REGULATION OF PSC-SU(Z)2 GENES

IN DROSOPHILA MELANOGASTER

by

SUNG-YEON PARK

A Dissertation submitted to the

Graduate school–New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Biochemistry

written under the direction of

Dr. Vincenzo Pirrotta

and approved by

_____

_____

_____

_____

New Brunswick, New Jersey

May, 2008

ABSTRACT OF THE DISSERTATION

REGULATION OF *PSC-SU(Z)2* GENES IN *DROSOPHILA MELANOGASTER*

by

SUNG-YEON PARK

Dissertation Director:

Dr.Vincenzo Pirrotta

The *Psc* and *Su(z)2* genes belong to the Polycomb Group (PcG) and Psc itself is one of the core components of the PRC1 repressive complex and genetic evidence suggests that it is autoregulated by PcG mechanisms. Recently, the *Psc-Su(z)2* region was also found to contain several putative Polycomb Response Elements that bind PcG proteins while the entire region is enriched for H3K27me3. Current model of PcG mechanism is all-or-none silencing paradigm derived from its role in homeotic gene regulation. However, it is likely that PcG regulation at many other target genes functions by down-regulating rather than silencing expression. This is certainly the case for *PcG* genes like *Psc* and *Su(z)2*. To understand how PcG mechanisms down-regulate a target gene, the nature of the PcG binding sites in the *Psc-Su(z)2* region and their repressive effects were analyzed, using reporter gene construct. There are at least two functional PREs that can silence a reporter gene in a PcG-dependent manner and one of them can also show anti-silencing activity, depending on the chromosomal context. In addition, we found a down-regulation module

in the vicinity of *Psc* promoter, whose effect is insensitive to the dosage of *PcG* genes. Probably, several different regulatory elements might be cooperative to down-regulate *Psc-Su(z)2* genes by PcG mechanism. I have also generated small deletions that remove one such binding peak. Deletion of one of the *Psc-Su(z)2* PREs increases the expression level of *Psc* and *Su(z)2* by 2-3 fold at late embryonic stage. Perhaps the increased expression of PSC can partially compensate by binding to the other PREs in the *Psc-Su(z)2* locus. On the contrary, the expressions of *CG13323* and *CG13324* genes behind of the PRE are decreased by 20 to 50 fold during entire embryonic stages. Also, the chromatin IP experiment showed that the loss of this fragment extends the domain of H3K27me3 around 10kb further, to a region that includes both of *CG13323* and *CG13324* transcripts. So, the fragment removed in both deletions may not possess only PRE but also a transcriptional enhancer for downstream genes or boundary element to block the PcG silencing.

ACKNOWLEDGEMENTS

I have to start by thanking my graduate advisor, Dr. Pirrotta, for his guidance, support and all his patience. He has a great scientific insight in pursuing the biological research and challenging against current paradigm. All of biologists recognize him as a hard worker. Yes, he is. He is the first comer in the morning and the last leaver in the evening in our lab. He has showed and taught me the attitude of a sincere scientist and a good advisor. He provided me wonderful opportunities to do biology in different countries. When I had to stop working in chromatin field in Korea, he rescued and supported me to continue the work with well-established system in Geneva, Switzerland; that is the most beautiful country in the world and in Rutgers. Finally, his sincere guidance worked out to get publication. Simultaneously, he opened my view into the PcG field and supported enormously to accomplish my thesis. His hard training, patience, and encouragement have built up my current statement. Also, he is the person who is far happier, when I am happy to get accomplishment in science like father. Specially, I really appreciate his teaching with extraordinary patience to improve my English skills. It has been a pleasure doing my graduate work in Dr. Pirrotta's lab.

I must thank the members of my thesis committee. Dr. Ruth, Dr. McKim, and Dr. Grant have been great advisors, with their suggestions for my research. Specially, I appreciate Dr. Ruth for her caring and supporting my work, while our lab started establishing in Rutgers.

Thanks must go to my precious and wonderful members of our lab from Geneva to US. Donna Mccabe, she has encouraged me sincerely, sometimes with fruits and sweet notes.

