combs on the meso- and metathoracic legs. In contrast, animals that are zygotically null for any other PcG subunit of the ESC-E(Z)

complex or PRC1 fail to survive beyond early pupal stages with most dying by the embryonic/L1 stage. Additional experiments with a conditional *esc* allele further delimited the main time of ESC function to a period of mid-embryogenesis extending from about the onset of gastrulation (about 3 hours at 25°C) until germ-band shortening (approximately 9-12 hours STRUHL and BROWER 1982). An independent study that measured phenotypic rescue by a heat-inducible esc^{+} transgene confirmed that the time of ESC action begins at about 3 hours of embryogenesis (SIMON *et al.* 1995). These genetically determined times of esc^{+} function coincide with the accumulation of ESC protein, which peaks during mid-embryogenesis and declines by the end of embryogenesis (FURUYAMA *et al.* 2003; GUTJAHR *et al.* 1995; NG *et al.* 2000).

However, full consideration of the genetic evidence also indicates that ESC does contribute to post-embryonic PcG repression, particularly in imaginal tissues. Analysis of esc^{-} larvae showed modest defects in *Hox* gene repression in imaginal discs as well as in the central nervous system (GLICKSMAN and BROWER 1988). In particular, this study attributed the extra sex combs phenotype of esc^{-} larvae to mis-expression of the *Scr Hox* gene in the T2 and T3 leg discs. In addition, production of extra sex combs from patches of esc^{-} tissue generated by somatic recombination during larval development indicates that the time of ESC action extends into the larval period, at least in leg discs (TOKUNAGA and STERN 1965). A post-embryonic role is consistent with the detection of ESC on the *Ubx* gene in wing discs (Fig. 5B and C) and with the overlapping roles of ESC and ESCL in *Ubx* repression in disc-derived MCW12 cells (Fig. 6E). This latter result might explain why esc^{-} wing discs did not produce

homeotic phenotypes even after sufficient passage to ensure depletion of maternal *esc*$^{+}$ product (STRUHL and BROWER 1982); presumably, both ESC and ESCL would need to be disrupted in this tissue to yield robust *Hox* mis-expression. Finally, although it is much less abundant at late developmental times as compared to in embryos, ESC is detected by Western blot in larval and pupal extracts (NG *et al.* 2000). Thus, the sum of the genetic and molecular data indicate that ESC does function during post-embryonic stages, albeit with a more modest overall contribution as compared to its critical role in embryos. These considerations imply that the developmental division of labor between ESC and ESCL is not simply that ESC functions only in embryos and ESCL takes over for subsequent stages. Rather, although ESC does predominate early, as evidenced by the global loss of H3-K27 methylation in *esc*$^{-}$ embryos (KETEL *et al.* 2005), post-embryonic development appears to involve both ESC and ESCL. We note that Struhl and Brower originally hypothesized that late developmental functions of the *esc* locus might be executed by an *esc*$^{+}$ isoform distinct from the embryonic version (STRUHL and BROWER 1982). Our data confirm that multiple ESC-related proteins do operate during fly development, with a late-acting version supplied by a second copy of the *esc* gene.

The functional context for ESCL during post-embryonic development is presumably as a subunit in E(Z)-containing complexes with histone methyltransferase (HMTase) activity. The fact that ESCL can assemble in place of ESC and restore HMTase activity to a reconstituted E(Z) complex (Fig. 7) indicates that the biochemical roles of ESCL and ESC are similar. ESCL/ESC functional overlap could reflect a mixture of post-embryonic E(Z) complexes, with some containing ESCL and others containing ESC.

The simplest version of this scenario would entail four-subunit post-embryonic HMTase complexes similar to the embryonic core complex (E(Z), SU(Z)12, NURF-55 and ESCL or ESC). However, post-embryonic E(Z) complexes have yet to be purified so their molecular compositions are not yet known. In fact, there is evidence that larval E(Z) complexes may differ from embryonic E(Z) complexes in features besides the ESCL/ESC subunit (FURUYAMA *et al.* 2004; FURUYAMA *et al.* 2003). For example, the SIR2 histone deacetylase has been reported to associate with larval but not embryonic E(Z) complexes (FURUYAMA *et al.* 2004). Much remains to be determined about post-embryonic E(Z) complexes, including subunit compositions and characterization of presumed HMTase activity.

Contributions of the ESC/ESCL subunit to histone methyltransferase complexes

Although the catalytic subunit, E(Z), contains the conserved SET domain, studies on fly, worm and mammalian homologs reveal that the ESC subunit is also critical for HMTase function. The single loss of ESC from the fly complex or loss of its homolog, MES-6, from the *C. elegans* complex yields subcomplexes with little or no HMTase activity *in vitro* (KETEL *et al.* 2005; NEKRASOV *et al.* 2005). In agreement with this, genetic removal of ESC eliminates most or all methyl-H3-K27 in fly embryos (KETEL *et al.* 2005), loss of MES-6 eliminates most or all methyl-H3-K27 in worm germlines and early embryos (BENDER *et al.* 2004), and loss of EED removes most or all methyl-H3-K27 from embryonic mouse cells (MONTGOMERY *et al.* 2005; SILVA *et al.* 2003). The mechanism by which ESC and its relatives potentiate the activity of HMTase complexes is not known. An *in vitro* study argues against a role for fly ESC in

mediating stable contacts with nucleosome substrate (NEKRASOV *et al.* 2005). On the other hand, loss of ESC by RNA interference in fly S2 cells leads to dissociation of E(Z) from chromatin targets (CAO *et al.* 2002).

A biochemical analysis of the human EED-EZH2 complex (also called PRC2) has revealed an intriguing difference in the HMTase depending upon the subtype of EED subunit present in the complex (KUZMICHEV *et al.* 2004). Multiple isoforms of EED are expressed in HeLa cells which differ in the extents of their N-terminal tails through use of alternative translation start sites. Incorporation of particular EED isoforms into EZH2 complexes can shift the enzyme specificity so that K26 of histone H1 is methylated in addition to H3-K27 (KUZMICHEV *et al.* 2004). Taken together with other studies, this suggests that EED is a regulatory subunit that can influence both substrate specificity and catalytic efficiency of the HMTase. In light of this finding, it seems possible that ESCL-E(Z) complexes might also have HMTase activity with altered lysine specificity. However, both ESCL-E(Z) and ESC-E(Z) recombinant complexes showed similar specificity for H3-K27 in H3/H4 tetramers (Fig. 7C) and we were unable to detect methylation of mammalian histone H1 by either of these recombinant complexes *in vitro* (data not shown). We note that the human H1-K26 methylation site is embedded in an AR<u>K</u>S sequence, which is also present surrounding H3-K27. This sequence is not conserved in *Drosophila* histone H1, suggesting that the ability of certain EZH2 complexes to methylate H1 may not be conserved in the fly system. However, there may well be other relevant methylation substrates besides histone H3

and it remains possible that alternative ESC isoforms could alter lysine specificities for these other substrates.

Functional relationship between ESC and ESCL proteins

Based upon their temporal expression profiles, it seems clear that *esc* and *escl* have distinct functions in a developmental context. Their temporal division of labor is most clearly demonstrated by *esc*$^{-}$ *escl*$^{+}$ embryos which show extreme homeotic transformations (STRUHL 1981) accompanied by dramatically reduced levels of methylated H3-K27 (KETEL *et al.* 2005). This division could be entirely a consequence of differential transcriptional controls built into their divergent promoters. That is, ESC and ESCL could be functionally identical proteins that are just expressed at peak levels at different times. Alternatively, the two proteins may possess intrinsic differences that are also important during development but which are not revealed by the assays we have applied so far (i.e. Fig. 7). One possibility, as mentioned above, is that ESC and/or ESCL may play a role in methylation of nonhistone proteins. The only nonhistone proteins yet identified that fly E(Z) complexes can methylate are two subunits of the core complex itself: E(Z) and SU(Z)12 (MULLER *et al.* 2002). It is not clear if this self-methylation is functionally relevant and, in any case, it occurs at comparable levels with the ESC- and ESCL-containing recombinant complexes (data not shown).

It is also possible that ESC and ESCL could differ in contributions to E(Z) complexes besides HMTase activity. These other functions could include interacting with and recruiting histone deacetylases (TIE *et al.* 2001; VAN DER VLAG and OTTE 1999),

mediating physical interactions with PRC1 components, recruiting E(Z) complexes to target loci, and/or influencing the way E(Z) complexes interact with other (non-K27) histone tail modifications. Indeed, there is evidence for differential association of HDACs with E(Z) complexes at embryonic versus larval stages (FURUYAMA *et al.* 2004), which parallels temporal changes in ESC and ESCL abundance. At the same time, ESC and ESCL functions must overlap enough to account for the sufficiency of either one to maintain *Ubx* repression in at least some post-embryonic cells (Fig. 6E). We envision that definitive answers will require promoter swap experiments where ESCL is placed under control of the ESC promoter, and vice versa, to determine which combinations provide genetic rescue of *esc* and *escl* mutations *in vivo*. Along with this approach, a complete understanding of the developmental role of ESCL will require generation of *escl* mutant alleles and systematic analysis of the phenotypic consequences of *escl* loss-of-function.

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Chapter Three

Tethering the PRC2 Histone Methyltransferase Complex to Ectopic Loci in *Drosophila*

The work presented in this chapter was performed in collaboration with Diane Cryderman, a member of Lori Wallrath's Laboratory at the University of Iowa, who performed the germline transformations to produce the transgenic ESC fusion expressor stocks and partially contributed to their characterization.

I. Introduction

The *in vitro* binding of the chromodomain of the Polycomb (PC) subunit of Polycomb Repressive Complex 1 (PRC1) to trimethyl-H3-K27 (FISCHLE *et al.* 2003c; MIN *et al.* 2003) has led to the suggestion that this binding activity is the primary mechanism for the recruitment of PRC1 to target sites. However, there are several other factors present at the PRE, including the PRC2 complex itself, and any of them might also play a role in the recruitment of PRC1 to target loci. Additionally, trimethyl-H3-K27 is often broadly and fairly evenly distributed along a silenced gene, but PRC1 is more concentrated at peaks which are not reflected in the trimethyl-H3-K27 distribution (KAHN *et al.* 2006; SCHWARTZ *et al.* 2006). This suggests that additional mechanisms are involved in determining PRC1 distribution.

How the broad distribution of H3-K27 methylation is itself generated is another unresolved question about the Polycomb system, since PRC2 is enriched at discrete locations in the PRE and promoter of a target gene. Presumably, some mechanism brings PRC2 into contact with distant regions of the silenced gene.

To help resolve these questions, it would be desirable to create a system where H3-K27 methylation could be applied to a locus independent of a PRE, and then to determine which other factors are subsequently recruited and whether H3-K27 methylation spreads away from the target site. Such a system would target the H3-K27 HMTase to an ectopic site that is not usually subject to Polycomb repression. More than the E(Z) catalytic core of the PRC2 HMTase would need to be repositioned, however, because E(Z) is catalytically inactive unless it is physically associated with the other subunits of PRC2. Thus, creating ectopic sites of H3-K27 methylation requires a system that will target the entire PRC2 core complex to chromatin sites.

To attempt production of ectopic H3-K27 methylation *in vivo*, we sought to use a tethering system developed by the Andrew Belmont group at the University of Illinois at Urbana-Champaign and further extended by our collaborators in Lori Wallrath's group at the University of Iowa. The Belmont group inserted arrays of lac operator (lacO) sequence from *E coli* into cultured mammalian cells and yeast, and found that they could track the positions of those insertions by imaging an engineered protein consisting of green fluorescent protein (GFP) fused to the DNA-binding domain (DBD) of the *E coli* Lac repressor (LacI; ROBINETT *et al.* 1996). Subsequently, the Wallrath

group adapted this system to study Heterochromatin Protein 1 (HP1) in *Drosophila* (DANZER and WALLRATH 2004; LI *et al.* 2003). Part of this adaptation involved designing reporter constructs containing lacO arrays so that they could be used to monitor local changes to chromatin state caused by LacI fusion protein binding. By inserting a reporter construct consisting of the lacO array adjacent to a reporter gene or genes, and fusing a chromatin-modifying protein to the LacI-DBD, one could determine the effect on nearby genes when the experimental protein is bound to chromatin. This system is isolated from confounding effects due to endogenous proteins because *Drosophila* proteins are unable to recognize the *E coli* lacO sequence.

The reporter constructs generated by the Wallrath lab come in two varieties. An early version contains the mini-*white* gene in association with the lacO array, and gene silencing can be assessed by examining eye color (LI *et al.* 2003). A more sophisticated reporter construct places the lacO array upstream of two genes, a heterologous plant-derived sequence and the *white* gene, both under the control of heat shock promoters (DANZER and WALLRATH 2004). The heterologous sequence is a partial cDNA from the barley *sip1* gene, and so its expression produces a unique mRNA sequence that is not normally produced in *Drosophila*. Heat shock promoters were chosen to drive these genes because they are well-characterized and are inducible in all tissues. The elements of the tethering system are combined by crossing an independently-created LacI expressor stock with a reporter stock, so the LacI fusion protein can be controlled by any chosen promoter.

In this system, a LacI-GFP fusion protein can serve as a control to demonstrate that regulatory effects are not caused by the LacI-DBD on its own. However, the LacI-DBD is a sizable domain, so researchers need to verify that the fusion constructs they create retain the biological activity they are interested in examining.

Previous studies have described silencing of reporter genes when individual PcG components are tethered to particular genomic sites by a variety of methods (BUNKER and KINGSTON 1994; MULLER 1995; ROSEMAN *et al.* 2001; VAN DER VLAG *et al.* 2000). In a study that fused PcG proteins to a zinc-finger DNA-binding domain from the Suppressor-of-Hairy-Wing (SU(HW)) protein (ROSEMAN *et al.* 2001), PC and SCM were found to be effective repressors when tethered near reporter genes, and ESC was found to be partially effective. Although this study also produced evidence that recruitment of additional PcG components was required for effective Polycomb repression, its conclusions were limited by rudimentary knowledge of PcG mechanisms at the time, particularly because it was not yet known that ESC was part of an HMTase complex. Additionally, the use of a DBD from an endogenous *Drosophila* protein meant that the tethering system could be compromised by confounding endogenous factors. In particular, the requirement that the endogenous SU(HW) protein be removed by mutation (ROSEMAN *et al.* 2001) contributed to a genetically complicated tethering system.

The Wallrath group developed the LacI tethering system to investigate mechanisms of silencing by HP1 (DANZER and WALLRATH 2004; LI *et al.* 2003). The first study (LI *et*

al. 2003) showed that the LacI-DBD could target HP1 to ectopic sites on *Drosophila* chromosomes where, in most cases, it could silence a nearby reporter gene. It appeared that the potency of the silencing effect was influenced by the local chromatin environment into which the reporter construct had inserted. They also concluded that tethered HP1 could silence the reporter gene without recruiting additional methylation by the SU(VAR)3-9 HMTase. The subsequent study (DANZER and WALLRATH 2004) showed that HP1 targeting could silence reporters driven by heat shock promoters within euchromatic regions and that the target site served as a nucleation point that could initiate the spread of HP1 to the reporter genes. Assays measuring the accessibility of DNA to restriction enzymes and micrococcal nuclease demonstrated that chromatin structure at the reporter genes was influenced by the addition of tethered HP1. Additionally, they found that while SU(VAR)3-9 was not required for silencing reporters in the immediate vicinity of the target site, it was required for silencing more distant reporters.

The LacI-DBD tethering system has also been used in *Drosophila* to study the effects of histone H3 serine-10 (H3-S10) phosphorylation. H3-S10 phosphorylation has been established as a negative regulator of HP1 binding and heterochromatin formation (DENG *et al.* 2008; FISCHLE *et al.* 2005; HIROTA *et al.* 2005; ZHANG *et al.* 2006) and is also associated with transcriptional activation (LABRADOR and CORCES 2003; NOWAK and CORCES 2000; NOWAK *et al.* 2003). In this case, the JIL-1 H3-S10 kinase (WANG *et al.* 2001b) was fused to the LacI-DBD and expressed under ubiquitous GAL4-UAS control to discern the effects of ectopic H3-S10 phosphorylation on chromatin structure

(DENG *et al.* 2008). These experiments produce H3-S10 phosphorylation at target sites on *Drosophila* polytene chromosomes, demonstrating that the LacI-DBD tethering system is capable of producing ectopic histone modifications in conjunction with an appropriate enzyme. Ectopic H3-S10 phosphorylation caused chromatin decondensation in both band and interband regions of *Drosophila* polytene chromatin, but was not associated with increased transcriptional activity as measured by immunodetection of the elongating form of RNA polymerase II (DENG *et al.* 2008). Strikingly, when reporter chromosomes were hemizygous for the reporter construct, decondensation could be observed in the chromatid that contained the targeting site, but not in its sister.

In light of these successful applications of LacI tethering in *Drosophila*, we wished to apply their methodology to study the PRC2 HMTase. Specifically, we wished to fuse the LacI-DBD to a PRC2 subunit to generate and study ectopic sites of PRC2 localization and H3-K27 methylation.

II. Materials and Methods

Plasmids. Tailed PCR was used to create a plasmid construct, called pFB-FLAG-ESC-trimmed, consisting of a full-length *esc* cDNA with an N-terminal FLAG tag inserted into the baculovirus expression vector pFastBac1 (Invitrogen). The FLAG-ESC insert was flanked on the 5' side by side by Not I and Nsi I sites and on the 3' side by Nhe I

and Xba I sites. To create a LacI-ESC fusion, a 1.1kb Not I-Nsi I fragment encoding the LacI-DBD was PCR-amplified and inserted into pFB-FLAG-ESC-trimmed just 5' to the FLAG tag (see Figure 1). The resulting LacI-ESC fusion gene was then excised as a Not I-Xba I fragment and inserted into the pCaSpeR-hs-act *Drosophila* germline transformation vector (THUMMEL and PIRROTTA 1992; map available at http://thummel.genetics.utah.edu/thummel_OLD_SITE/vectormaps/pcasper-hs-act.html) for expression in *Drosophila* (see below). To create the alternative ESC-LacI fusion, a 1.1kb Nhe I-Xba I fragment encoding the LacI-DBD was inserted into pFB-FLAG-ESC-trimmed at the 3' end of the ESC ORF. The resulting ESC-LacI fusion gene was excised as a Not I-Xba I fragment and inserted into pCaSpeR-hs-act.

***Drosophila* Stocks.** Stocks expressing the ESC-LacI and LacI-ESC fusion proteins were produced in the Wallrath lab using standard transformation procedures (RUBIN and SPRADLING 1982). The LacI-HP1 expressor stock and the 179.1 and 157.4.112 reporter stocks are described in (LI *et al.* 2003). The GFP-LacI expressor stock and the 4D5 and 87C reporter stocks are described in (DANZER and WALLRATH 2004).

Antibodies. The antibodies used and their sources were: Mouse monoclonal α-FLAG M5 (Sigma); Mouse monoclonal α-LacI (Upstate); Rabbit polyclonal α-trimethyl-H3-K27 is described in (PETERS *et al.* 2003) and obtained from Upstate; Rabbit polyclonal α-SU(Z)12, described in (MULLER *et al.* 2002), was raised against SU(Z)12 amino acids 448-802 and is referred to as the "SAC" antibody.

Recombinant Protein Complexes. Protein expression was performed using the Bac-to-Bac system (Invitrogen) using procedures described in (KETEL *et al.* 2005). Concentration of eluted protein complexes was measured by Bradford dye-binding assay (Bio-Rad) according to manufacturers instructions. HMTase assays were performed as described (MULLER *et al.* 2002), using polynucleosome arrays from HeLa nuclei prepared as described (KETEL *et al.* 2005).

Western Blots. Adult flies were heat shocked as described (ROSEMAN *et al.* 2001). Protein extracts were prepared according to (BORNEMANN *et al.* 1998) except that the 2X SDS sample buffer contained both 1mM PMSF and 1μg/ml leupeptin. Protein loading was balanced by prior Coomassie staining of protein samples after resolving on SDS-PAGE. For blotting, protein samples were resolved by SDS-PAGE on 10% gels, and transferred to Protran (Whatman) membranes. Membranes were blocked in PBS with 5% nonfat dry milk for one hour before incubating in primary antibody overnight at 4°C. For Western blots on recombinant complexes, α-FLAG was diluted to 1:5000 and the secondary antibody, goat-α-mouse conjugated to horseradish peroxidase (HRP), was diluted 1:10,000. For Western blots on *Drosophila* protein extracts, α-LacI was diluted to 1:500 and the secondary antibody, goat-α-mouse conjugated to HRP, was diluted 1:3000. Blots were incubated in secondary antibody for one hour at room temperature. Signals were developed with an ECL chemiluminescence detection kit (Amersham).

Rescue of Lethality. Stocks, crosses and procedures for the rescue of *esc* null *Drosophila* embryos by a heat-shock inducible transgene were as described (SIMON *et al.* 1995). Embryos were collected on agar plates for two hours, aged at 25°C for two hours, heat shocked at 36°C for two hours and then incubated at 25°C. To evaluate rescue, embryos were mechanically dechorionated approximately 24 hours after deposition, and cuticle patterns were scored for differentiation of head, thoracic, and abdominal segments.