She is a good listener and counselor whenever I was disappointed at science and attacked by unexpected situations. I would like to thank Yuri and Tanya who are always willing to help me and give advice to pursue my experiments. I have enjoyed all discussions with Yuri and he made me more inspired in PcG world. He has influenced a lot to wide-open my scientific view and attitude. I would like to thank Katsu who is always ready to help me and encourage me. I appreciate Greg for his generous help to write my proposal and thesis. I must thank Pavartha and Huabing for their generous help and encouragement. Also, I appreciate Dr. Kusch, our neighbor for his advice and exciting conversations about life as well as science. I must thank members from university of Geneva in Switzerland: Giovanna, Rafaella, Maxim, Alexey, Ilham, and Louisa. Specially, I would like to thank Giovanna for friendship and encouragement. When I spent hard time to be adjusted in European life, she was the person who introduced me wonderful Italian foods and made me more excited. We used to say " God made us meet, right?"

I must thank very important person, Dr. Spoerel who was my first advisor in Korea. He taught a lot of techniques for molecular biology, when I started grabbing micro-pipette. When left Korea, he introduced me Dr. Pirrotta to continue my thesis.

Thanks must go to all Korean friends from Geneva-Korean church, Rutgers, and Praise Presbyterian church in New Jersey. Their friendship and sincere prayer have pulled out me from desperate situation and made me more fun in foreign life.

How do I express thanks to my family?

My mother and father always support and encourage me with strong confidence and prayer to finish up my PhD, even though it took far more time than expected. Even when I hesitate to move or go further, my mother always gave the verse: Do not fear, for I am

with you; Do not anxiously look about you, for I am your God. I will strengthen you, surely I will help you, Surely I will uphold you with My righteous right hand (Isaiah 41:10). My father is as proud of me as anyone can be. My older brother, sister-in-law, older sister, younger brother, and my niece are great supporters for my continuation of study in abroad. Specially, I really appreciate my sister who always prays for me and advises me.

Finally, thanks must go to my Lord. He is my shepherd whenever I am, wherever I am, whatever I am doing as written in Psalm 23:1 “The Lord is my shepherd, I shall not be in want.” By the graces of God, I am what I am. Shall be all glory to Him!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Polycomb silencing

Polycomb group (PcG) proteins were found first in *Drosophila melanogaster* as the products of genes that are required for the appropriate expression of homeotic (Hox) genes. Hox genes in Drosophila are organized in two complexes. First, the Bithorax Complex (BX-C) contains three homeotic genes, *Ultrabithorax (Ubx)*, *Abdominal A (Abd-A)* and *Abdominal B (Abd-B)*, which are involved in the development of the third thoracic segment and all the abdominal segments (Lewis, 1951). The other complex called the Antennapedia Complex (ANT-C) includes five genes that are essential for the development of the head and the two first thoracic segments (Lewis, 1978) (Fig.1A). These homeotic genes are activated by segmentation genes in early embryonic stages and they are repressed by specific segmentation gap genes. During gastrulation, when the early effectors disappear, the action of PcG proteins becomes detectable. The PcG functions are responsible for maintaining the silenced state of target genes that were initially repressed (Pirrotta, 1997).

PcG protein complexes

The *Polycomb (Pc)* gene was found by analyzing a dominant mutation that produced ectopic sex combs on the second and the third legs of adult male flies (Lewis, 1947). These genetic screens identified other PcG genes whose individual mutations show phenotypes similar to those of *Pc* mutations, or which can enhance the phenotypes of *Pc* mutant alleles (Jürgens, 1985). These genetic evidences suggested that PcG proteins can

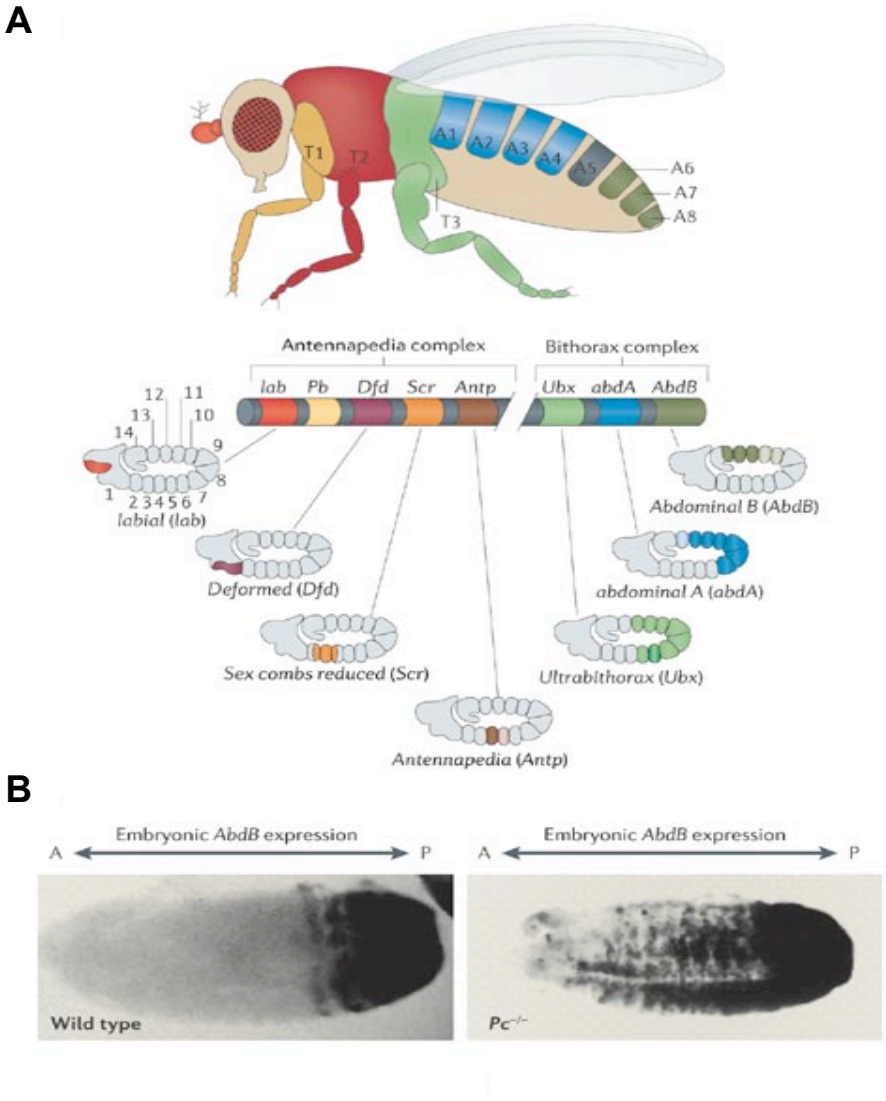


Figure 1. Polycomb proteins function as repressors of Hox genes.

(A) Drosophila Hox (homeotic) genes are organized in two complexes: the Antennapedia complex and the Biothorax complex, which are involved in the development of the head, thoracic segments and all the abdominal segments.

(B) Polycomb group (PcG) proteins are required for the appropriate expression of Hox genes. For example, in the presence of *Pc* mutation, *AbdB* is ectopically expressed in anterior part in which this gene should be repressed in wild type. It leads to homeotic transformation (Sparmann and van Lohuizen, 2006).

form multimeric complexes and also core proteins of PcG complex colocalize strongly on polytene chromosome (Decamillis et al., 1992, Franke et al., 1992). To date, biochemical and genetic analysis elucidated three types of PcG complexes in *D. melanogaster*: PRC1, PRC2 and PhoRC complexes (Table 1). The PRC1 complex contains a core quartet of PcG proteins: Polycomb (PC), Posterior sex combs (PSC), polyhomeotic (PH) and dRING as well as Zeste, several TAFs and number of other proteins (Shao et al., 1999; Saurin et al., 2001). In this complex, PC contains a chromodomain that binds specifically to trimethylated lysine 27 of histone H3 (H3 K27) in PcG target genes (Fischle et al., 2003). *Drosophila* RING possesses a RING domain that supposes to function as E3 ligase that mono-ubiquitylates lysine 119 of histone H2A (Wang et al., 2004). Another RING-domain protein in mammals, BMI1 which is the homologue of PSC in flies can increase the E3 ligase activity of RING 1B, *in vitro* (Li et al., 2006). When the ubiquitylation in a RING protein is removed, PcG-dependent silencing is said to be disrupted. But, the detail mechanisem remains to be understood. The PRC2 complex is composed of Enhancer of *zeste* (E(Z)), SU(Z)12, ESC and P55 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). The SET domain of E(Z) protein is responsible for trimethylation of H3K27 in PcG target genes and wide-spread monomethylation and dimethylation of H3K27 in *Drosophila* (Ebert et al., 2004). All these components are necessary for the proper histone methyl transferase activity (Ketel et al., 2005). The PhoRC complex contains PHO and PHOL which can bind directly to DNA (Brown et al., 1998), and an MBT-domain protein, SFMBT that is known to bind specifically to mono- and