Polytene Chromosome Immunostaining. Adult flies were allowed to seed vials with embryos for two days. After removing the adults, vials were heat shocked daily at 36°C throughout development until larvae were harvested for chromosome squashes. HP1-LacI and ESC-LacI larvae shown in Figure 3 were heat shocked once daily for 45 minutes. LacI-ESC larvae shown in Figures 4 through 6 were heat shocked twice daily for one hour. Preparation and staining of larval polytene chromosomes generally followed the method described in (PLATERO *et al.* 1995). Salivary glands were dissected from crawling third-instar larvae in buffer (COHEN and GOTCHEL 1971) containing 10mM $MgCl_2$, 25mM disodium glycero-phosphate, 3mM $CaCl_2$, 10mM KH_2PO_4, 0.5% NP40, 30mM KCl, and 160mM Sucrose. Within 8 minutes glands were transferred to fix buffer containing 0.1M NaCl, 2mM KCl, 10mM sodium phosphate pH 7.0, 2% Triton-X 100, and 2% formaldehyde. Glands were incubated in fix for two minutes followed by two minutes of incubation in squash buffer containing 45% glacial acetic acid and 2% formaldehyde. Glands were then squashed using siliconized cover slips (Hampton Research) and flash frozen in liquid nitrogen. After removal of the

cover slip, slides were immersed in TBST (10mM Tris-HCl pH 8.0, 0.15M NaCl, 0.05% Tween 20) before thawing. If slides were not to be immunostained immediately, they were stored at -20°C in a solution containing two parts glycerol to one part TBS. Stored slides were washed three times for five minutes each in TBST before proceeding to blocking. Slides were blocked for one hour in TBST with 10% normal goat serum (Vector Laboratories). Primary antibodies were diluted in 100µl of blocking buffer per slide. Incubation proceeded under a cover slip in a humid chamber at 4°C overnight. α-LacI was used at 1:200, α-trimethyl-H3-K27 was used at 1:100, α-SU(Z)12 was used at 1:25, and α-PH was used at 1:50. After incubation, slides were washed three times for 5 minutes each in TBST. Secondary antibodies were goat-α-mouse conjugated to Alexa568 and goat-α-rabbit conjugated to Alexa488 (Invitrogen). Both secondary antibodies were diluted 1:2000 in 100µl blocking buffer per slide and, incubation proceeded under cover slips for one hour at room temperature in a humid chamber. After three more washes of 5 minutes in TBST, slides were mounted in Vectashield (Vector Laboratories) containing 1µg/ml DAPI. Digital pictures were taken with a Leica DMRB microscope and AxioCam MRc camera with MRGrab software (Zeiss).

III. Results

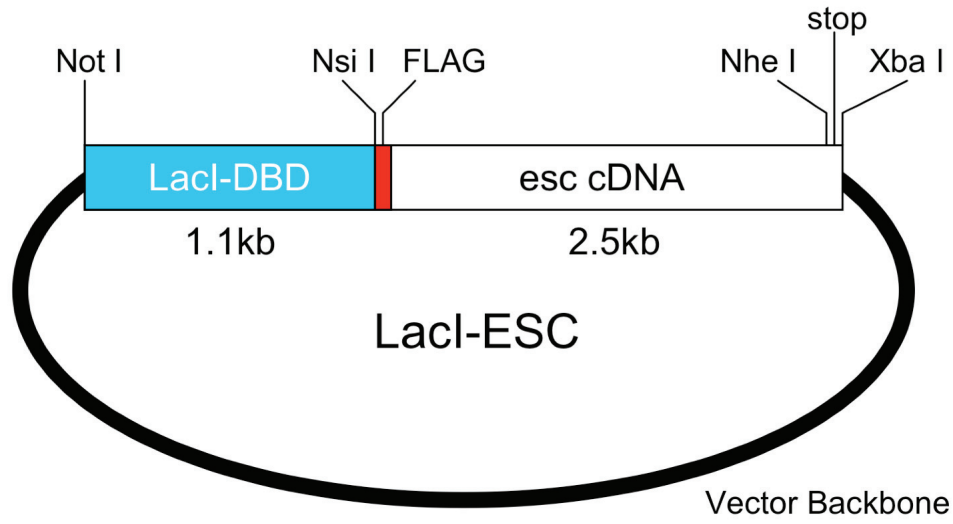
Fusion Proteins ESC-LacI and LacI-ESC can Assemble into Catalytically Active Complexes *in Vitro*.

Because the PRC2 catalytic subunit E(Z) is essentially inactive by itself (KETEL *et al.* 2005), it is necessary to tether the entire PRC2 complex to chromatin in order to produce H3-K27 methylation at target sites. The ESC subunit was chosen for fusion to the LacI-DBD because rescuing ESC protein fusions but not E(Z) protein fusions have been described previously in *Drosophila* (MULLER *et al.* 2002; NG *et al.* 2000). We created two fusion constructs by ligating the sequence for the LacI-DBD in-frame to either the N-terminal or C-terminal ends of the *esc* cDNA (Figure 1, see Materials and Methods). These constructs were created in the pFastBac1 vector plasmid for expression in a baculovirus system. Both constructs contain FLAG tags for affinity purification. We refer to the construct where LacI-DBD is fused to the N-Terminal of ESC as "LacI-ESC," and the construct that fuses the LacI-DBD to the C-terminal of ESC as "ESC-LacI."

Each construct was separately co-expressed with wild-type versions of the other PRC2 core components E(Z), SU(Z)12, and NURF55. The FLAG tag on the fusion proteins was used for affinity purification from Sf9 cell lysates. Both fusion proteins were able to assemble into catalytically active complexes as shown in Figure 2A. Although the LacI-ESC contains an internal FLAG tag, this position still allowed affinity purification, as shown in the Coomassie stained gel in Figure 2A. The HMTase assays (Figure 2A,

Figure 1: Expression constructs for ESC fusion proteins. The coding sequence for the LacI-DBD was ligated in-frame to the 5' end of FLAG-tagged *esc* cDNA to produce LacI-ESC (top) and to the 3' end to produce ESC-LacI (bottom). The vector backbone was pFastBac1 for baculovirus expression and pCaSpeR-hs-act for expression in *Drosophila* (see Materials and Methods).

Figure 1


Not I
Nsi I
FLAG
Nhe I
stop
Xba I
LacI-DBD
esc cDNA
1.1kb
2.5kb
LacI-ESC
Vector Backbone

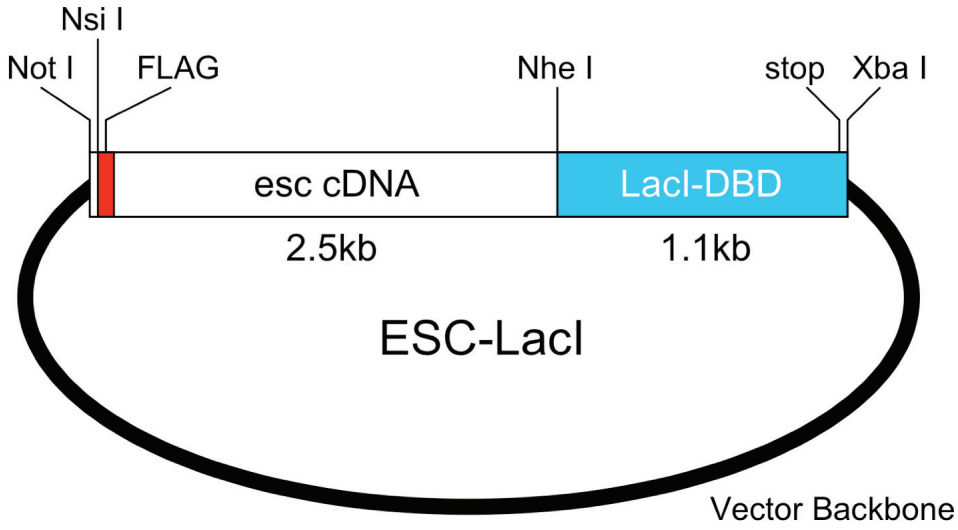
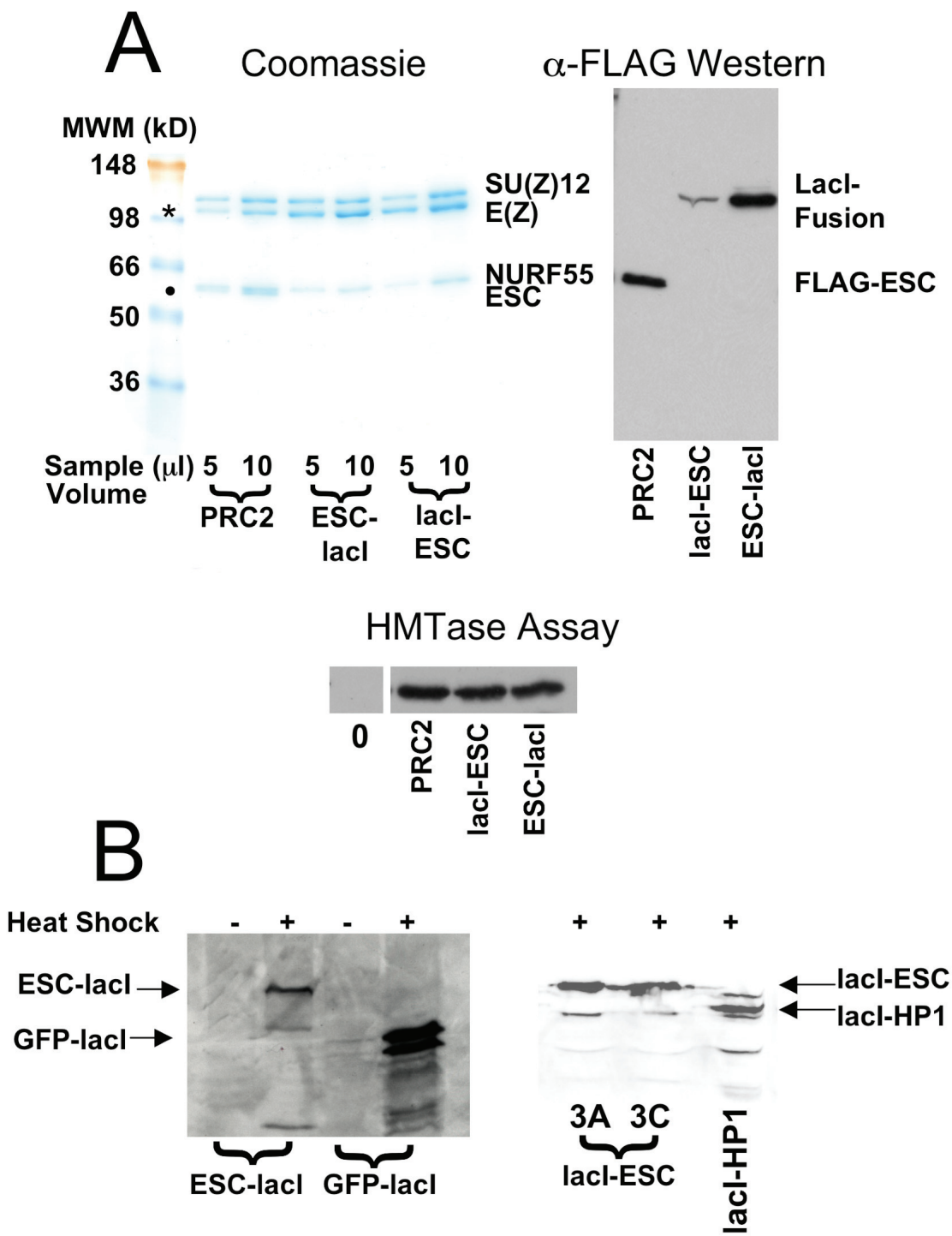

Nsi I
Not I
FLAG
Nhe I
stop
Xba I
esc cDNA
LacI-DBD
2.5kb
1.1kb
ESC-LacI
Vector Backbone

Figure 2: Expression of ESC fusion proteins. (A) Assembly and activity of recombinant PRC2 bearing ESC fusion proteins. Both fusion proteins were separately co-expressed with the PRC2 components E(Z), SU(Z)12, and NURF55 in a baculovirus/Sf9 cell system. FLAG-tagged ESC, which can assemble with the other subunits into catalytically active recombinant PRC2 (MULLER *et al.* 2002) was included as a control. Complexes were purified by anti-FLAG affinity (see Materials and Methods), and samples were resolved by SDS-PAGE and stained with coomassie. The presence of the E(Z), SU(Z)12 and NURF55 indicate that the fusion proteins were incorporated into modified PRC2 complexes. In wild-type PRC2, FLAG-ESC and NURF55 run as a doublet (•). The LacI fusion proteins co-migrate with E(Z) (*), as verified by Western blot (right panel). The lighter signal for LacI-ESC in the Western blot presumably results because the antibody does not recognize the internal FLAG tag as well as the N-terminal FLAG tags in the other samples, since coomassie staining and Bradford assay both indicated that the lanes were equally loaded. Each complex was tested at 50nM in the HMTase reaction at bottom. Polynucleosome arrays prepared from HeLa cells were used as a substrate. The "0" lane contains polynucleosome substrate and radiolabelled SAM but no enzyme complex.

(B) Expression of fusion proteins in *Drosophila*. Flies were heat shocked and protein extracts were immunoblotted with α-LacI (see Materials and Methods). At left, a blot of an ESC-LacI expressor stock compared to GFP-LacI. At right a blot kindly provided by Diane Cryderman (Wallrath Lab) showing expression of the LacI-ESC 3A and 3C inserts compared to LacI-HP1. Expression in the LacI-HP1 and GFP-LacI stocks is

sufficiently strong to produce chromosome accumulation detectable by immunostaining in previous studies (LI *et al.* 2003).

Figure 2


A
Coomassie
α-FLAG Western
MWM (kD)
148
98
66
50
36
SU(Z)12
E(Z)
NURF55
ESC
LacI-
Fusion
FLAG-ESC
Sample (μl) 5 10 5 10 5 10
Volume
PRC2
ESC-
lacI
lacI-
ESC
PRC2
lacI-ESC
ESC-lacI
HMTase Assay
0
PRC2
lacI-ESC
ESC-lacI
B
Heat Shock - + - +
ESC-lacI
GFP-lacI
ESC-lacI
GFP-lacI
+ + +
lacI-ESC
lacI-HP1
3A 3C
lacI-ESC
lacI-HP1

bottom) indicate that the recombinant complexes containing the fusion proteins can methylate polynucleosomes as robustly as wild type PRC2 complex *in vitro*.

ESC-LacI Does Not Target Correctly to lacO Arrays *In Vivo*.

After verification that both ESC-LacI and LacI-ESC fusions could assemble into catalytically active PRC2 complexes *in vitro*, we transferred the fusion construct cassette to the pCaSpeR-hs-act vector for expression in *Drosophila* (see Materials and Methods). Creation of the transgenic *Drosophila* lines was performed by our collaborators in Lori Wallrath's laboratory at the University of Iowa.

We selected three lines because they expressed the ESC fusions at levels similar to control LacI fusion proteins used previously in the Wallrath lab (see Figure 2B). These included one ESC-LacI line with an insert on the second chromosome, and two LacI-ESC lines, one with an insert on the X chromosome (LacI-ESC 3A) and one with an insert on the second chromosome (LacI-ESC 3C).

Salivary chromosome squashes were prepared to assess whether the ESC fusion proteins were targeted correctly to chromosome sites bearing reporters with lacO arrays. The location of the fusion proteins was visualized by immunostaining with a monoclonal antibody raised against LacI protein. The ESC-LacI line was crossed into reporter chromosome stocks so as to produce larvae homozygous for both the ESC-LacI expression construct and the reporter. When the ESC-LacI stock carrying a reporter at 87C was heat shocked, squashed, and stained in parallel with a lacI-HP1 line previously

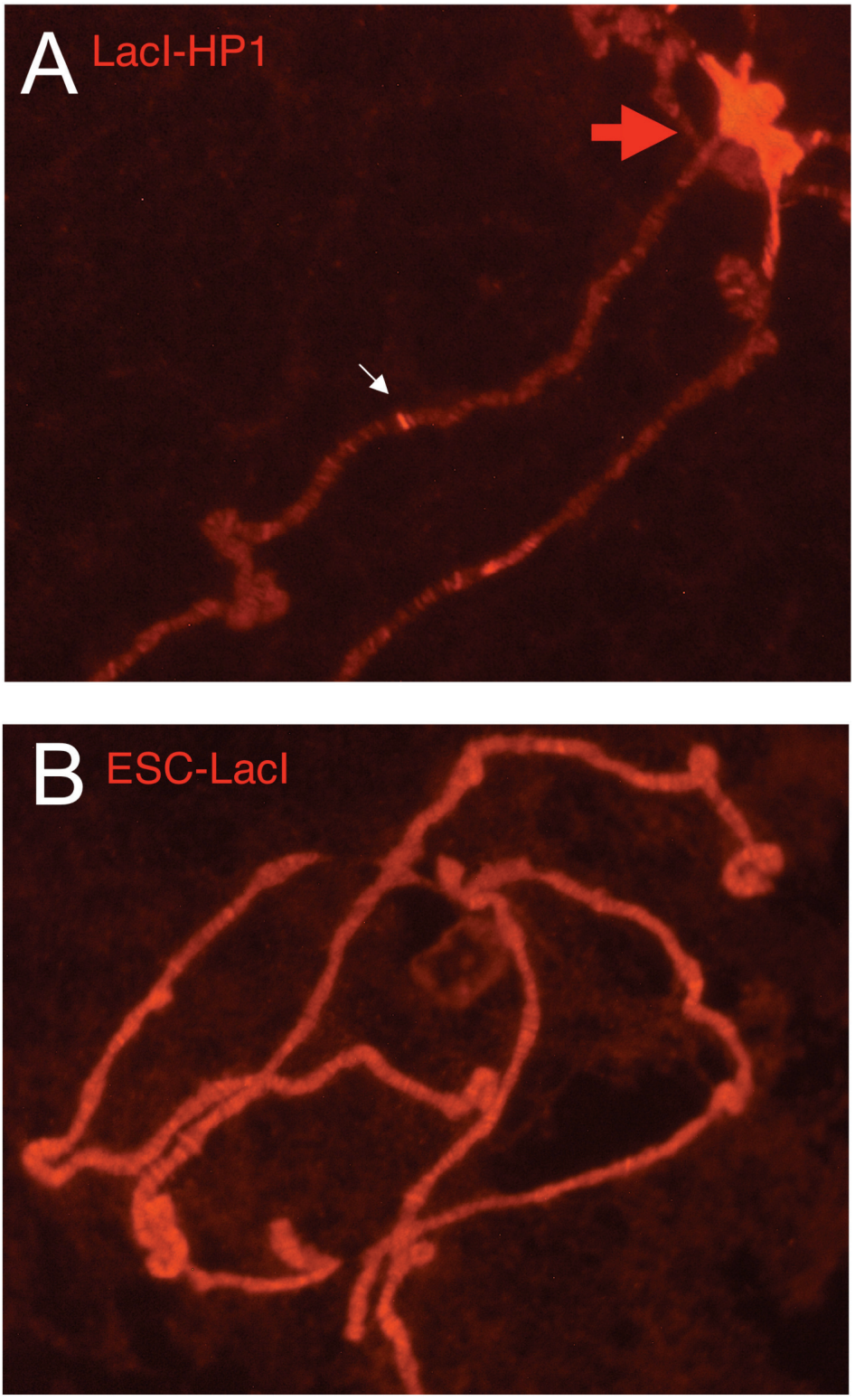
known to target correctly (LI *et al.* 2003), we observed that ESC-LacI was broadly distributed rather than targeted to the lacO array, as shown in Figure 3. The LacI-HP1; 87C control animals showed accumulation of staining at the heterochromatic chromocenter and a bright band at the 87C targeting site, but few other signals along the chromosomes, as expected from previous results (LI *et al.* 2003). In contrast, the ESC-LacI staining covered the entire lengths of all chromosomes, indicating that it is not tethered specifically to the target site. For this reason, we did not perform further experiments with the ESC-LacI fusion protein.

LacI-ESC 3A Partially Rescues ESC Loss-of-Function in Embryos.

Complete loss of ESC protein is embryonic lethal, but it has been demonstrated that a pulse of transgenic ESC driven by heat shock early in development can rescue to surviving first instar larvae (SIMON *et al.* 1995). To test whether the LacI-ESC fusion protein is functional *in vivo*, we attempted to repeat this rescue experiment with LacI-ESC substituting for ESC. Because *esc* is on the second chromosome, we were unable to easily test the 3C insertion, which is also on the second chromosome. Instead, we created embryos with one copy of the X-linked LacI-esc 3A insert in an esc^{10}/esc^{2} genetic background and heat shocked them from 2-4 hours of development to assess rescue. No larvae emerged from the esc^{10}/esc^{2} control embryos that lacked the transgene. In heat shocked embryos that possessed the LacI-esc 3A transgene, approximately 5-10% of the embryos emerged as larvae over the two days after fertilization. This represents a lower rate of survival compared to previous experiments

Figure 3: LacI-HP1 and ESC-LacI on Drosophila larval polytene chromosomes. Stocks homozygous for both the ESC-LacI or LacI-HP1 expressor and the 87C reporter were heat shocked, prepared, and stained with α-LacI in parallel (see Materials and Methods). (A) LacI-HP1 accumulates at the chromocenter (red arrow). This region is highly heterochromatic and is known to accumulate wild-type HP1, indicating that LacI-HP1 is properly targeted in chromatin. HP1 staining elsewhere in the nucleus is limited, but an intense band is visible at the reporter location (white arrow). These results replicate those described in (Li *et al.* 2003) and validate our experimental procedures. (B) In contrast, ESC-LacI is broadly distributed over the entire genome.