Complex	Proteins			Domains	Functions
	Fly	Human	Mouse		
PRC1	PC	CBX2/HPC1 CBX4/HPC2 CBX8/HPC3	CBX2/M33 CBX4/MPC2 CBX8/PC3	chromo	binding to H3K27me3
	PH	EDR1/HPH1 EDR2/HPH2 EDR3/HPH3	EDR1/RAE28 EDR2/MPH2 EDR3	Zinc-finger-SPM	
	RING	RING1/RING1A RNF2/RING1B	RING1/RING1A RNF2/RING1B	RING-finger	Ubiquitin ligase
	PSC	BMI1 RNF110/ZFP144/PCGF2 ZNF134	BMI1 RNF110/ZFP144/MEL18 ZNF134	RING-finger	cofactor for RING
	SCM	SCML1	SCMH1	Zinc-finger-SPM	
PRC2	E(Z)	EZH1 EZH2	EZH1/ENX2 EZH2/ENX1	SET	histone methyl-transferse
	ESC	EED	EED	WD40 repeats	cofactor for E(Z)
	SU(Z)12	SUZ12	SUZ12	Zinc-finger	
	PCL	PHF1	PHF1/PCL1	PHD-finger	
PhoRC	PHO	YY1	YY1	Zinc-finger	DNA binding
	SFMBT	hSFMBT	L3MBTL2, MBTD1	MBT,SAM	binding to mono-& di-methyl H3K9, H4K20
?	SU(Z)2			RING-finger	
	PHOL			Zinc-finger	DNA binding

Table1. PcG proteins in different species

The main components of PRC complexes are conserved from fly to mammals.

ESC, extra sex comb; E(Z), Enhancer of zeste; PC, Polycomb; PCL, Polycomb-like; PH, polyhomeotic; PHO, pleiohomeotic; PHOL, pleiohomeotic-like; PSC, Posterior sex-combs; SU(Z), Suppressor of zeste; SCM, Sex comb on midleg; SFMBT, Scm-related gene containing four MBT domains. A RING finger is a conserved cysteine-rich domain named after the really interesting new gene (RING). The PHD domain stands for plant homeodomain. The SET domain stands for the three poteins: SU(VAR)3-9, E(Z) and TRX. SPM refers to the presence of this domain in SCM, PHO and MBT proteins. WD repeats are a conserved domain that usually ends with TrpAsp (WD).

(Schwartz and Pirrotta, 2006; Sparmann and van Lohuizen, 2006).

di-methylated H3K9 and H4K20 (Klymenko et al., 2006), but the role of these methylation-marks remains to be understood.

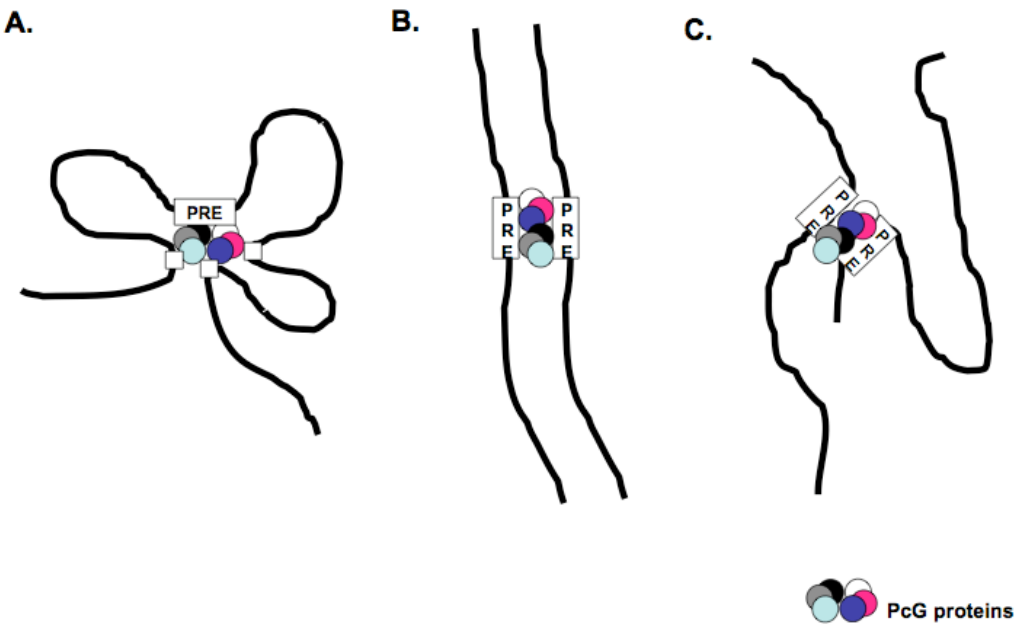
The Polycomb response element

The specific elements for PcG-mediated repression were identified from the functional analysis of the regulatory regions of several PcG target genes in *D. melanogaster*. PcG complexes bind to Polycomb response elements (PREs), several hundred base pairs long in the vicinity of target genes although sometimes, they are found to be located tens of kilobases away from the promoter of target gene. PREs do not have a conserved sequence. But, PREs often contain conserved short motifs that are recognized by known DNA-binding proteins, such as GAGA factor, Zeste, Pipsqueak, DSP1, PHO and PHOL (Horard et al., 2000; Hodgson et al., 2001; Faucheux et al., 2003; Dejardin et al., 2005). Although the role of these factors in PRE function is not well understood, they might recruit the components of the PRC1, the PRC2 or the PhoRC complexes. A number of PREs have been characterized using transgenic constructs in which a PRE flanks a reporter gene such as *mini-white* or *lacZ*. PREs can induce repression of the reporter genes, leading to a variegated phenotype. This variegated phenotype is dependent on the PcG proteins, because variegation is suppressed in a *Pc-G* mutant background (Fauvarque and Dura, 1993; Chan et al., 1994; Kassis, 1994; Pirrotta and Rastelli, 1994; Gindhart and Kaufman, 1995; Zink and Paro, 1995). However, even with the same transposon, the silencing may be sensitive to mutations in one PcG gene but not in another, depending on the site of insertion (Pirrotta, 1997), suggesting that different kinds of PcG complexes can be assembled. Also, the sequence flanking the insertion site could

influence the PRE activity in a positive or negative way (Sigrist and Pirrotta, 1997). PcG complexes might cooperate with other weak PREs in flanking regions (Muller and Bienz, 1991; Poux et al., 1996; Pirrotta, 1997). Similar cooperation can occur between two PREs brought together by homologous chromosome pairing and between transposons inserted at different sites even in different chromosomes (Vazquez et al., 1993). When the fly containing the transposon is homozygous, the repressive effect is enhanced, which is called pairing-sensitive repression (PSR). It demonstrates that the paired PREs interact to produce a more stable PcG complex (Fauvarque and Dura, 1993; Chan et al., 1994; Kassis, 1994) (Fig.2). Additionally, PRE-containing transposon prefers to be inserted in the vicinity of other PRE containing chromosomal sites (Fauvarque et al., 1993; Kassis, 1994). This 'homing' tendency suggests that PcG complexes tend to associate in the nucleus and bias the probability of insertion of a transposon (Pirrotta, 1997).

The effect of PRE-initiated silencing is potentially dangerous to adjacent genes if it cannot be prohibited from spreading of PcG silencing to genes that should be activated. In past decades, the transgenic studies have shown that insulating elements can act as barriers not only to the interaction of enhancers and promoters but also to the interaction of silencing elements and targets. Recently, Kahn et al. have shown that the Su(HW) insulator prevents the spreading of H3K27 trimethylation, which is necessary for the stable binding of PcG complexes to target genes (Kahn et al., 2006).

The other function known to be associated with the PRE is the Trithorax Element (TRE), the site of action of Trithorax (TRX) and other proteins such as ASH1. The TRE involves sequences either partially or identically overlapping with sequences important for the



(Pirrotta, 1997)

Figure 2. Cooperative interactions of PREs

(A) Multiple weak PRE sites can interact through looping to lead to the formation of a stable silencing complexes.

(B) Interactions between two PREs brought by chromosome pairing

(C) This cooperative interaction can occur betwen PREs at different sites even on different chromosomes.

silencing function (Tillib, S. et al., 1999). TrxG proteins function as anti-repressors rather than activators for PcG target genes. So, the same DNA sequences seem to behave as a PRE or a TRE, depending on the early events that set the epigenetic state of the gene (Schwartz and Pirrotta, 2006).