Figure 3



Genotype		Heat	Rescue		
ESC null	Transgene	Shock	++	+	-
+	-	+	0	0	62
+	+	-	0	0	33
+	+	+	36	18	44

Table 1: Rescue of ESC loss in embryos by LacI-ESC 3A transgene. All embryos were the progeny of parents that were esc^{2}/esc^{10} on the second chromosome (ie, null for ESC protein). Rescue was evaluated as follows: ++ indicates that dead embryos showed well-formed mouth parts and differentiated thoracic and abdominal segments, + indicates that embryos showed partially formed mouth parts and intermediate segment patterning, - indicates that mouth parts had not formed and there was only abdominal segmentation.

with heat-inducible ESC (SIMON *et al.* 1995). Thus, there is some rescue to larval viability, but this may be reduced compared to wild type ESC.

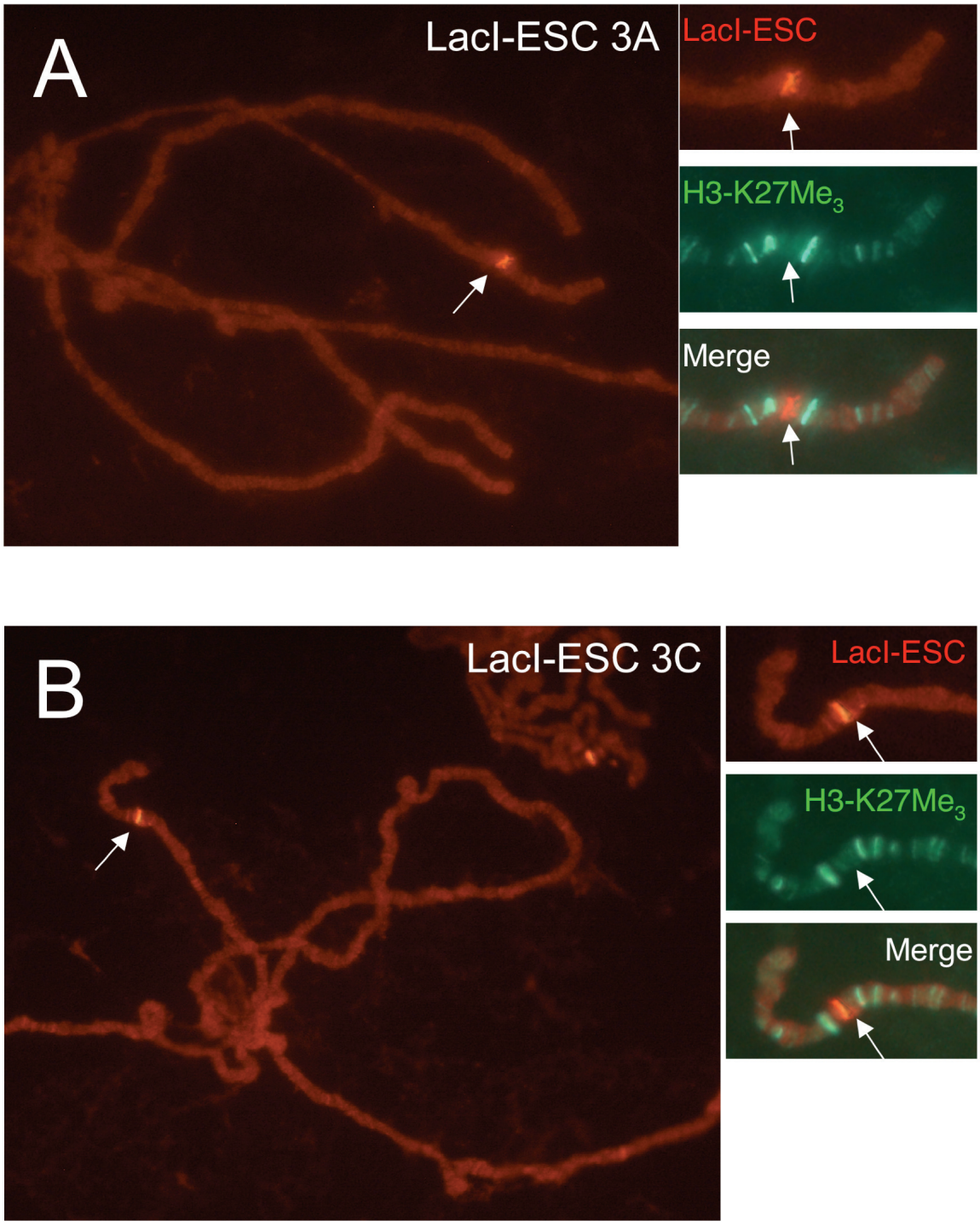
To further investigate the nature of the LacI-ESC rescue, we examined the segmental patterns in embryos that failed to hatch. Table 1 shows a tabulation of results from scoring of LacI-ESC 3A; esc^{10}/esc^{2} and control esc^{10}/esc^{2} embryos randomly selected approximately 24 hours after deposition. As shown, rescue is dependent on both the presence of the LacI-ESC transgene and its induction by heat shock. In heat-shocked embryos that contain the transgene there was at least some rescue in 55% of the observed embryos. Since the cross scheme used for this assay (SIMON *et al.* 1995) provides the transgene to only half of the embryos, we conclude that most or all of the embryos that received the transgene show some rescue.

LacI-ESC Targets Correctly to lacO Arrays *In Vivo*.

The ability of the LacI-ESC 3A insert to rescue ESC loss in *Drosophila* embryos (Table 1) motivated investigation into LacI-ESC targeting and function at later developmental stages. Rather than producing stable homozygous lines containing both the LacI-ESC expressors and the reporter chromosomes, LacI-ESC expressors were crossed to the reporter stocks and the heterozygous progeny were heat shocked, squashed and stained. Both LacI-ESC lines produced visible accumulations specifically at the 4D5 (Figure 4) and 87C (data not shown) target sites. While there is some weaker staining at sites elsewhere along the chromosomes, the signal at the targeting site is very bright, indicating that the LacI-ESC fusion protein is recruited to the lacO

Figure 4: LacI-ESC distribution on polytene chromosomes. Polytene chromosomes from larvae containing the 4D5 (X chromosome) reporter and either LacI-ESC 3A (panel A) or 3C (panel B) inserts. In contrast to the ESC-LacI fusion protein (Figure 3) these fusions accumulate specifically at the reporter location (arrows). Insets show detail of staining for trimethyl-H3-K27 at the reporter site. Despite the recruitment of LacI-ESC to this location robust ectopic methylation is not detected.

Figure 4


A
LacI-ESC 3A
LacI-ESC
H3-K27Me3
Merge
B
LacI-ESC 3C
LacI-ESC
H3-K27Me3
Merge

reporter *in vivo*. The weaker bands suggest that LacI-ESC may also be assembling at normal PRC2 sites *in vivo*. However, when squashes from LacI-ESC expressing larvae were double-stained for trimethyl-H3-K27 (see insets in Figure 4), there is no apparent concentration of H3-K27 methylation that coincides with the LacI-ESC targeting.

LacI-ESC is Incorporated into PRC2 *in Vivo*.

We considered two possibilities that might explain why we did not detect ectopic methylation at the reporter construct insertions despite the functional activity of LacI-ESC fusion protein as demonstrated by the rescue assay (Table 1). First, we wanted to establish whether the PRC2 complex is assembling at the reporter site, since tethering LacI-ESC in itself would not produce methylation unless it was bound to the other PRC2 components needed to create an active complex. Second, we were concerned that the reporter constructs we were using did not provide a suitable context for methylation by PRC2. Because LacI-ESC is under heat shock control in the 3A insert, and because the reporter constructs in the 4D5 and 87C stocks both contain heat shock promoters, the times of maximum expression for LacI-ESC would also be times when the reporter genes would be the most actively transcribed. Thus it is possible that a complex containing LacI-ESC would be unable to methylate the target site because of high levels of transcription during periodic heat shocks.

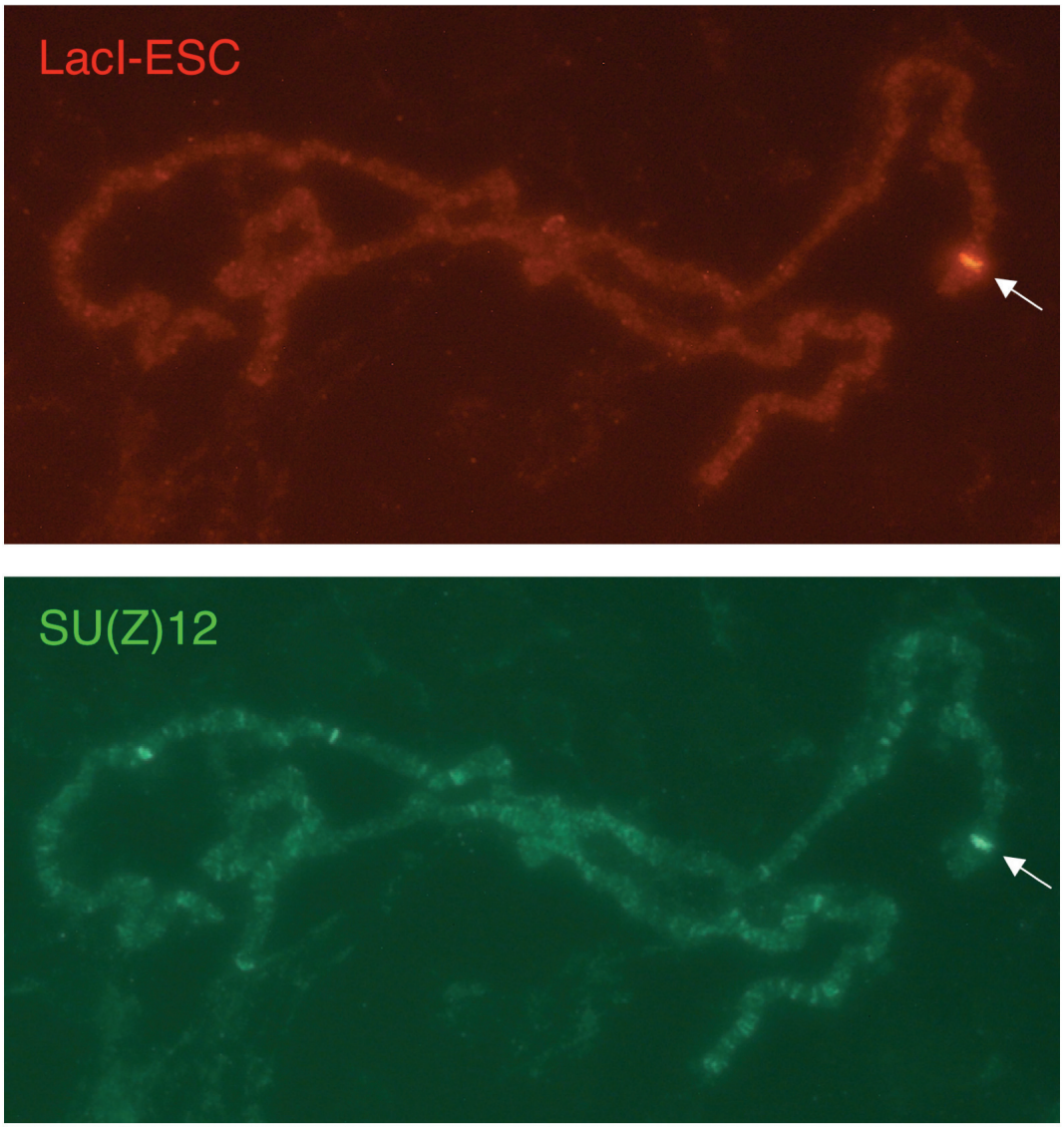
To remove this latter complication, we switched to independent lacO reporter lines (LI *et al.* 2003) that do not contain heat shock promoters in the reporter construct. We crossed the LacI-ESC 3A expressor to these "lacO-plus-*miniwhite* only" lines and

prepared chromosome squashes on larvae after twice-daily heat shock throughout development. Slides were stained with α-SU(Z)12 to assess PRC2 assembly or α-trimethyl-H3-K27 to assess methylation activity. All slides were double-stained with α-LacI to verify that LacI-ESC was present at the lacO array.

LacI staining at the target site indicates that the LacI-ESC fusion protein is present at the target site, but LacI-ESC could conceivably arrive at the target site without any other PRC2 subunits. To investigate whether the entire PRC2 complex is present at the target site, we stained chromosomes for the SU(Z)12 subunit of PRC2, since it would not be attracted on its own to lacO binding sites. Moreover, because SU(Z)12 contacts ESC only indirectly through E(Z) (KETEL *et al.* 2005; see Chapter One, Figure 1), a SU(Z)12 signal at the target site would suggest that the all of the enzymatically significant subunits of PRC2 are being tethered to the lacO array. Figure 5 shows a robust SU(Z)12 signal coincident with the LacI signal at a target located at 61F on the third chromosome. In fact, this is the strongest SU(Z)12 band detected on the chromosomes. When 20 random chromosomes were examined for this staining, in every instance the LacI signal was coincident with the strongest SU(Z)12 band. In addition, similar stainings with a different reporter stock (159.1, target at 86D) showed a similar unique concentration of SU(Z)12 at the lacO array along with LacI-ESC (data not shown). Unfortunately, 61F is also the location of a weak endogenous Polycomb site (DECAMILLIS *et al.* 1992). However, robust SU(Z)12 staining is not present on this band in wild-type chromosomes. Furthermore, examination of 12 chromosomes from a preparation in which control GFP-LacI was targeted to the 61F region showed there was

Figure 5: LacI-ESC and SU(Z)12 distributions on polytene chromosomes. Polytene chromosome from a larva containing the 157.4.112 reporter and lacI-ESC 3A inserts. LacI staining is shown in red (top panel), SU(Z)12 is shown in green (bottom panel). The lacO array is shown by the arrow, at cytological position 61F on the third chromosome. The robust accumulation of SU(Z)12 at the reporter site is the brightest signal in the entire nucleus. Wild type chromosomes show only a weak signal at this position.

Figure 5



no enhancement of the SU(Z)12 signal above other endogenous bands on the chromosomes (data not shown). We infer from the robust enhancement of SU(Z)12 staining at lacO arrays when LacI-ESC is present that LacI-ESC is being incorporated into PRC2 complexes in salivary gland cells.

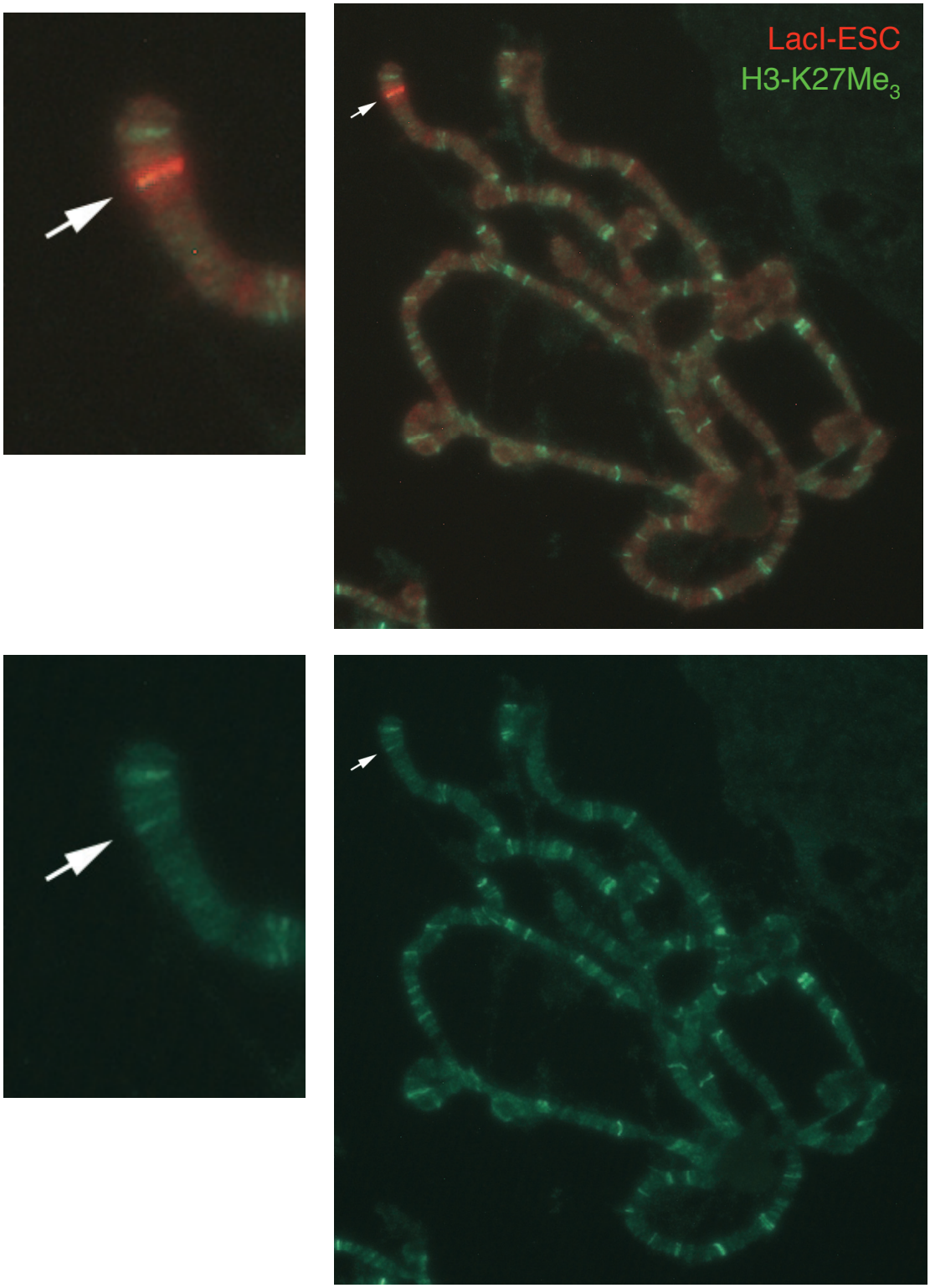
However, we still do not see evidence of robust H3-K27 methylation on polytene chromosomes when PRC2 is tethered to these LacO arrays. As Figure 6 shows, there is no enhancement of methyl-H3-K27 signal proportional to the LacI-signal at the targeting site. We interpret the faint band of H3-K27 methylation at the site (see inset) as the signal associated with an endogenous Polycomb site nearby within 61F. If there is any HMTase activity from the tethered PRC2 complex located at this site, it is evidently too weak to distinguish from this endogenous signal.

IV. Discussion

The experiments performed with the LacI-DBD tethering system for PRC2 do not show evidence for *in vivo* methylation at the lacO array target site to date. The tools that have been used so far do not rule out the possibilities that weak methylation at the target locus is masked by the methylation at nearby endogenous Polycomb sites, or indeed that methylation from any tethered PRC2 complex incorporating LacI-ESC is so weak as to be undetectable by immunostaining. However, in order to address the questions that motivated this approach, *in vivo* methylation will need to be demonstrated.

Figure 6: LacI-ESC and trimethyl-H3-K27 distribution on polytene chromosomes. Chromosomes from a larva heterozygous for both the 157.4.112 reporter and the LacI-ESC 3A inserts. LacI is stained in red and trimethyl-H3-K27 is stained in green. Arrows show the location of the reporter site. Since the reporter site is close to a weak endogenous Polycomb site, the faint band of H3-K27 methylation visible in the lower panel is probably the result of endogenous PcG activity.

Figure 6



Although PRC2 may play multiple functions during Polycomb repression, H3-K27 methylation is the major biochemical activity that has been described. Consequently, it is difficult to imagine how the LacI-ESC protein could rescue ESC null embryos if it was completely unable to support HMTase activity, and it would be useful to conduct further tests using reporter constructs in which the target site is clearly distant from any endogenous Polycomb sites. The reporter stocks F3.1.1 (second chromosome), S4.5 and S9.2 (both third chromosome) carry chromosomes in which the reporter constructs are at some distance from reported Polycomb sites (DECAMILLIS *et al.* 1992). These stocks (LI *et al.* 2003) are from the earlier version of reporter that does not carry the heat shock promoter, which might make the locus easier to methylate.

This study does present evidence indicating that LacI-ESC is being incorporated into PRC2 complex *in vivo*. Again, the rescue assay indicates that LacI-ESC is able to support Polycomb repression *in vivo*, and a previous study (ROSEMAN *et al.* 2001) observed reporter gene silencing when ESC was tethered to ectopic locations with the Suppressor-of-Hairy-Wing-DBD. Since we now understand that ESC functions within the PRC2 complex during Polycomb repression, these results are both consistent with the ability of PRC2 to incorporate LacI-ESC *in vivo*. In addition, the ability of recombinant LacI-ESC to incorporate with recombinant PRC2 components in Sf9 cells as revealed by affinity purifications *in vitro* (Figure 2A) suggests that there is nothing to prevent assembly under physiological conditions. Finally, the strongly enhanced SU(Z)12 signal consistently colocalizing with LacI at the target site on polytene chromosomes indicates that SU(Z)12 is physically interacting with LacI-ESC. On the

chromosomes observed, the expression of LacI-ESC produces the strongest SU(Z)12 signal in the entire genome (Figure 5). The geometry of the PRC2 complex argues that an interaction between SU(Z)12 and LacI-ESC must include E(Z) because the SU(Z)12 and ESC subunits have stable contacts with E(Z) but not with each other (KETEL *et al.* 2005; see Chapter One; Figure 1). It is conceivable that the addition of the LacI-DBD to ESC might alter the assembly of PRC2 components so that it cannot support Polycomb repression, but the robust methylation observed by recombinant LacI-ESC containing PRC2 *in vitro* (see Figure 2A), makes this unlikely. Incorporation of LacI-ESC into PRC2 *in vivo* seems the most straightforward interpretation of our results.

Considering the difficulty of drawing conclusions from negative results, it is disappointing that the experiments performed to date have not produced robust ectopic methylation on polytene chromosomes *in vivo* (Figure 6). Evidently the robust HMTase activity that PRC2 retains when incorporating LacI-ESC *in vitro* (Figure 2A, bottom panel) is somehow severely compromised *in vivo*. Possible changes that might increase the intrinsic ability of tethered PRC2 complex to attain robust HMTase activity are discussed below. One possibility that should be considered is that endogenous demethylases might efficiently removing methyl marks produced by the tethered PRC2. H3-K27-specific demethylases have been described recently in vertebrate systems and in *Drosophila* (BURGOLD *et al.* 2008; DE SANTA *et al.* 2007; LAN *et al.* 2007; SMITH *et al.* 2008). The described demethylases can impact Polycomb repression and the studies include examples where they act in response to both developmental and environmental cues. It is conceivable that H3-K27 demethylases could preferentially remove methyl

marks from ectopic locations, where genomic and molecular cues might not be present to sustain the methylation. If methylation by tethered PRC2 is being removed as fast as it is applied by competing demethylases, this would represent a significant technical barrier to evaluating the functional consequences of ectopic methylation. Thus, it might be worth exploring experiments using a demethylase-deficient genetic background, treatment of larvae with demethylase-inhibitory molecules, or specifically knocking down demethylase activity in salivary glands.

Until H3-K27 trimethylation at the target site is demonstrated it is unclear whether it can be technically achieved. This raises the question of how much further effort should be spent on further developments to this system. As before, the ability of LacI-ESC to substitute for ESC in the genetic rescue experiment presented here and the ability of tethered ESC to silence reporter genes in previous work (ROSEMAN *et al.* 2001) argue that LacI-ESC supports PRC2 function *in vivo*, at least to some degree. It may be that the LacI-DBD tethering system has some value even if it cannot produce robust ectopic methylation. Experiments tethering LacI-HP1 to reporter sites observed a short-range silencing effect independent of SU(VAR)3-9 and H3-K9 methylation as well as a long-range silencing effect that did depend on SU(VAR)3-9 (DANZER and WALLRATH 2004). This raises the possibility that H3-K27 methylation serves to transmit Polycomb silencing activity over long distances (such as from PREs to distant promoters and coding regions), and that the ability of LacI-ESC to partially rescue (Table 1) and of tethered ESC to silence reporters (ROSEMAN *et al.* 2001) might be due to a short-range, methylation-independent effect. However, these speculations are not bolstered by

negative results and are of questionable value unless validated by experimental demonstration. There is an intriguing possibility that PRC1 might be recruited by protein-protein interactions with PRC2, possibly mediating the activity we have observed. However, pilot experiments designed to detect a PRC1 component at target sites when LacI-ESC is expressed (data not shown) have also yielded negative results so far, similar to our results for H3-K27 methylation. As with methylation, a weak effect might be better demonstrated by testing additional reporter locations.

Assuming that it is possible in principle for PRC2 to methylate H3-K27 when tethered to chromatin by the LacI-DBD *in vivo*, there two modifications to the tethering system that are worth considering. Despite the previous arguments in favor of LacI-ESC supporting PcG function, more robust methylation might be achieved by fusing the LacI-DBD to a different subunit of PRC2. SU(Z)12 would seem to present the best possibility, since the multiple contributions of NURF55 to chromatin metabolism (MARTINEZ-BALBAS *et al.* 1998) mean that directly tethering NURF55 would be expected to bring a number of different factors besides PcG proteins to the target site. Another alternative would be to express the fusion protein continuously over a developmental period with the GAL4-UAS system (BRAND and PERRIMON 1993). This strategy was successful for experiments that tethered the H3-S10 kinase (DENG *et al.* 2008). This method requires a more challenging cross strategy to bring the UAS expressor, GAL4 driver, and reporter chromosomes together, but it eliminates the need for heat shocking. Moreover continuous expression of the fusion protein from the UAS promoter may facilitate application of histone modifications during the DNA replication

cycle and might better out-compete any endogenous demethylases that are preventing methyl-H3-K27 from accumulating at the target site. If the technical challenge of robust, ectopic H3-K27 methylation *in vivo* can be overcome, it will be interesting to test the consequences that attend targeting of PcG complexes, possibly including ectopic gene silencing at the reporter sites.

Chapter Four

Chromatin recruitment of the Sex comb on Midleg (SCM) repressor independent of other Polycomb group proteins

Liangjun Wang, Neal Jahren, Ellen L. Miller, Carrie S. Ketel, Daniel R. Mallin and Jeffrey A. Simon

Department of Genetics, Cell Biology and Development, University of Minnesota.

This chapter is in preparation for submission to a journal for publication. I contributed the characterization of the *scm*^{sz3} mutant shown in figure 4, the material for the ChIP experiments in Figure 5, and the characterization of the Pc-R4 stock and material for the resulting ChIP experiments, both of which are shown in Figure 6.

I. Introduction

The Polycomb group (PcG) proteins are a set of conserved chromatin regulators that work together to execute gene silencing during development (see MULLER and KASSIS 2006; SCHWARTZ and PIRROTTA 2007; and SPARMANN and VAN LOHUIZEN 2006 for reviews). Most PcG proteins were initially identified in *Drosophila* based upon their roles in *Hox* gene silencing along the anterior-posterior body axis (BREEN and DUNCAN 1986; JONES *et al.* 1998; JÜRGENS 1985; LEWIS 1978; SIMON *et al.* 1992; STRUHL and AKAM 1985). Subsequently, genome-wide studies revealed that PcG proteins collaborate to control hundreds of target genes in both flies and mammals (BOYER *et al.*

2006; BRACKEN *et al.* 2006; LEE *et al.* 2006; NEGRE *et al.* 2006; SCHWARTZ *et al.* 2006; SQUAZZO *et al.* 2006; TOLHUIS *et al.* 2006). In mammalian systems, PcG proteins play key regulatory roles in X-chromosome inactivation (HEARD 2005; PLATH *et al.* 2003; SILVA *et al.* 2003; ZHAO *et al.* 2008) and in maintenance of both embryonic and lineage-restricted stem cells (BOYER *et al.* 2006; LEE *et al.* 2006; PARK *et al.* 2003; SPARMANN and VAN LOHUIZEN 2006). The common theme that links their diverse biological functions is the role of PcG proteins in modifying chromatin to perpetuate gene silencing. In addition, human PcG proteins have been linked to oncogenesis in many different tissue types (SIMON and LANGE 2008; SPARMANN and VAN LOHUIZEN 2006 for reviews), suggesting that they contribute to abnormal chromatin states in cancer cells (OHM *et al.* 2007; SCHLESINGER *et al.* 2007; TING *et al.* 2006; YU *et al.* 2007). Thus, there is great interest in determining molecular mechanisms by which PcG proteins alter chromatin and control gene expression in both normal development and disease.

Biochemical studies have defined subunit compositions and revealed molecular activities of several PcG complexes purified from *Drosophila* embryos or from human cells (CAO *et al.* 2002; CZERMIN *et al.* 2002; KUZMICHEV *et al.* 2002; LEVINE *et al.* 2002; MULLER *et al.* 2002; SHAO *et al.* 1999). Three *Drosophila* PcG complexes have been purified and characterized so far, termed Polycomb repressive complex 1 (PRC1), PRC2, and PHO-RC (CZERMIN *et al.* 2002; FRANCIS *et al.* 2001; KLYMENKO *et al.* 2006; MULLER *et al.* 2002; SHAO *et al.* 1999). PHO-RC contains two subunits, PHO and SFMBT (KLYMENKO *et al.* 2006). PHO is the best-characterized sequence-specific

DNA-binding protein in PcG silencing (BROWN *et al.* 2003; BROWN *et al.* 1998; FRITSCH *et al.* 1999; SHIMELL *et al.* 2000) and there is evidence that it plays a key role in recognizing PcG target genes and recruiting other PcG complexes to these loci (MOHD-SARIP *et al.* 2005; WANG *et al.* 2004b). SFMBT contains putative protein interaction domains (see below) but its precise biochemical role in PcG silencing has yet to be elucidated.

PRC2 contains four core subunits, E(Z), ESC, SU(Z)12 and NURF55 (CZERMIN *et al.* 2002; MULLER *et al.* 2002). PRC2 possesses intrinsic histone methyltransferase (HMTase) activity, which is provided by the SET domain of E(Z). Contributions from the ESC and SU(Z)12 subunits are also required for robust enzyme activity (KETEL *et al.* 2005; NEKRASOV *et al.* 2005). The PRC2 HMTase primarily methylates histone H3 on lysine 27 (K27) (CAO *et al.* 2002; KETEL *et al.* 2005; MULLER *et al.* 2002) and this histone modification is commonly associated with PcG-silenced genes *in vivo* (EBERT *et al.* 2004; PAPP and MULLER 2006; SCHWARTZ *et al.* 2006). *In vitro* binding studies (FISCHLE *et al.* 2003c; MIN *et al.* 2003), together with *in vivo* PRC2 loss-of-function studies (BOYER *et al.* 2006; CAO *et al.* 2005; WANG *et al.* 2004b), suggest that methylation of H3-K27 creates a binding site that helps recruit PRC1 to local chromatin. Besides PC, PRC1 contains three other core subunits, PSC, PH and dRING1 (FRANCIS *et al.* 2001; SHAO *et al.* 1999). Among these, PSC is a central contributor to transcriptional silencing by PRC1 *in vitro* (KING *et al.* 2005; KING *et al.* 2002) and dRING1 harbors ubiquitin ligase activity that can modify histone H2A on K119 (CAO *et al.* 2005; WANG *et al.* 2004a). In general, the subunit compositions and

biochemical activities of PRC1 and PRC2 are conserved between the fly and human versions. There are also mammalian homologs of both PHO-RC subunits (SHI *et al.* 1991; USUI *et al.* 2000) although a mammalian PHO-RC complex has not been described. Thus, the core machinery used for PcG chromatin modification and gene silencing is strikingly similar in flies and mammals.

Although individual biochemical activities have been assigned to particular PcG complexes, it is not yet clear how their multiple functions are integrated to achieve stable gene silencing. A hierarchical pathway for recruitment of PcG complexes to target genes has been proposed based upon their known activities and interactions (reviewed in CAO and ZHANG 2004b; WANG *et al.* 2004b). In this scheme (see Fig. 7), PHO-RC and PRC2 play primary roles in recognizing target genes and recruiting PcG components to the local chromatin. The end result is recruitment of PRC1, which is thought to be most directly responsible for executing gene silencing. The mechanism of silencing by PRC1 has not yet been determined. Previous studies have suggested that PRC1 silencing may involve histone ubiquitylation (CAO *et al.* 2005; WANG *et al.* 2004a), inhibition of nucleosome remodeling (FRANCIS *et al.* 2001; SHAO *et al.* 1999), compaction of nucleosome arrays (FRANCIS *et al.* 2004), blocked initiation after RNA polymerase II is bound (DELLINO *et al.* 2004), and/or arrested transcription elongation (STOCK *et al.* 2007).

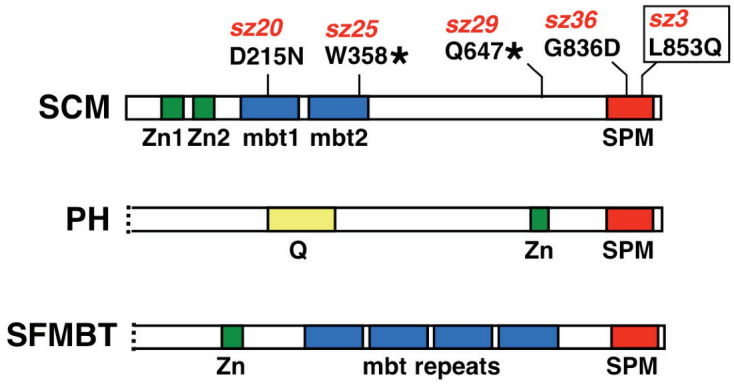
The PcG protein Sex comb on midleg (SCM) is just as critical as any subunit of PHO-RC, PRC1, or PRC2 for *Hox* gene silencing in fly embryos (BORNEMANN *et al.* 1998;

BREEN and DUNCAN 1986). However, the biochemical role of SCM in the context of these well-characterized PcG complexes has not been determined. *In vitro* interactions between SCM and a PRC1 subunit, PH, initially suggested that SCM might function as a component of PRC1 (PETERSON *et al.* 1997). Indeed, SCM can assemble into a recombinant PRC1 complex via this PH interaction (PETERSON *et al.* 2004). However, several biochemical tests with fly embryo extracts revealed that SCM does not behave as a core subunit of PRC1. First, gel filtration chromatography reveals separable peaks for SCM and PRC1 (PETERSON *et al.* 2004). Second, coimmuneprecipitations detect little or no SCM association with PRC1 subunits (KLYMENKO *et al.* 2006; PETERSON *et al.* 2004). Finally, purified preparations of PRC1 contain some SCM, but it is consistently detected in substoichiometric quantities (LEVINE *et al.* 2002; SAURIN *et al.* 2001; SHAO *et al.* 1999). Thus, the sum of biochemical tests so far indicate that SCM is not a core subunit of PRC1, PRC2, or PHO-RC *in vivo* (KLYMENKO *et al.* 2006; NG *et al.* 2000; PETERSON *et al.* 2004; SAURIN *et al.* 2001).

Based upon its domain content (Fig. 1, top), SCM appears to lack intrinsic enzymatic or DNA recognition functions. However, SCM domain organization does offer some functional clues and intriguing similarities with other PcG components. In particular, SCM shares a C-terminal domain, called the SPM domain, with the PRC1 subunit, PH, and with the PHO-RC subunit, SFMBT (Fig. 1, shown in red). This domain is a subtype within the larger family of SAM domains (PONTING 1995) and, like other family members, it is a multihelix bundle that mediates homotypic and heterotypic protein interactions (reviewed in QIAO and BOWIE 2005). Indeed, this domain mediates

Figure 1: Domain organizations of SCM and two related PcG proteins. PH is a subunit of PRC1 (FRANCIS and KINGSTON 2001; SHAO *et al.* 1999) and SFMBT is a subunit of PHO-RC (KLYMENKO *et al.* 2006). All three proteins share a C-terminal SPM domain (red) and copies of particular subtype of Cys2-Cys2 zinc finger (green). In addition, SCM and SFMBT share multiple mbt repeats (blue). "Q" represents a glutamine-rich domain. N-terminal portions of PH and SFMBT lacking homology domains are not shown. The positions and mutant lesions of five newly characterized *Scm* alleles are shown. Two are nonsense mutations (*sz25*, *sz29*) and three are missense mutations (*sz3*, *sz20*, *sz36*). The *sz3* allele (boxed) is a pupal-lethal hypomorphic mutant used for wing disc ChIPs in this study.

Figure 1


sz20
D215N
sz25
W358*
sz29
Q647*
sz36
G836D
sz3
L853Q
SCM
Zn1 Zn2 mbt1 mbt2
SPM
PH
Q
Zn
SPM
SFMBT
Zn
mbt repeats
SPM

the *in vitro* SCM-PH binding described previously (PETERSON *et al.* 1997). Thus, although SCM is not stably associated with either PRC1 or PHO-RC in fly embryo extracts, this shared domain provides the capacity, or at least the potential, to interact with these other PcG complexes (GRIMM *et al.* 2007; PETERSON *et al.* 2004).

We reasoned that previous analyses of soluble nuclear extracts does not provide a chromatin context, which may be required to fully assess the functional relationship of SCM to other PcG components. To address this, we have employed chromatin immuneprecipitation (ChIP) to directly test SCM in the context of a bona fide chromatin target *in vivo*. Using both *Drosophila* S2 cells, and larval wing discs, we find that SCM colocalizes with components of PHO-RC, PRC2, and PRC1 on a well-characterized Polycomb response element located upstream of the Hox gene *Ultrabithorax* (*Ubx*). To investigate functional relationships between SCM and these PcG complexes, we extend the ChIP analyses to interrogate chromatin from cells and/or tissues where levels of SCM, PHO, SU(Z)12, or PC have been reduced by RNA interference. We also test PcG associations in chromatin from wing discs bearing a newly-described *Scm* loss-of-function allele. Our results provide further evidence that SCM is not an integral subunit of the three defined fly PcG complexes and they suggest that SCM targeting to chromatin sites can occur, to significant extent, independently of these PcG complexes.

II. Materials and Methods

Fly stocks and crosses. The *Scm*^{sz3} mutant was kindly provided by Henrik Gyurkovics (Biological Research Center, Szeged, Hungary). *Scm*^{H1} is a null allele that converts W248 to a stop codon (BORNEMANN *et al.* 1998). Both Scm mutations were maintained over a TM6B third chromosome balancer carrying the Tb dominant marker. *Scm*^{sz3}/TM6B Tb males were crossed to *Scm*^{H1}/TM6B Tb females and the *Scm*^{sz3}/ *Scm*^{H1} progeny were selected as non-Tb larvae. The fly stock Pc-R4 (National Institute of Genetics, Japan, Stock number 32443R-4, http://www.shigen.nig.ac.jp/fly/nigfly/rnaiDetailAction.do?stockId=32443R-4) contains a transgene to express a short-hairpin (sh) RNA, covering approximately 400bp near the 5' end of the PC coding region, under UAS control. Expression of the *Pc* shRNA is driven by GAL4 activator provided from a second construct in trans (BRAND and PERRIMON 1993). For phenotypic analysis in adults (Fig. 6B), homozygous Pc-R4 males were crossed to females homozygous for the A9-GAL4 wing disc driver (HAERRY *et al.* 1998) and the progney were reared at 25°C. To produce wing discs for analysis by Western blotting, RT-PCR, and ChIPs (Figs. 6C-E), homozygous Pc-R4 males were crossed to females homozygous for the ubiquitous da-GAL4 driver (WODARZ *et al.* 1995) and the progeny were raised at 29°C prior to harvesting of wing discs from third instar larvae. Consistent with temperature-sensitivity of GAL4-UAS expression in *Drosophila* (BRAND and PERRIMON 1993), we found that the extent of PC loss from the *Ubx* PRE was slightly greater if this cross was performed at 29°C rather than 25°C. A *y Df(1)w*^{67c2} fly stock was used routinely as an essentially wild-type

control in Western blots, immunostainings, RT-PCR, and ChIP assays. This stock was raised at 25°C for all experiments, except when used as a control in experiments with Pc-R4 (Figs. 6C-E), when it was raised at 29°C.

Antibodies. Primary antibodies used to detect PcG proteins in Western blots and chromatin immuneprecipitations were the following rabbit polyclonals described previously: α-PHO (BROWN *et al.* 2003), α-E(Z) (CARRINGTON and JONES 1996), α-PC (WANG *et al.* 2004b), α-SU(Z)12 (MULLER *et al.* 2002), and α-SCM (BORNEMANN *et al.* 1998).

Western blots. Protein extracts from whole larvae were prepared as described (BORNEMANN *et al.* 1998) using 5-10 µl of 2X SDS sample buffer per larva with 1 mM PMSF and 1µg/ml leupeptin added to the sample buffer. Wing disc protein samples were prepared using approximately 50 discs disrupted in 15 µl of 2X SDS sample buffer. Samples were boiled for three minutes, chilled on ice, and centrifuged briefly at ~10,000 RPM to remove particulate material before loading. Protein extracts from S2 cells were prepared as described (WANG *et al.* 2004b). Proteins were resolved by SDS-PAGE on 10% gels and transferred to Protran (Whatman) membranes. Blots were blocked in 5% nonfat dry milk for one hour before incubating in primary antibody overnight at 4°C. Antibodies against PHO, PC, SCM and SU(Z)12 were each used at 1:500 dilutions. Antibody against E(Z) was used at 1:1000. A monoclonal antibody against α-tubulin (DM1A, Sigma), used to gauge equivalence of lane loadings, was diluted 1:2000. After washing, blots were incubated with a horseradish peroxidase-

conjugated goat α-rabbit secondary antibody (Jackson) at 1:5000, except for the anti-tubulin blots which were incubated with a horseradish peroxidase-conjugated goat α-mouse secondary antibody at 1:10,000. Secondary antibody incubations were at room temperature for one hour and signals were developed using an ECL chemiluminescence detection kit (Amersham).

Detection of Hox gene expression. Reverse transcription PCR (RT-PCR) to detect *Ubx* mRNA levels, and control *RpII140* mRNA levels, was performed as described (WANG *et al.* 2004b), using total RNA extracted from S2 cells or larval wing discs with Trizol reagent (Invitrogen). Immunostaining to detect ABD-A in embryos was performed as described (SIMON *et al.* 1992) using a rabbit polyclonal antibody (KARCH *et al.* 1990). Immunostaining to detect UBX in larval discs was performed essentially as described (BROWER 1987) using a mouse monoclonal primary antibody (FP.3.38; WHITE and WILCOX 1984). For initial analysis of *Scm* mutant disks, α-UBX was diluted 1:1000 and immunodetection was performed using a biotinylated goat α-mouse secondary antibody (1:1000) and the VectaStain ABC kit (Vector Laboratories). For the immunostainings in Figure 4A, α-UBX was diluted 1:500 and a goat α-mouse secondary antibody conjugated to Alexa568 (Invitrogen) was used at 1:2000. Tissues were mounted in Vectashield (Vector Laboratories) and images were captured using a Leica DMRB microscope and AxioCam MRc camera with MRGrab software (Zeiss).

Tissue culture and RNA interference. *Drosophila* S2 cell culture and harvesting of total protein for Western blot analyses were performed as described (WANG *et al.*

2004b). Double-stranded RNAs specific for PHO, E(Z), PC, and GFP were produced using templates described previously (WANG *et al.* 2004b). For SCM knockdown, an *Scm* cDNA was used as template to generate a 578 bp dsRNA extending from 37 bp upstream of the ATG to 541 bp downstream. Double-stranded RNAs were synthesized and transfected into S2 cells as described (CAO *et al.* 2002).

Chromatin immunoprecipitation (ChIP) assays. Formaldehyde cross-linked chromatin was prepared from fly S2 cells or wing imaginal discs (typically ~200 per batch) and immunoprecipitations were performed as described (WANG *et al.* 2004b) using 5 µl of antiserum against PHO, SU(Z)12, PC, or SCM per ChIP. Amplification of *Ubx* PRE fragments and a control *RpII140* fragment by endpoint PCR was performed as described (WANG *et al.* 2004b). Amplification of *Ubx* PRE fragments from wing disc ChIPs by real-time Q-PCR used the primer pairs 5'-CGC ACT CAA AAT CCG AAA AT-3' and 5'-CGC ACG TCA GAC TTG GAA TA-3' for fragment "PRE1", and 5'-GGG CTA TTC CAA GTC TGA CG-3' and 5'-GGC CAT TAC GAA CGA CAG TT-3' for fragment "PRE2". Q-PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to the manufacturer's instructions, except the total reaction volume was 15µl. Q-PCR was performed using 45 cycles consisting of 95°C for 30s, 55°C for 30s, 72°C for 30s on a Mastercycler RealPlex 2S (Eppendorf).

III. Results

SCM colocalizes with other PcG components at the Ubx PRE.

One of the best-characterized Polycomb response elements in *Drosophila* is located approximately 25 kb upstream of the *Hox* gene *Ubx*, within the *bxd* regulatory region. Previous studies demonstrated that this PRE can confer PcG silencing of reporters *in vivo* (CHAN *et al.* 1994; SIMON *et al.* 1993) and this operational assay has delimited this PRE to an approximately 0.5 kb region (FRITSCH *et al.* 1999; HORARD *et al.* 2000). Furthermore, numerous studies have mapped the associations of PcG proteins with this *Ubx* PRE using chromatin immuneprecipitation assays performed on cultured fly cells, embryos, or larval imaginal discs (CAO *et al.* 2002; KAHN *et al.* 2006; KLYMENKO *et al.* 2006; NEKRASOV *et al.* 2007; PAPP and MULLER 2006; SAVLA *et al.* 2008; WANG *et al.* 2004b). Thus, it is well-established that subunits of PHO-RC, PRC2, and PRC1 associate with this *Ubx* PRE region *in vivo*, with peak association detected on the b4 and b5 fragments (WANG *et al.* 2004b), as depicted here in Fig. 2A.

To determine if SCM tracks with these other PcG components on the *Ubx* PRE, we performed ChIP assays using *Drosophila* Schneider line 2 (S2) cells. Fig. 2B compares the distributions of SCM (left panel) and PHO (right panel) across four fragments (b3-b6) from the *Ubx* PRE region and on a negative control fragment (Rp), derived from the gene encoding an RNA polymerase II subunit. This analysis shows that the SCM distribution coincides with PHO (Fig. 2B), and with PRC2 and PRC1 (CAO *et al.* 2002; WANG *et al.* 2004b), within this *Ubx* PRE region.

Figure 2: SCM associates with a Polycomb response element (PRE) upstream of *Ubx*. (A) The map depicts the *Ubx* transcription start region and a PRE located ~25 kb upstream within the *bxd* regulatory region. Numbered fragments above the map have been described previously (CAO *et al.* 2002; WANG *et al.* 2004b; WANG *et al.* 2006) and were used in ChIP assays with signals obtained by endpoint-PCR. Fragments "1" and "2", below the map, were used in ChIP assays employing Q-PCR signal detection. (B) ChIP analyses to detect distributions of PHO (left panel) and SCM (right panel) on the *Ubx* PRE in *Drosophila* S2 cells. Antibodies used for immunoprecipitations are indicated at the top and amplified fragments are indicated to the left. "Mock" indicates control immunoprecipitation with protein A-agarose beads alone and "Rp" indicates a control fragment from the *RpII140* locus.

Figure 2

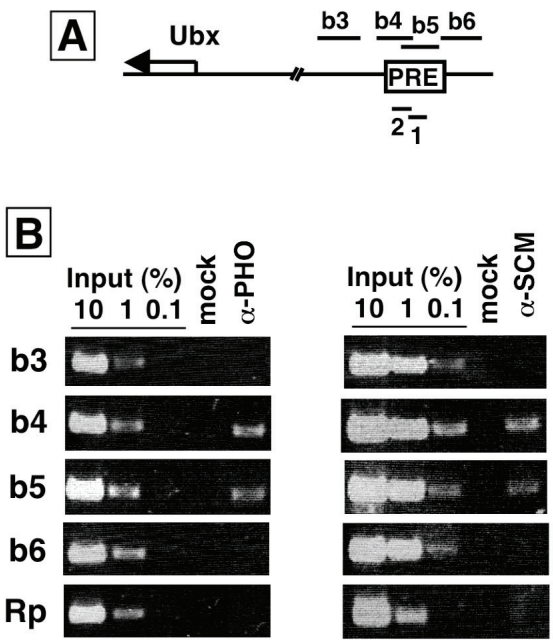
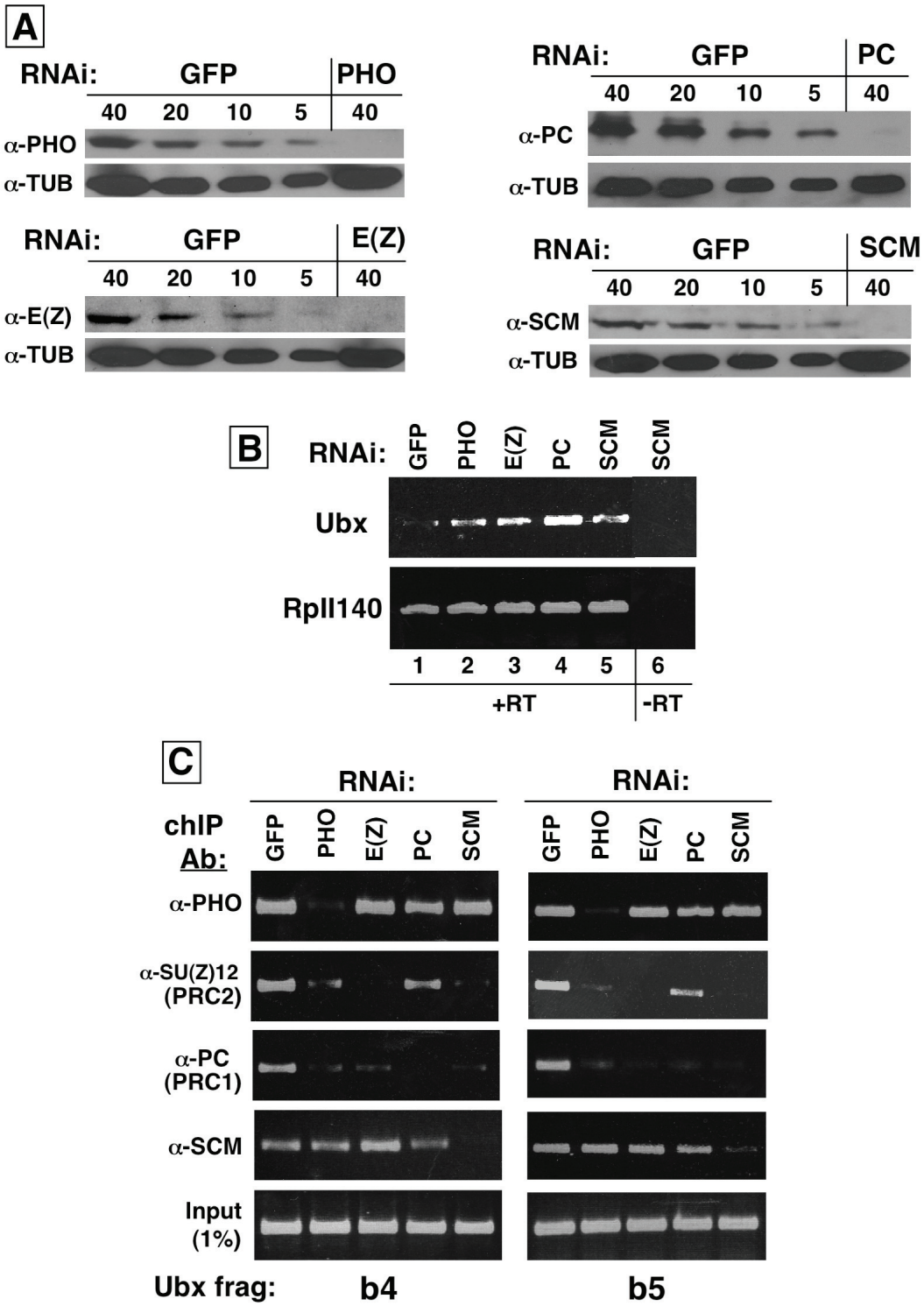

A
Ubx
b3
b4
b5
b6
PRE
2
1
B
Input (%)
10 1 0.1
mock
α-PHO
Input (%)
10 1 0.1
mock
α-SCM
b3
b4
b5
b6
Rp

Figure 3: Consequences of SCM depletion and other PcG protein depletions in S2 cells. (A) Western blots to detect levels of individual PcG proteins after depletion by RNA interference. Labels at the left of each panel denote antibodies to detect indicated PcG proteins or α-tubulin, which was used as a loading control. Labels at the top of each panel identify samples from mock-treated (GFP) or PcG-depleted S2 cells containing indicated amounts of protein in μg. Greater than 8-fold reductions in levels of PHO, E(Z), PC, or SCM were obtained. (B) RT-PCR analysis of *Ubx* and *RpII140* expression after treatment with mock (GFP) or indicated PcG double-stranded RNAs. "+RT" indicates reverse transcriptase added and "-RT" indicates control with reverse transcriptase omitted from the reaction. (C) ChIP analyses to detect associations of indicated PcG proteins with *Ubx* PRE fragments b4 (left panel) and b5 (right panel). Labels at left indicate antibodies used in each chromatin immunoprecipitation and labels at top identify samples with individual PcG proteins depleted by RNAi. Lanes labelled "GFP" identify mock-depleted samples. PHO is a subunit of PHO-RC, SU(Z)12 is a subunit of PRC2, and PC is a subunit of PRC1.

Figure 3


A
RNAi: GFP PHO
40 20 10 5 40
α-PHO
α-TUB
RNAi: GFP E(Z)
40 20 10 5 40
α-E(Z)
α-TUB
RNAi: GFP PC
40 20 10 5 40
α-PC
α-TUB
RNAi: GFP SCM
40 20 10 5 40
α-SCM
α-TUB
B
RNAi: GFP PHO E(Z) PC SCM SCM
Ubx
RpII140
1 2 3 4 5 6
+RT -RT
C
RNAi:
RNAi:
chIP Ab:
GFP PHO E(Z) PC SCM
GFP PHO E(Z) PC SCM
α-PHO
α-SU(Z)12 (PRC2)
α-PC (PRC1)
α-SCM
Input (1%)
Ubx frag: b4 b5

SCM functions in Ubx silencing in fly S2 cells.

Previous studies have shown that *Hox* gene mRNA levels are elevated in fly S2 cells when subunits of PHO-RC, PRC2, or PRC1 are depleted by RNA interference (BREILING *et al.* 2001; CAO *et al.* 2002; WANG *et al.* 2004b). Although it is not certain that precise *in vivo* mechanisms are preserved, these studies indicate that PcG silencing operates at *Hox* targets in S2 cells. To determine if SCM is also functionally engaged in *Ubx* gene silencing in S2 cells, we transfected short double-stranded RNAs to knock down SCM and assayed for changes in *Ubx* mRNA levels by RT-PCR. Fig. 3A (bottom) shows that our RNAi conditions yield greater than eight-fold reduction in the level of SCM. Separate RNAi treatments produce similar degrees of knockdown in the levels of PHO, E(Z), or PC (Fig. 3A). Figure 3B shows that each one of these RNAi knockdowns causes *Ubx* desilencing, as measured relative to a negative control sample treated with a nonspecific (GFP) double-stranded RNA. This degree of *Ubx* desilencing is similar to that observed previously after RNAi depletion of PHO, E(Z), or PC in S2 cells (WANG *et al.* 2004b). These results demonstrate that, like other PcG proteins, SCM functions in *Ubx* gene silencing in S2 cells. Thus, fly S2 cells provide a valid platform for investigating functional interdependence among SCM and the previously defined PcG complexes.

Chromatin associations after depletion of PHO-RC, PRC2, and PRC1 subunits.

ChIP assays were performed using cross-linked chromatin from S2 cells after RNAi depletion of PHO, E(Z), or PC. For comparison, parallel mock-depleted samples were obtained after treatment with nonspecific GFP dsRNA. In addition to Western blotting

(Fig. 3A), RNAi depletions were verified by loss of corresponding ChIP signals on the b4 (Fig. 3C, left panel) and b5 (Fig. 3C, right panel) fragments. For example, the PHO ChIP signals are substantially reduced in the PHO RNAi samples (Fig. 3C, top row). In these assays, PRC2 chromatin association was gauged by ChIP using antibody against the SU(Z)12 subunit and PRC1 association was tracked using antibody to PC.

As reported previously (WANG *et al.* 2004b), association of both PRC2 and PRC1 with the *Ubx* PRE is reduced after PHO depletion, consistent with the proposed roles of PHO-RC in target recognition and recruitment of other PcG complexes. However, PHO depletion has little or no effect upon the association of SCM, which is retained at both the b4 and b5 fragments (Fig. 3C, fourth row, second column in each panel). Similarly, E(Z) knockdown (third column) dislodges PRC2 and PRC1 but not SCM, and PC knockdown (fourth column) dislodges PRC1 but not SCM. Taken together, these results suggest that none of the three characterized fly PcG complexes is critically required for SCM chromatin targeting in S2 cells. We note that, aside from the surprising independence of SCM, the ChIP results in Figure 3C are as expected from earlier work which suggested a PcG recruitment hierarchy featuring sequential association of PHO, PRC2, and PRC1 (WANG *et al.* 2004b).

Chromatin associations after SCM knockdown.

ChIP assays were also performed using S2 cells with SCM depleted. SCM chromatin association is significantly reduced, as expected, after SCM RNAi (Fig. 3C, fifth column, fourth row in both panels). This result verifies the specificity of the SCM antibody in generating the SCM ChIP signal.

To assess if SCM plays a role in chromatin association of other PcG components, the SCM-depleted samples were tested by ChIP for PHO, SU(Z)12, and PC (fifth columns). We found that PHO association with b4 and b5 appears unaffected by SCM loss, whereas associations of both PRC2 and PRC1 appear compromised. Thus, this matrix of ChIP assays suggest parallels between PHO and SCM in their effects upon PcG complex recruitment; both PHO and SCM can associate with the Ubx PRE independently of other PcG components and loss of either PHO or SCM appears to reduce or dislodge PRC2 and PRC1.

Characterization of a pupal-lethal Scm allele.

In addition to S2 cell experiments, we wished to extend our analysis of SCM by interrogating chromatin derived from fly tissues *in vivo*. Several studies have successfully exploited *Drosophila* wing imaginal discs to study PcG chromatin associations (CAO *et al.* 2002; PAPP and MULLER 2006; WANG *et al.* 2004b). A key feature of wing discs is that *Ubx* is kept off in this tissue due to PcG silencing. Thus, in contrast to intact embryos, where *Ubx* is expressed in about half the cells and silenced in the other half, larval wing discs provide material for *Ubx* ChIP studies where PcG silencing greatly predominates.

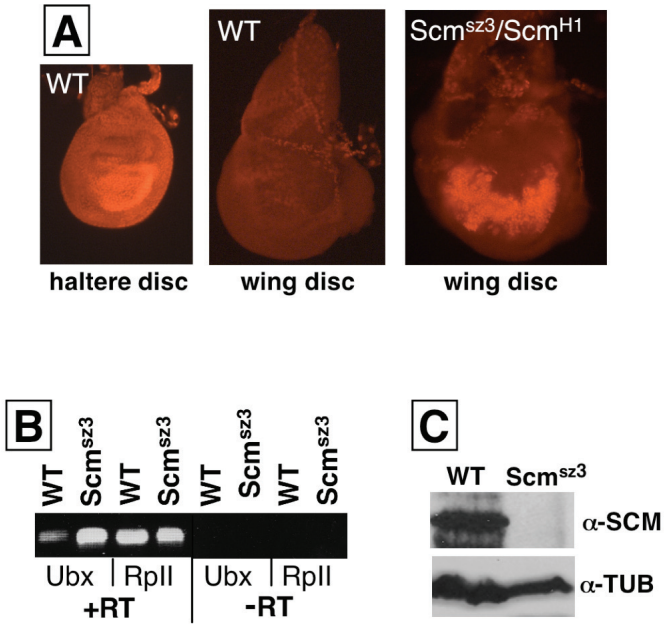
A key challenge to testing *Scm* loss-of-function in larval wing discs is that *Scm* null mutants die as embryos (BORNEMANN *et al.* 1998; BREEN and DUNCAN 1986). Thus, we required a hypomorphic *Scm* mutant that survives to late larval or pupal stages that,

nevertheless, displays substantial *Ubx* desilencing in wing discs. Hypomorphic pupal-lethal *Scm* alleles have been described but their relatively mild phenotypes and retained SCM targeting to *Hox* loci (BORNEMANN *et al.* 1998) suggested that these alleles were too weak for our envisioned ChIP analysis. Consequently, we investigated a set of nineteen novel *Scm* fly mutants to identify at least one that might supply wing discs with robust desilencing of *Ubx*. These nineteen new mutants, kindly provided by Henrik Gyurkovics (Biological Research Center, Szeged, Hungary), were isolated in a screen for modifiers of *miniwhite* silencing by linked PREs. This screen has previously been reported to yield PcG mutations (SHANOWER *et al.* 2005; VAZQUEZ *et al.* 1999).

The nineteen *Scm* alleles were first categorized as putative nulls versus hypomorphs using two criteria: spatial expression of the ABD-A HOX protein in embryos and determination of lethal phase. *Scm* nulls die by late embryogenesis with robust ectopic accumulation of ABD-A, whereas hypomorphs survive to larval or pupal stages and display little or no ABD-A mis-expression in embryos (BORNEMANN *et al.* 1996; BORNEMANN *et al.* 1998; BREEN and DUNCAN 1986). These tests identified seven putative hypomorphic *Scm* alleles that survive to pupal stages and show normal ABD-A patterns in embryos. These hypomorphic alleles were then tested for *Ubx* desilencing in larval imaginal discs. Each mutant was crossed to an *Scm* null, Scm^{H1} (BORNEMANN *et al.* 1998), and tissues dissected from the $Scm^{hypomorph}/Scm^{H1}$ larval progeny were immunostained to reveal patterns of UBX accumulation. As shown in Fig. 4A, UBX normally accumulates in the haltere disc but is absent in wild-type wing discs, where it is subject to PcG silencing (BEUCHLE *et al.* 2001). Of the seven new *Scm* hypomorphs,

Figure 4: Ubx desilencing in *Scm^{sz3}* mutant larvae. (A) Patterns of UBX accumulation revealed by immunostaining of a wild-type haltere disc (left), wild-type wing disc (middle), and an *Scm^{sz3}*/*Scm^{H1}* wing disc (right). (B) RT-PCR analysis of *Ubx* and *RpII140* mRNA levels in wing discs isolated from wild-type (WT) or *Scm^{sz3}*/*Scm^{H1}* mutant larvae. "+RT" indicates reverse transcriptase added and "-RT" indicates control with reverse transcriptase omitted from the reaction. (C) Western blots to detect SCM or α-tubulin (loading control) in extracts from wild-type (WT) or *Scm^{sz3}*/*Scm^{H1}* mutant larvae.

Figure 4


A
WT
WT
Scm^sz3/Scm^H1
haltere disc
wing disc
wing disc
B
WT
Scm^sz3
WT
Scm^sz3
WT
Scm^sz3
WT
Scm^sz3
Ubx
RpII
Ubx
RpII
+RT
-RT
C
WT
Scm^sz3
α-SCM
α-TUB

most showed only subtle UBX mis-expression consisting of isolated cells or small patches of cells. In contrast, a single mutant, Scm^{sz3}, displayed substantial ectopic UBX in the wing disc (Fig. 4A, right panel). The UBX pattern varied from disc to disc, but typically encompassed >30% of the disc territory with preferred accumulation in the wing pouch area. Similarly, RT-PCR analysis revealed *Ubx* desilencing at the mRNA level in Scm^{sz3}/ Scm^{H1} mutant wing discs (Fig. 4B).

By these criteria, Scm^{sz3} was by far the most attractive *Scm* mutant for use in ChIP analysis of wing discs. Furthermore, Western blot analyses of larval extracts detected substantially reduced levels of SCM in the Scm^{sz3} mutant (Fig. 4C). In contrast, SCM accumulates at levels comparable to wild-type in previously characterized *Scm* hypomorphs, such as $Scm^{Su(z)302}$ and Scm^{R5-13} (BORNEMANN *et al.* 1998). Intriguingly, sequence analysis revealed that *sz3* is a missense mutation (L853Q) within the SPM domain (Fig. 1, top). Presumably, the alteration within this conserved protein interaction domain destabilizes SCM, perhaps through reduced ability to bind partners *in vivo*. Indeed, we note that the same *sz3* missense change was independently isolated in a targeted two-hybrid screen for binding-defective SPM domain mutants (L48Q in PETERSON *et al.* 2004). The map locations of the *sz3* allele, plus one other hypomorph (*sz20*) and three other nulls (*sz25*, *sz29*, and *sz36*) determined in this study, are displayed in Fig. 1.

PcG chromatin associations in Scm mutant wing discs.

Wing discs were harvested from Scm^{sz3}/ Scm^{H1} larvae and were subjected to ChIP analysis in parallel with control discs from wild-type larvae. Fig. 5A (left panel) shows that, in wild-type discs, PHO, SU(Z)12, PC, and SCM are all detected in association with the b4 and b5 fragments of the *Ubx* PRE but not on the flanking b6 fragment or on the control fragment from the *RpII140* locus. Thus, the normal wing disc distributions of these PcG proteins, including SCM, mirror their distributions in S2 cells (Figs. 2 and 3C). Fig. 5A, right panel, shows ChIP results from the Scm^{sz3} mutant. In agreement with Western blot analysis (Fig. 4C), little or no SCM is detected on the *Ubx* PRE in this mutant (rightmost column). The remaining ChIP samples show that PHO is retained on the *Ubx* PRE in the *Scm* mutant discs but the signals for SU(Z)12 and PC are reduced. Thus, PHO association appears unaffected by SCM loss-of-function whereas PRE binding by both PRC2 and PRC1 is diminished. These results are in close agreement with PcG associations detected after SCM knockdown in S2 cells (Fig. 3C).

In addition to endpoint-PCR analysis of the wing disc samples (Fig. 5A), we also performed real-time PCR assays to obtain more quantitative readouts (Fig. 5B). These quantitative-PCR (Q-PCR) assays relied upon amplification of *Ubx* PRE fragments 1 and 2, depicted in Fig. 2A, which are subfragments of the b5 and b4 fragments, respectively. The Q-PCR results confirm that there is substantial loss of SCM signal in the *Scm* mutant discs and that PHO is unaffected by this SCM disruption (Fig. 5B, top). In addition, the Q-PCR assays detect reduced SU(Z)12 and PC signals but they do not appear eliminated (Fig. 5B, bottom). We conclude that PHO is retained and there is

Figure 5: PcG chromatin associations at the Ubx PRE in Scm mutant wing discs. ChIP assays to detect PRE binding of PHO, SU(Z)12, PC, and SCM in wild-type or Scm^{sz3}/Scm^{H1} wing discs as revealed by endpoint-PCR (A) and quantitative (real-time) Q-PCR (B). (A) Left panel shows ChIP analysis of wild-type wing discs and right panel shows analysis of Scm^{sz3}/Scm^{H1} wing discs. Antibodies used for immunoprecipitations are indicated at the top of each panel and amplified fragments are indicated to the left. "Mock" indicates control immunoprecipitation with protein A-agarose beads alone. "Rp" indicates a control fragment from the *RpII140* locus. (B) Bar graphs depict Q-PCR ChIP signals obtained using antibodies against the indicated PcG proteins and chromatin samples from wild-type (blue) or Scm^{sz3}/Scm^{H1} (maroon) wing discs. Error bars show standard deviations from the mean determined using at least six independent Q-PCR reactions. For the SU(Z)12 and PC ChIP data (bottom panels), Student's t-test yields $p \leq 0.02$ for all *Scm* mutant versus wild-type comparisons. "PRE1" and "PRE2" correspond to fragments 1 and 2, respectively, in Fig. 2A.

Figure 5

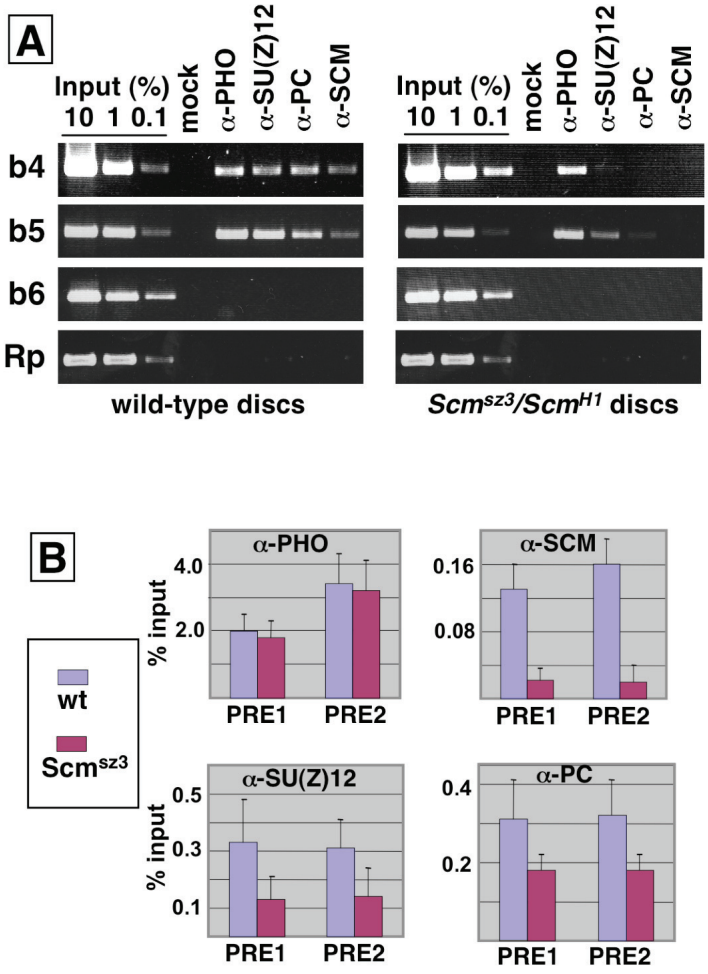
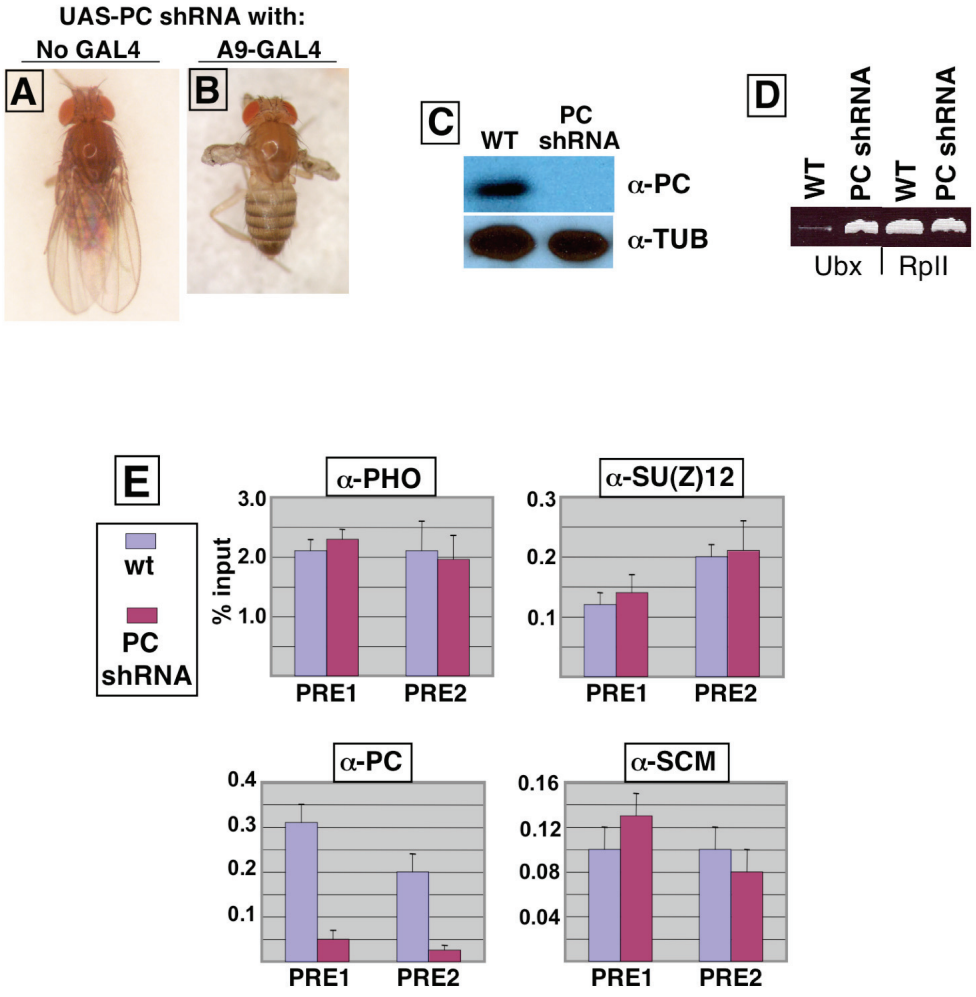

A
Input (%)
10 1 0.1
mock
α-PHO
α-SU(Z)12
α-PC
α-SCM
b4
b5
b6
Rp
wild-type discs
Scm^sz3/Scm^H1 discs
B
wt
Scm^sz3
% input
α-PHO
α-SCM
α-SU(Z)12
α-PC
PRE1
PRE2

Figure 6: Consequences of PC knockdown in wing discs. Phenotypes of adult flies bearing the UAS-Pc shRNA construct without a GAL4 driver (A) or combined with the A9-GAL4 wing disc driver (B). (C) Western blots to detect PC or α-tubulin (loading control) in wing discs from wild-type (WT) or *UAS-Pc shRNA; da-GAL4* larvae. (D) RT-PCR analysis of *Ubx* and *RpII140* mRNA levels in wing discs isolated from wild-type (WT) or *UAS-Pc shRNA; da-GAL4* larvae. (E) Bar graphs depict Q-PCR ChIP signals obtained using antibodies against the indicated four PcG proteins and wing disc chromatin samples from wild-type (blue) or *UAS-Pc shRNA; da-GAL4* (maroon) larvae. Error bars show standard deviations from the mean determined using at least six independent Q-PCR reactions. "PRE1" and "PRE2" correspond to fragments 1 and 2, respectively, in Fig. 2A.

Figure 6


UAS-PC shRNA with:
No GAL4
A9-GAL4
A
B
C
WT
PC shRNA
α-PC
α-TUB
D
WT
PC shRNA
WT
PC shRNA
Ubx
RpII
E
wt
PC shRNA
% input
α-PHO
α-SU(Z)12
α-PC
α-SCM
PRE1
PRE2

partial loss of PRC2 and PRC1 from the *Ubx* PRE when Scm function is compromised *in vivo*.

PcG chromatin associations in wing discs after PC knockdown.

In order to conduct similar ChIP analyses on wing discs bearing loss of PRC1 function, we investigated the feasibility of depleting the PC subunit via an RNA interference approach. Specifically, we tested if conditional expression of a short-hairpin (sh) RNA targeted against Pc mRNA could produce Pc loss-of-function in wing discs. A similar shRNA approach has been used to create *in vivo* knockdown of the PcG protein PCL (SAVLA *et al.* 2008). We obtained a transgenic fly line, called Pc-R4, from among a collection of fly lines (http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp; National Institute of Genetics, Japan) that express individual shRNAs under control of the GAL4 upstream activating sequence (UAS). Crosses were performed to generate progeny bearing both the UAS-Pc shRNA construct and a "driver" construct that expresses GAL4. As shown in Fig. 6B, wing-to-haltere transformations were produced when the A9-GAL4 wing disc driver (HAERRY *et al.* 1998) was used. Thus, targeted expression of Pc shRNA produced the canonical phenotype expected for Pc loss-of-function in wing discs. When the ubiquitous daughterless-GAL4 driver (WODARZ *et al.* 1995) was used instead, the consequence was pupal lethality, presumably reflecting more widespread PC knockdown. Western blot analysis showed that PC levels are dramatically reduced in wing discs dissected from these *da-GAL4*/+; *UAS-Pc shRNA*/+ larvae (Fig. 6C). Likewise, RT-PCR analysis revealed *Ubx* desilencing in these wing

discs (Fig. 6D), verifying that this genotype disrupts PcG silencing at the *Ubx* target locus.

Figure 6E shows the results of ChIP assays performed using wing discs harvested from *da-GAL4*/+; *UAS-Pc shRNA*/+ larvae. Q-PCR analysis of the ChIP samples revealed that this shRNA approach yields 6 to 8-fold reductions of PC bound to the *Ubx* PRE when compared to wild-type (third panel). In contrast, levels of PHO, SU(Z)12, and SCM bound to the PRE appear unaltered (Fig. 6E). Thus, SCM chromatin association at this PRE is retained *in vivo* despite substantial removal of a core PRC1 subunit. This result is consistent with our findings using S2 cells (Fig. 3C). The retention of PHO and SU(Z)12 despite PC knockdown (Fig. 6E) is also consistent with previously described tests on PcG complex recruitment (WANG *et al.* 2004b), which implied that PHO and PRC2 act upstream to target PRC1 to this PRE.

IV. Discussion

SCM and PcG complex recruitment to the PRE

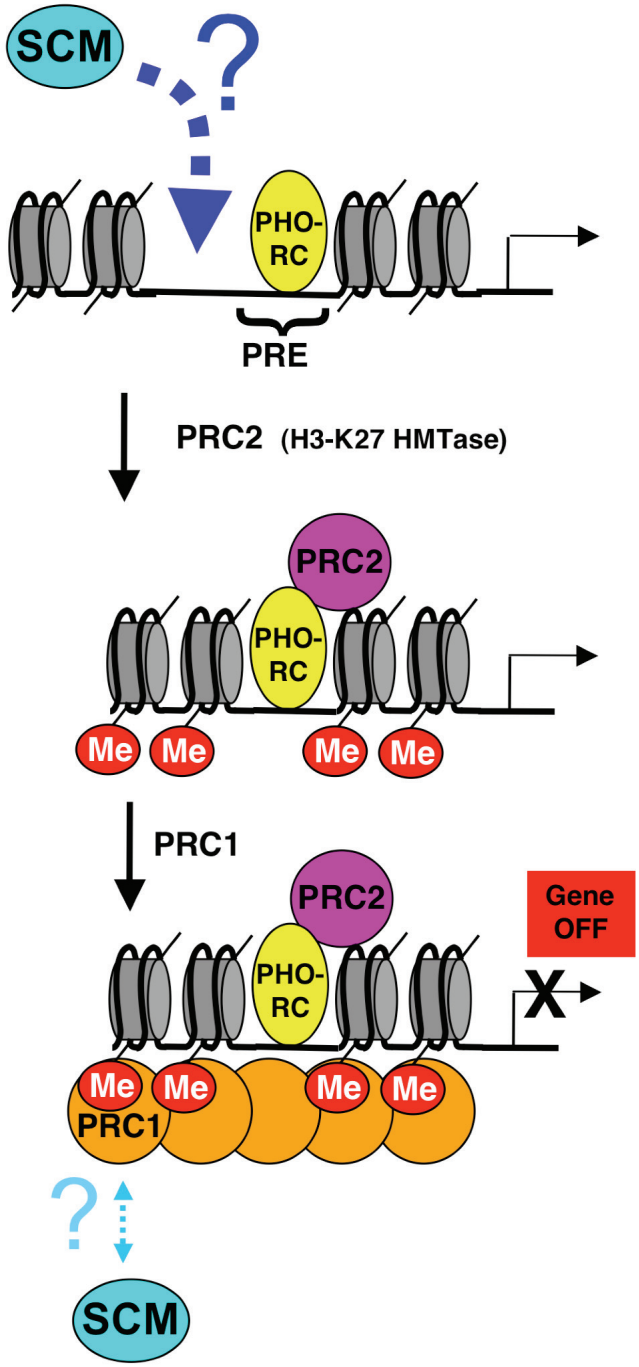
Chromatin immuneprecipitation studies using fly S2 cells, embryos, and wing discs indicate that the DNA-binding protein PHO can recognize PREs *in vivo* and play a key role in recruiting other PcG components to target sites (KLYMENKO *et al.* 2006; WANG *et al.* 2004b; also KWONG *et al.* 2008; OKTABA *et al.* 2008; PAPP and MULLER 2006). The molecular mechanisms of PRC2 and PRC1 recruitment are not fully understood,

but could involve physical interactions between PHO and subunits of these complexes (MOHD-SARIP *et al.* 2005; MOHD-SARIP *et al.* 2002; WANG *et al.* 2004b). Thus, most models for recruitment of fly PcG complexes to target chromatin feature an early step involving PHO binding to PRE DNA, as depicted in Fig. 7. Since PHO exists in a stable heterodimer with the PcG protein SFMBT, which colocalizes with PHO at PREs *in vivo*, the PRE-bound species is the two-subunit PHO-RC complex (KLYMENKO *et al.* 2006).

Once PRC2 has arrived at target chromatin, it is thought to tri-methylate H3-K27 on local nucleosomes (CAO *et al.* 2002; CZERMIN *et al.* 2002; KETEL *et al.* 2005; KUZMICHEV *et al.* 2002; MULLER *et al.* 2002). Since the PRC1 subunit, PC, can specifically recognize trimethylated H3-K27 via binding to its chromodomain (FISCHLE *et al.* 2003c; MIN *et al.* 2003), it has been hypothesized that this interaction helps recruit PRC1 to target loci. Indeed, the loss of PRC1 from target loci following knockdown or inactivation of PRC2 subunits in either *Drosophila* or mammalian cells is consistent with this view (BOYER *et al.* 2006; CAO *et al.* 2005; WANG *et al.* 2004b). Taken together, these results support a proposed multistep recruitment hierarchy (WANG *et al.* 2004b), consisting of initial binding by PHO-RC, then PRC2, and then PRC1 which executes gene silencing (Fig. 7). However, the overall contribution of PC-Me-K27 binding to PRC1 targeting *in vivo* is yet to be determined. An alternative view is that binding of Me-H3-K27 to PRC1 promotes formation of intralocus loops that bring PRE-bound PcG complexes into contact with the body of the gene to be silenced (MULLER and KASSIS 2006; SCHWARTZ and PIRROTTA 2007).

Figure 7: Recruitment of Polycomb Group Complexes. According to this model (CAO and ZHANG 2004b; WANG *et al.* 2004b), recruitment proceeds hierarchically, beginning with PhoRC binding to the PRE, which is depleted of nucleosomes (top). Recruitment of PRC2 is dependent on PhoRC. Once at the target site, PRC2 methylates H3-K27 (middle). Finally PRC1 is recruited to chromatin and promotes gene silencing (bottom). PRC1 is known to bind trimethylated H3-K27, but protein-protein interactions with PhoRC, PRC2, or other factors may also play an important role in PRC1 recruitment. *In vitro* interactions between SCM and the PRC1 component PH initially suggested that SCM might be recruited to chromatin at later stages, with PRC1 (shown at bottom). However, our ChIP data suggest that SCM may instead play a role at an earlier step (shown at top), possibly in parallel with PhoRC and/or in an as-yet undescribed complex with a DNA-binding factor.

Figure 7


SCM
?
PHO-
RC
PRE
PRC2 (H3-K27 HMTase)
PRC2
PHO-
RC
Me
Me
Me
Me
PRC1
PRC2
Gene
OFF
PHO-
RC
X
Me
Me
Me
Me
PRC1
?
SCM

How might SCM fit in molecularly with the other PcG components depicted in Figure 7? Although *in vitro* associations of SCM with PRC1 subunits have been described (PETERSON *et al.* 1997; PETERSON *et al.* 2004; SAURIN *et al.* 2001; SHAO *et al.* 1999), the ChIP analyses here indicate that SCM can associate with the *Ubx* PRE despite the loss of PRC1 (Figs. 3C, 6E). Similarly, although SCM can bind to the PHO-RC subunit SFMBT *in vitro* (GRIMM *et al.* 2007), SCM localization at the PRE does not appear dependent upon PHO (Fig. 3C). Taken together, these ChIP results are consistent with biochemical studies that reveal SCM separability from PHO-RC, PRC1, and PRC2 in fly embryo extracts (KLYMENKO *et al.* 2006; NG *et al.* 2000; PETERSON *et al.* 2004).

An intriguing finding from our matrix of molecular epistasis tests is that SCM exhibits very similar recruitment properties as compared to PHO. Specifically, both SCM and PHO can localize to the *Ubx* PRE independent of all other PcG components tested and loss of either SCM or PHO diminishes PRC2 and PRC1 association with the PRE (Figs 3C, 5A and B, 6E). This similarity suggests that SCM may function, like PHO-RC, at an early step in PcG recruitment. In this context, it is worth emphasizing the striking overall similarities between SCM and the PHO-RC subunit, SFMBT (Fig. 1). Perhaps SCM partners with a yet-to-be identified PcG DNA-binding protein, akin to the functional partnership of SFMBT with PHO (KLYMENKO *et al.* 2006). Indeed, since PHO-binding sites are insufficient for PRE function *in vivo* (FRITSCH *et al.* 1999; SHIMELL *et al.* 2000) and many other DNA-binding proteins have been implicated in *Drosophila* PcG silencing (MULLER and KASSIS 2006), there is abundant evidence that PRE sequence recognition involves more than just PHO-RC. The consensus view is

that PREs contain a composite of PHO sites plus additional types of factor-binding motifs (MULLER and KASSIS 2006). At present, little is known about the nature of SCM-containing complexes beyond the detection of an approximately 500 kD complex in fly embryo extracts (PETERSON *et al.* 2004). It will be informative to characterize stably associated SCM partner proteins and evaluate their potential roles in binding to PRE DNA.

Although the ChIP assays presented here, together with previous biochemical tests (KLYMENKO *et al.* 2006; NG *et al.* 2000; PETERSON *et al.* 2004), emphasize SCM separability from other PcG components, SCM must still be integrated with its PcG cohorts to achieve gene silencing. This interdependence is highlighted by *in vivo* assays where robust silencing of a *miniwhite* reporter by a tethered form of SCM is disrupted if the PRC1 subunit, PH, is compromised by mutation (ROSEMAN *et al.* 2001). If PRC1 is the PcG complex most central to the silencing mechanism (Fig. 7), then the most likely molecular roles for SCM are to help recruit PRC1 to target loci and/or to potentiate the PRC1 activity that directly impedes transcription. To address these possibilities, it will first be necessary to establish the rate-limiting steps impacted in PcG silencing.

Functional domains shared in SCM and SFMBT

Ultimately, a precise understanding of SCM function requires deciphering the mechanistic contributions of each of its three identified domains. As depicted in Figure 1, SCM contains a C-terminal SPM domain, two mbt repeats, and two Cys2-Cys2 type zinc fingers. Strikingly, each of these domains is present also in SFMBT (Fig. 1), suggesting that the overall biochemical roles of these two PcG components are very

similar. In addition, the PH PcG protein possesses two of these three homology domains (Fig. 1). This presents the curious situation where three different PcG proteins are related by shared domains yet none reside in a stable common complex, at least not in soluble nuclear extracts (KLYMENKO *et al.* 2006; NG *et al.* 2000; PETERSON *et al.* 2004).

There is currently *in vitro* and *in vivo* data on roles of the SPM domain and mbt repeats, but little knowledge yet about the zinc fingers. The SPM domain is a subtype within the broader category of SAM domains that mediate protein interactions (BORNEMANN *et al.* 1996; PONTING 1995; QIAO and BOWIE 2005). The SCM version of this domain is capable of robust self-binding and cross-binding to the PH version *in vitro* (PETERSON *et al.* 1997) and SCM-SFMBT interactions have also been noted (GRIMM *et al.* 2007). The importance of SPM domain interactions *in vivo* is emphasized by PcG phentoypes observed after over-expressing a dominant-negative isolated SPM domain in developing flies (PETERSON *et al.* 2004). Yet it remains unclear precisely what SPM interactions contribute to the PcG silencing mechanism. The simple idea that they constitutively glue PcG complex subunits together is at odds with their separabilities in embryo extracts. Perhaps SPM interactions primarily function directly at chromatin targets, where they could sponsor contacts among different PcG complexes rather than among subunits within the same complex. Such chromatin-specific interactions could contribute to intralocus loops that have been hypothesized at PcG silenced loci (MULLER and KASSIS 2006; SCHWARTZ and PIRROTTA 2007).

The functional significance of the SCM mbt repeats is reflected by partial loss-of-function alleles that alter the first repeat (BORNEMANN *et al.* 1998) and by Hox gene silencing defects observed after disruption of the second repeat (GRIMM *et al.* 2007). Recent structural determinations and *in vitro* binding studies have revealed that mbt repeats are modules for binding to methylated lysines (GRIMM *et al.* 2007; KLYMENKO *et al.* 2006; SANTIVERI *et al.* 2008; SATHYAMURTHY *et al.* 2003). Since trimethylated-H3-K27 is a prominent feature of PcG-silenced chromatin, the mbt repeats, at first glance, could play a role akin to the PC chromodomain. However, there are important differences between the substrate-binding properties of these mbt repeats versus the PC chromodomain. First, the mbt repeats prefer mono- and, to a lesser, extent di-methylated lysines whereas the chromodomain prefers the tri-methyl form (FISCHLE *et al.* 2003c; MIN *et al.* 2003). An intriguing hypothesis is that this mono/di preference could reflect a "grappling hook" function whereby hypomethylated nucleosomes are recognized and brought into proximity for tri-methylation by PRC2 (KLYMENKO *et al.* 2006; MULLER and KASSIS 2006). Another distinction is that the binding mode of mbt repeats is largely independent of peptide sequence context whereas chromodomain binding depends upon residues flanking the methylated lysine (GRIMM *et al.* 2007; SANTIVERI *et al.* 2008). Consistent with this, the SCM mbt repeats lack binding preference for any of the histone tail lysines (GRIMM *et al.* 2007; SANTIVERI *et al.* 2008). Thus, mbt repeats provide a pocket for methyl-lysine binding, but it is not yet clear if the relevant substrate is a particular methylated histone or even a nonhistone protein. Certainly, the *in vitro* binding preferences could be modified by additional mbt-associated factors *in vivo*.

Figure 8: Alignment of Zinc Finger Domains in SCM, SFMBT, and PH. Amino acid residues from the two zinc finger domains in *Drosophila* SCM, with the zinc finger domains in *Drosophila* SFMBT and PH, and three human PH homologs. Two human SCM homologs and two human SFMBT homologs have no zinc fingers. Zinc co-ordinating cysteines are shown in bold. The amino acid residues shown from each protein are indicated in the column on the right. The consensus sequence is shown at bottom.

Figure 8

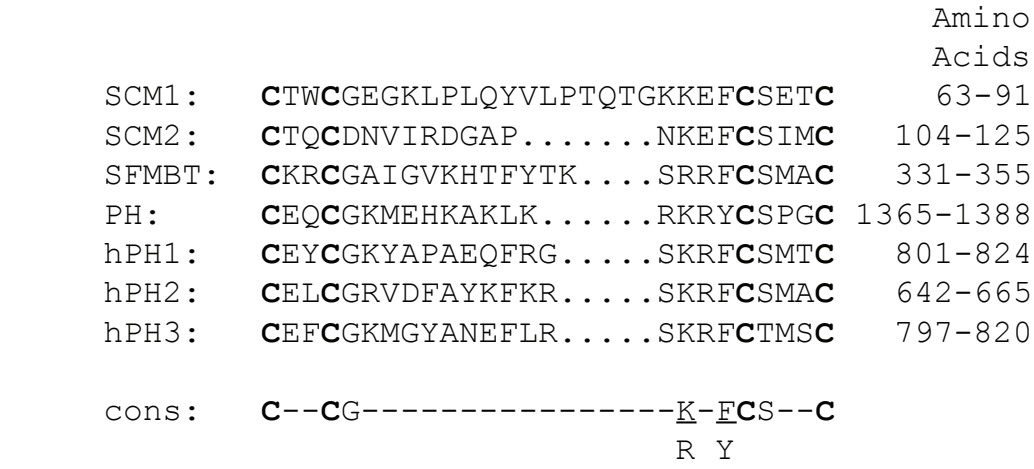

Amino
Acids
SCM1: CTWCGEGKLPLQYVLPTQTGKKEFCSETC 63-91
SCM2: CTQCDNVIRDGAP.....NKEFCSIMC 104-125
SFMBT: CKRCGAIGVKHTFYTK....SRRFCSMAC 331-355
PH: CEQCGKMEHKAKLK.....RKRYCSPGC 1365-1388
hPH1: CEYCGKYAPAEQFRG.....SKRFCSMTC 801-824
hPH2: CELCGRVDFAYKFKR.....SKRFCSMAC 642-665
hPH3: CEFCGKMGYANEFLR.....SKRFCTMSC 797-820
cons: C--CG----------K-FCS--C
R Y

Figure 8 shows a sequence alignment of the Cys2-Cys2 fingers present in SCM, SFMBT and PH. It seems clear that this Zn finger is a distinct subtype that adheres to the consensus sequence CXXC GX_nF/Y CSXXC. These fingers do not appear to function by binding DNA since sequence-specific binding is not observed *in vitro* for any of them (BORNEMANN *et al.* 1996; BORNEMANN *et al.* 1998; KLYMENKO *et al.* 2006). Thus, their molecular role is unknown but their common inclusion in fly PcG proteins that also share SPM domains and mbt repeats suggests some key contribution to PcG chromatin function. Curiously, both the SCM and SFMBT human homologs appear to have lost their Cys2-Cys2 fingers, whereas all three human PH homologs have retained them (Fig. 8). Thus, if these fingers are critical in PcG silencing, then they apparently can be supplied from different combinations of PcG proteins in flies versus mammals. It will be important to test the genetic requirement for the SCM Zn fingers in *Drosophila* and to define the mechanistic contributions of all three SCM functional domains to PcG chromatin silencing.

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Appendix

Phosphorylation of histone H3 Serine-28 in *Drosophila*

I. Introduction

Covalent modification to histone tail domains is in important factor determining chromatin structure and the transcriptional state of nearby genes (BHAUMIK *et al.* 2007; ITO 2007). The histone code hypothesis promotes the idea that these covalent modifications constitute marks that signal chromatin modifying factors and help determine chromatin dynamics (JENUWEIN and ALLIS 2001; STRAHL and ALLIS 2000). The marks that can be applied to histone tails include acetylation, phosphorylation and methylation. Different modifications may influence each other and also influence the factors that apply and read modifications, opening the possibility for combinatorial regulation by higher-order interactions between systems of chromatin modifying factors (BERGER 2007; BUSZCZAK *et al.* 2009; FISCHLE 2008; FISCHLE *et al.* 2003b). Serine-28 on histone H3 (H3-S28) is adjacent to lysine-27 (H3-K27) which is methylated during Polycomb repression (MULLER *et al.* 2002; reviewed in SIMON 2009). Since H3-S28 can be phosphorylated, there is interest in determining whether H3-S28 phosphorylation is a regulator of Polycomb repression.

Many serine and threonine residues in histone tails accept phosphorylation (ITO 2007). Paradoxically, serine phosphorylation is associated with both the most condensed and

the most relaxed states of chromatin (NOWAK and CORCES 2004; PRIGENT and DIMITROV 2003). The phosphorylation of serine-10 on histone H3 (H3-S10) is the most extensively studied phosphorylation modification, and was originally observed during mitosis (HENDZEL *et al.* 1997; WEI *et al.* 1998), when chromosomes are most highly condensed. Although the correlation between mitotic condensation and H3-S10 phosphorylation is not perfect, it is widely regarded as a mitotic marker (JOHANSEN and JOHANSEN 2006). Subsequently, phospho-H3-S10 was also observed in transcriptionally active locations, where chromatin is highly relaxed (LABRADOR and CORCES 2003; NOWAK and CORCES 2000; NOWAK *et al.* 2003).

Several different kinases have been reported to apply phosphorylation to H3-S10 (Ito 2007). The Aurora B kinase is responsible for the phosphorylation that accompanies mitotic condensation (HSU *et al.* 2000), and the JIL-1 kinase phosphorylates H3-S10 during interphase in *Drosophila* (JIN *et al.* 1999; WANG *et al.* 2001b) and is associated with chromatin decondensation at transcriptionally active loci. Recent work that targets JIL-1 to ectopic locations on *Drosophila* polytene chromosomes (DENG *et al.* 2008) produces chromatin decondensation, even in normally heterochromatic regions, but is not accompanied by transcriptional activation. This suggests that chromatin relaxation and transcription are separable phenomena and that H3-S10 phosphorylation is causally connected only to the former. Because the phosphorylation marks are applied by different kinases, it is possible that the subsequent condensation or relaxation is determined by factors associated with the specific kinase that applies the mark. There is also interest in whether the total pattern of chromatin marks, including acetylation and

methylation events on other residues, influences the changes to chromatin structure that result from phosphorylation (NOWAK and CORCES 2004).

The preponderance of serine residues adjacent to lysine residues in histones has motivated a hypothesis that transitions between chromatin states can be mediated by a binary "methyl/phos switch" (FISCHLE *et al.* 2003a). In this model, lysine methylation and phosphorylation of the adjacent serine are mutually antagonistic modifications, and so the lysine-serine pair are generally found in a bistable state where one or the other, but not both, residues are modified. The presence of two distinct modifications is hypothesized to increase the robustness of the signal to chromatin modifying factors that read these marks. This theory also predicts that there should be modules on chromatin-associated factors that specifically recognize phosphorylated serine residues, but so far none have been reported. An alternative mechanism for a methyl-phos switch is simply that the presence of a phosphate group on the adjacent residue disrupts binding of a module such as the chromodomain that specifically recognizes methyl-lysine (JOHANSEN and JOHANSEN 2006). Because methylation tends to be a stable modification, the application and removal of the adjacent phosphate would provide a mechanism for transient changes to the chromatin state over short transition times.

The phosphorylation of H3-S10 provides some support for the existence of a methyl-phos switch. Lysine-9 of histone H3 (H3-K9) is immediately adjacent to H3-S10. H3-K9 accepts methylation from the SET domain histone methyltransferase SU(VAR)3-9, and the methylated lysine is recognized by the chromodomain of HP1

(BANNISTER *et al.* 2001; LACHNER *et al.* 2001). This methylation and subsequent binding by HP1 promotes the formation of heterochromatin and gene silencing. Phosphorylation of H3-S10 has been observed to release HP1 binding in preparation for mitosis (FISCHLE *et al.* 2005; HIROTA *et al.* 2005) and to antagonize the formation of heterochromatin during interphase (DENG *et al.* 2007; ZHANG *et al.* 2006). This regulation of the SU(VAR)3-9/HP1 system by H3-S10 kinases can be interpreted as the operation of a methyl/phos switch. It also suggests a solution to the paradox of H3-S10 phosphorylation's association with such widely different chromatin states, since both mitotic chromosome condensation and chromatin relaxation require the dissociation of HP1 (JOHANSEN and JOHANSEN 2006).

The H3-K9/S10 example motivates speculation that H3-S28 phosphorylation might exert a similar regulatory effect on Polycomb repression. Both the H3-K9/S10 residues and the H3-K27/S28 residues appear within the amino acid sequence ARKS in the histone H3 tail (PRIGENT and DIMITROV 2003) suggesting that they may have similar functional properties. Moreover, like HP1, the Polycomb Repressive Complex 1 (PRC1) component Polycomb (PC), contains a chromodomain that binds to methyl-lysine, although in the case of PC, the binding is specific for H3-K27 (see Chapter One).

H3-S28 phosphorylation was discovered serendipitously in mammalian cells by researchers who were creating antibodies to phosphoproteins when they noticed that one sample of antibodies reacted with an antigen on histones (GOTO *et al.* 1999).

Further investigation revealed that the specific antigen recognized was phospho-H3-S28. In this initial work, the modification was detected only on mitotic chromosomes. Subsequent work established that Aurora B kinase phosphorylates H3-S28 as well at H3-S10 (GOTO *et al.* 2002), but while heavy H3-S10 phosphorylation initiates just prior to mitosis, (HENDZEL *et al.* 1997), H3-S28 is not phosphorylated until early prophase (GOTO *et al.* 1999). Later, H3-S28 phosphorylation was also observed on interphase chromosomes in mammalian cells (DUNN and DAVIE 2005; DYSON *et al.* 2005). A recent study that fractionated chicken erythrocyte chromatin found that phosphorylated H3-S28 was enriched in fractions associated with transcriptional activation, such as the promoters of transcriptionally active and competent genes (SUN *et al.* 2007). They also presented evidence that nucleosomes phosphorylated at H3-S28 dissociate from DNA more easily, suggesting that H3-S28 phosphorylation promotes chromatin relaxation. Both H3-S10 and H3-S28 are observed to be phosphorylated in response to stimulating Mitogen Activated Protein Kinase pathways. However, immunostaining and immunoprecipitation reveal that the two phosphorylations are applied independently. A MAP triple kinase has actually been demonstrated as an H3-S28 kinase in response to both stress and EGF signaling (CHOI *et al.* 2005).

As a first step towards investigating whether there is a regulatory interaction between H3-S28 phosphorylation and Polycomb repression, we performed a series of immunostaining experiments in *Drosophila* cells and tissues. Our purpose was to survey the prevalence and behavior of H3-S28 phosphorylation in *Drosophila*.

II. Materials and Methods

Fly Stocks. All flies used for these experiments .were from a pseudo wild type stock, *y,Df(1)w*^{67c2}.

S2 cell staining. Cultured cells were stained in glass chamber slides pre-treated with 0.5mg/ml concanavilin A for one hour to promote cell attachment. Cells were allowed to attach for 1-2, then culture media was then removed and wells were rinsed with PBS. The fixation and staining method is described in (MESSMER *et al.* 1992). Monoclonal rat α-phospho-H3-S28 (HTA28, Sigma) was used at 1:100. Goat-α-rat secondary antibody conjugated to Cy3 (Jackson) was preadsorbed against fixed cells and then used at 1:2000.

***Drosophila* embryo and larval immunostainings.** Embryos were collected for 24 hours on agar plates and then dechorionated, fixed and devitellinized according to a procedure as described (MITCHISON and SEDAT 1983) with changes suggested by G Struhl. Embryos were dechorionated in household bleach diluted to half strength for 30 seconds and washed thoroughly with water. Dechorionated embryos were placed in a glass tube. 900µl of a solution containing 0.1M PIPES, 2mM $MgSO_4$, and 1mM EGTA at pH9.6 was added, followed by 100µl of 37% formaldehyde and 2ml heptane. The embryos float in the interface between the aqueous solution and the heptane. Debris such as agar and hatched larvae sink to the bottom. Embryos were fixed and

permeablized in the interface for 20 min at room temperature with gentle agitation. After fixation, debris on the bottom of the tube and the fixation buffer in the (lower) aqueous phase was removed leaving the embryos behind in the heptane. 2ml of methanol was then added, followed by vigorous agitation for 15-30 seconds. Embryos then sank to the bottom, leaving ruptured vitelline membranes at the interface. The heptane (upper) phase and interface was removed, followed by removal of the methanol, leaving the fixed embryos in the tube. The embryos were then rinsed twice in methanol.

Staining was performed as described (KARCH *et al.* 1990), with modifications for use with fluorescent secondary antibodies. Embryos were rinsed briefly in BBT solution containing 55mM NaCl, 40mM KCl, 15mM $MgSO_4$, 5mM $CaCl_2$, 20mM glucose, 50mM sucrose, 10mM tricine, 0.1% Triton X-100 and 0.1% BSA at pH7.0 and then blocked in BBT for 10 min. Rat monoclonal α-phospho-H3-S28 was diluted 1:100 in BBT. Embryos were incubated in primary antibody overnight at 4°C. After primary antibody, embryos were washed in BBT for a total of three hours with six changes of solution. Goat-α-rat secondary antibody conjugated to Cy3 (Jackson) was used at 1:2000 in BBT containing 1µg/ml DAPI. Secondary staining was also overnight at 4°C, followed by three hours of washing in BBT as before. Embryos were mounted in Vectashield (Vector Laboratories). Digital pictures were taken with a Leica DMRB microscope and AxioCam MRc camera with MRGrab software (Zeiss).

Larval tissues were stained as described in Chapter Four using the same α-phospho-H3-S28 and secondary antibodies, at the same dilutions, as for embryo stainings.

Chromosome squashes. Chromosome squashes were prepared as described in Chapter Three. Rat monoclonal α-phospho-H3-S28 (see above) was diluted 1:100. Goat-α-rat secondary antibody conjugated to Cy3 (Jackson) was used at 1:2000.

III. Results

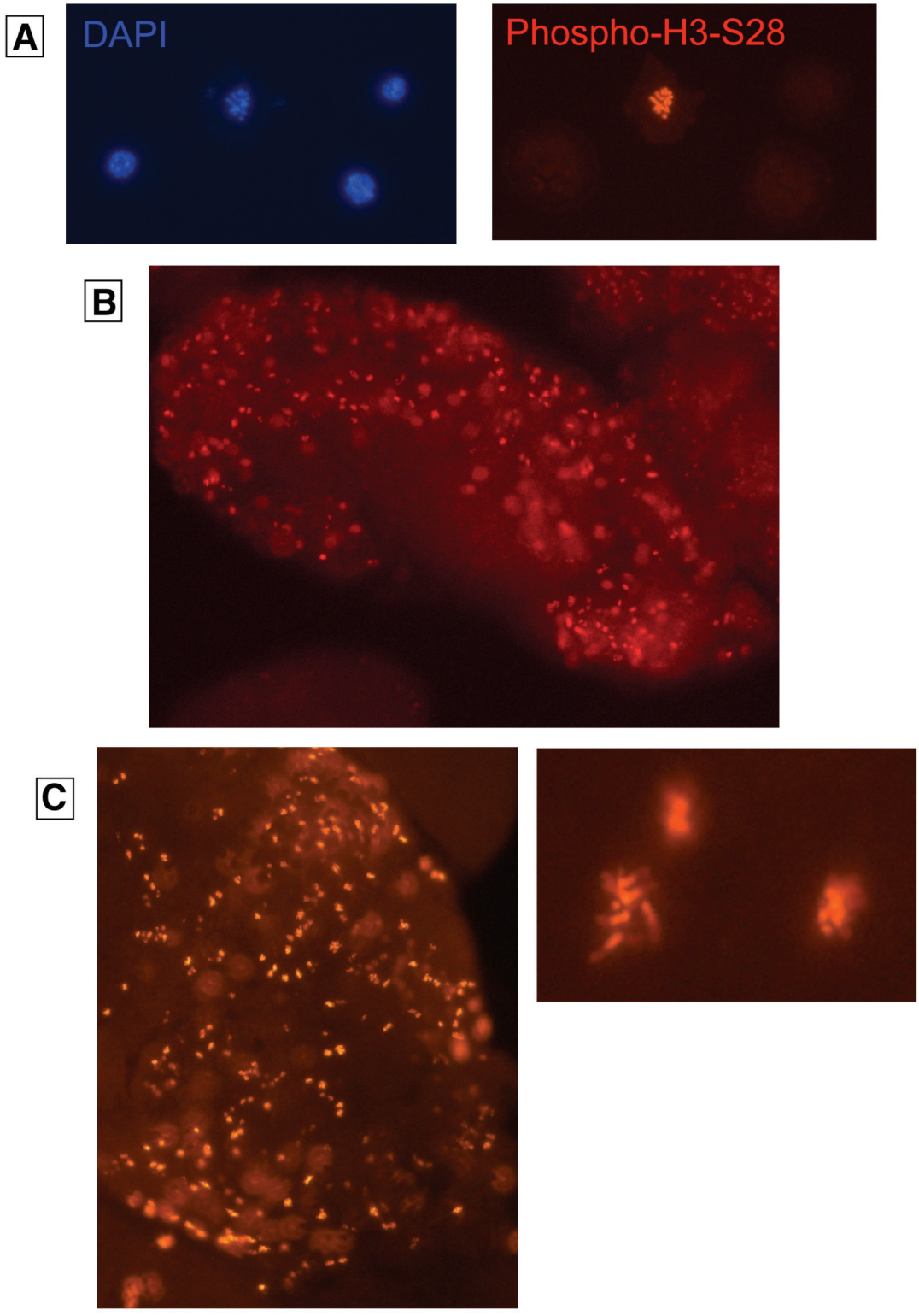
H3-S28 is phosphorylated on mitotic chromosomes in Drosophila.

Figure 1A shows cultured *Drosophila* S2 cells stained with a mouse monoclonal antibody that specifically recognizes phosphorylated H3-S28 (GOTO *et al.* 1999). There is no staining on the three interphase nuclei, but a strong signal on the condensed chromatin of the single nucleus in mitosis. Antibody concentrations that were high enough to produce a signal in interphase nuclei also resulted in equally strong staining of the cytoplasm (data not shown), suggesting that the nuclei of interphase cells do not stain above background level with this antibody.

We also stained whole mount embryos (Figure 1B) and larval tissues (Figure 1C). The nuclei that stained for phospho-H3-S28 in both cases were widespread throughout the tissue, but only a fraction of the total nuclei stained. The stained nuclei contain mitotic figures, which were visible at higher magnification (see Figure 1C, inset). Thus, we

Figure 1: Phospho-H3-S28 staining on mitotic chromosomes. Phosopho-H3-S28 is stained in red for cultured *Drosophila* S2 cells (A), a mid-stage embryo (B), and larval brain lobe (C). Higher magnification on the larval brain (inset) reveals mitotic figures. Ellen Miller assisted with embryo and larval stainings.

Figure 1



conclude in these tissues that the phospho-H3-S28 signal is accumulating specifically on mitotic chromosomes.

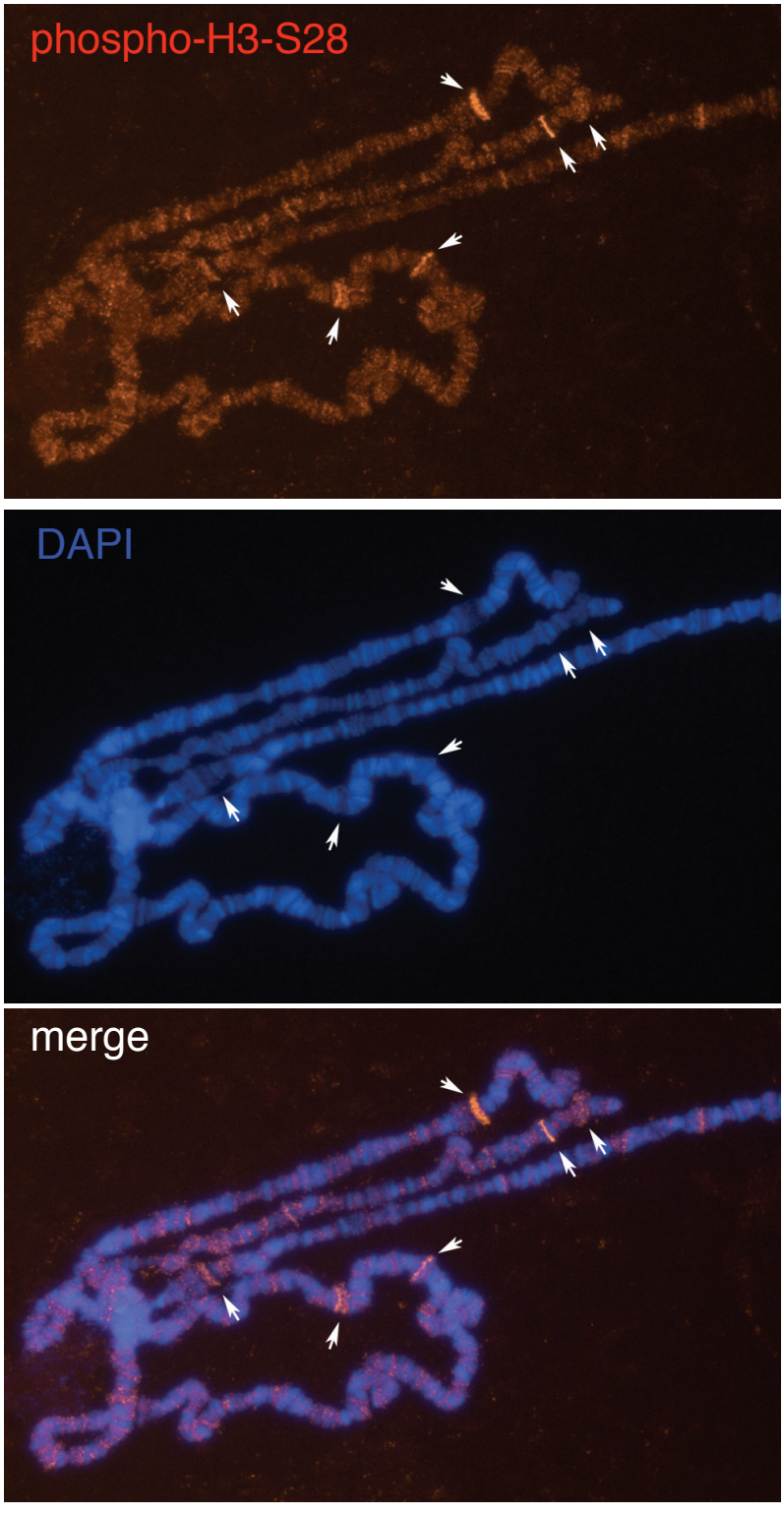
Phospho-H3-S28 accumulates at transcriptionally active loci on polytene chromosomes.

To survey the occurrence of phospho-H3-S28 on *Drosophila* polytene chromosomes, we prepared chromosomes squashes from wild-type larvae. As can be seen in Figure 2, there is some signal along the length of the chromosomes. However, the most striking feature of the staining pattern is the accumulation of signal at the transcriptional puffs. Transcriptional puffs are regions of decondensed chromatin that support heavy transcription of local genes. In normally developing larvae, these puffs represent genes activated by the hormone ecdysone (THUMMEL 2002) and occur at stereotypical locations along the chromosomes. The correlation between transcriptional puffs and strong bands of phospho-H3-S28 staining was not absolute, but it was consistent enough to suggest that phospho-H3-S28 was accumulating specifically at transcriptionally active loci on the chromosomes.

At the time these experiments were performed, H3-S28 phosphorylation had not yet been reported in interphase nuclei and a current review (PRIGENT and DIMITROV 2003) considered it exclusively a mitotic modification. Thus we were curious if the accumulations of phospho-H3-S28 that we observed on ecdysone puffs would be present at transcription sites induced by experimental manipulations. Such an experiment would provide stronger evidence that H3-S28 phosphorylation is involved

Figure 2: Phospho-H3-S28 staining on *Drosophila* polytene chromosomes. Larval salivary glands were squashed and immunostained for phospho-H3-S28. Arrows denote some of the most intensely staining regions, which correspond to chromosome puffs as revealed by DAPI counterstaining.

Figure 2

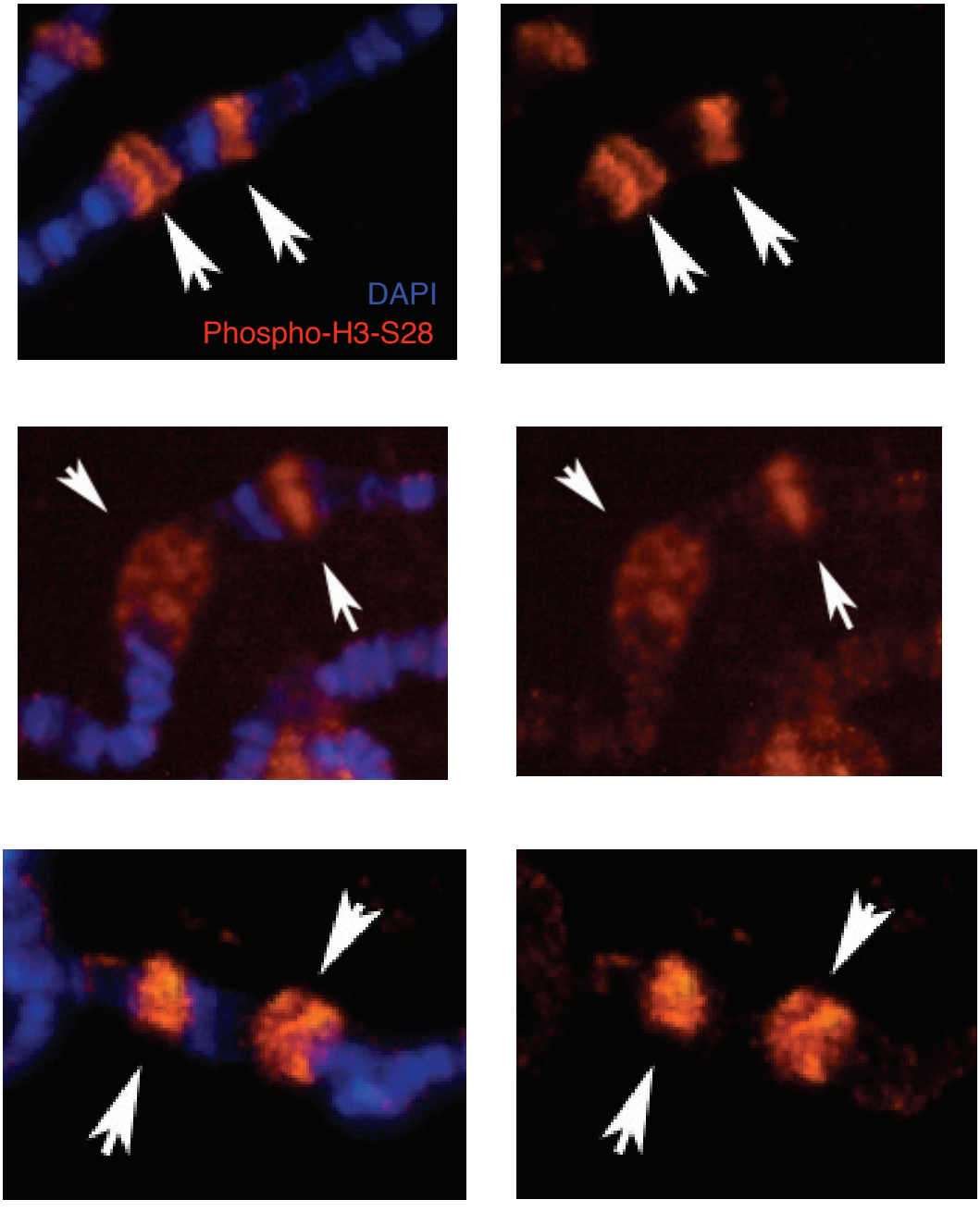


in the cause and effect processes of transcriptional activation. We chose to heat shock larvae and look for changes in the distribution of phospho-H3-S28. Heat shock produces dramatic changes in transcription as the genes for chaperone proteins are highly transcribed and other transcriptional activity is suspended (LINDQUIST 1986). On Polytene squashes, these changes can be visualized by observing transcriptional puffing at the loci for the genes encoding these chaperone proteins (ASHBURNER 1970). We heat shocked larvae for 20 to 30 minutes to produce maximal puffing (SIMON *et al.* 1985) and prepared chromosome squashes.

Figure 3 shows detailed views of the 87A and 87C heat shock loci of the third chromosome. Phospho-H3-S28 is now accumulating strongly at these loci under heat shock, suggesting that H3-S28 is being phosphorylated specifically at transcriptionally active loci. However, the overall pattern of weak basal staining along the length of the chromosomes punctuated by intense bands at discrete locations did not change, only the positions of some of the bands. Taken together, these results stand in contrast to the reported pattern of changes to the phospho-H3-S10 distribution on polytene chromosomes previously reported (NOWAK and CORCES 2000). H3-S10 is heavily phosphorylated over widespread areas of the genome under normal conditions and concentrates to discrete loci under heat shock. The contrast between reported results for phospho-H3-S10 staining and the results we observe for H3-S28 staining helps to validate the specificity of the antibody we are using.

Figure 3: Phospho-H3-S28 accumulates at heat shock puffs. Detailed views of heat shock puff loci from chromosome squashes prepared immediately after larvae were subjected to heat shock. Pictures show the 87A and 87C heat shock loci with abundant phospho-H3-S29 accumulation.

Figure 3



IV. Discussion

The evidence we present indicates that H3-S28 phosphorylation in *Drosophila* occurs in the general pattern that has been previously observed in vertebrate cells (CHOI *et al.* 2005; DUNN and DAVIE 2005; DYSON *et al.* 2005; GOTO *et al.* 1999; SUN *et al.* 2007). Mitotic chromosomes in *Drosophila* cells and tissues appear to be heavily phosphorylated at H3-S28 and we see accumulation of phospho-H3-S28 at actively transcribed loci on polytene chromosomes. Like in the mammalian cells (CHOI *et al.* 2005; DUNN and DAVIE 2005; DYSON *et al.* 2005) we see H3-S28 phosphorylated during transcription associated with the stress response and developmental signaling. It is not clear why we do not detect H3-S28 phosphorylation during interphase in proliferating cells, although the initial observations in cultured mammalian cells were also unable to detect this modification during interphase (GOTO *et al.* 1999; PRIGENT and DIMITROV 2003). It may be an artifact of the different fixation process involved in whole-mount tissue staining versus chromosome squashes, or it may be a biological difference between cells that are still in the mitotic cycle and larval cells that are undergoing endoreplication rather than mitosis.

The hypothesis that H3-S28 phosphorylation might be antagonistic to Polycomb repression is consistent with phospho-H3-S28 association with transcriptionally active loci. A chromatin fractionation study (SUN *et al.* 2007) found that phospho-H3-S28 was enriched in the same transcriptionally active and competent fractions that were also enriched for the H3 lysine-4 (H3-K4) methylation mark that is applied by the Trithorax

Group proteins (SIMON and TAMKUN 2002). However, the fractionation study did not establish whether phospho-H3-S28 and methyl-H3-K4 were both present on the same histone tails or even at the same genomic regions.

If H3-S28 phosphorylation is a negative regulator of Polycomb repression, there are at least two possible ways it could function. One would be that phospho-H3-S28 would prevent PRC2 from methylating H3-K27, and so it would help prevent Polycomb repression on transcriptionally active genes. Another possibility would be that H3-S28 becomes phosphorylated at loci that already carry the methyl-H3-K27 modification as a way of quickly and/or transiently relieving Polycomb repression. These hypotheses make predictions about the genomic distribution of these modifications. If phospho-H3-S28 prevents the establishment of Polycomb repression, then we should expect to see methyl-H3-K27 excluded from regions of H3-S28 phosphorylation, whereas if phospho-H3-S28 relieves pre-existing Polycomb repression, then we would expect to see not only overlap, but also doubly-modified histones. In pilot experiments that double-stained polytene chromosomes for both phospo-H3-S28 and trimethyl-H3-K27 (data not shown) we saw some coincidence of the two modifications, but we primarily observed regions where adjacent bands seemed to exclude each other. The low resolution of immunostaining experiments limits their ability to address these alternatives. In any case, it is not clear whether either of the antibodies used in the pilot experiment is capable of recognizing a doubly-modified histone. Moreover, since each nucleosome contains two copies of each core histone, there are two histone H3 tails in each nucleosome, which may carry different modifications. Final resolution of the

question of whether histones can carry both K27 and S28 modifications at the same time may require the generation of an antibody specific for the double modification or the analysis of histones by mass spectrometry, which can distinguish the modifications of individual histone residues (JOSHI *et al.* 2008; PETERS *et al.* 2003).

Finally, evolving ideas on the role of H3-K27 methylation in Polycomb repression affect the significance of hypothesized interactions with phospho-H3-S28. As we have discussed in previous chapters of this dissertation, H3-K27 has been theorized as the primary mechanism for initial recruitment and retention of PRC1 at Polycomb sites (CAO *et al.* 2002; SIMON 2003), whereas other researchers propose that trimethyl-H3-K27 functions primarily to stabilize intra-locus looping structures at target loci after its initial recruitment by some other mechanism, such as protein-protein interactions (PAPP and MULLER 2006; SCHWARTZ and PIRROTTA 2007). Thus, some models of PcG complex recruitment predict that PRC1 would completely dissociate from chromatin if H3-S28 were phosphorylated, but others predict that PRC1 might persist at its initial recruitment site even if trimethyl-H3-K27 binding was disrupted by H3-S28 phosphorylation. This limits the analogy to HP1, which appears to completely dissociate from chromosomes when H3-S10 is phosphorylated (FISCHLE *et al.* 2005; HIROTA *et al.* 2005; JOHANSEN and JOHANSEN 2006). Abolishing methyl-H3-K27 binding would presumably destabilize hypothesized looping structures at loci under Polycomb repression but the significance of these structures themselves in unclear. While no one doubts the importance of H3-K27 methylation in Polycomb repression, it may be necessary to further characterize its mechanistic role before we can develop a

testable model of how H3-S28 phosphorylation would exercise a regulatory influence on Polycomb repression.

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