PcG silencing mechanism

H3K27 tri-methylation by E(Z) in PRC2 complex has a crucial role in the stable binding of PcG complexes to target genes. If E(Z) is disrupted by a temperature-sensitive mutation during larval development, binding of PRC1 components is lost from polytene chromosomes (Czermin et al., 2002; Ebert et al., 2004). How do PcG complexes silence a target gene? First, the several DNA binding factors bind on the PRE and might recruit PRC1, PRC2 and PhoRC complexes. The E(Z) can provide trimethyl group on H3K27 residue nearby nucleosomes. Then, these PcG complexes might inhibit transcription of target gene by blocking transcriptional initiation complexes or inhibition of chromatin remodeling factors, although the mechanism of transcriptional interference is still under debate. In some cases, the PREs are found to be located tens of kilobases away from the promoter of target gene. How can PcG proteins reach to the target gene in long distance? Recently, quantitative PCR or microarray approaches with ChIP experiments have shown that PcG proteins have sharp binding peaks at known PREs or presumptive PREs in *D. melanogaster*, even if PC is distributed more broadly than other PcG proteins and it forms tails gradually from the PRE peak. On the contrary, the distribution of trimethylated H3K27 extends over the entire transcription unit, the promoter and the upstream regulatory region, frequently involving many tens of kilobases at a silenced

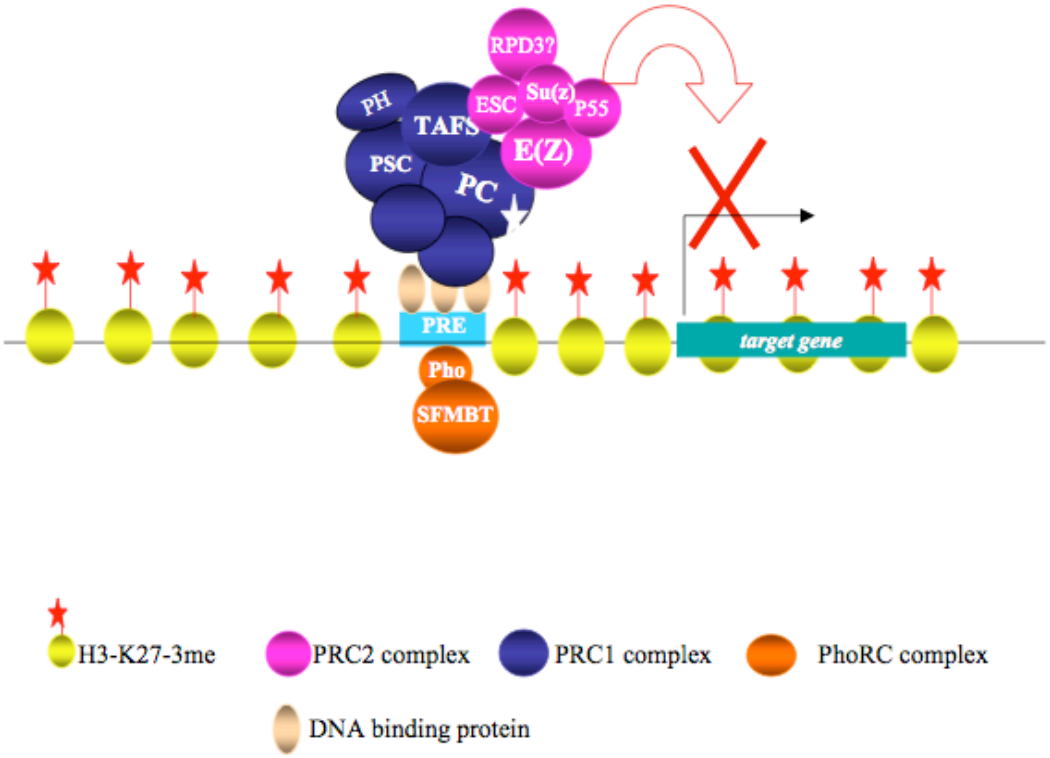


Figure 3. PcG silencing mechanism on the target gene

The PRC1, PRC2 and PhoRC complexes are recruited cooperatively by known DNA binding proteins through Polycomb response element (PRE). The H3K27 tri-methylation by the methyltransferase of E(Z) is extended over the entire transcriptional unit, the promoter and the upstream regulatory region. And, this spreading of methylation can be facilitated by the transient interaction of the PC chromo domain with H3K27me3. But, the mechanism of transcriptional interference remains to be elucidated (Schwartz and Pirrotta, 2007).

gene (Fig. 3) (Kahn et al., 2006; Papp and Muller, 2006; Schwartz et al., 2006). To explain the extended methylation domain in PcG silencing in a long range, Schwartz et al. proposed a looping model (Kahn et al., 2006; Schwartz et al., 2006). The PcG complexes are recruited to the known PRE or the presumptive PRE through the DNA binding proteins. Then, E(Z) bound to the PRE first methylate neighboring nucleosomes. Later, the whole PRE assembled complexes might loop out to scan the entire region, providing the opportunity for E(Z) to methylate all accessible nucleosomes (Kahn et al., 2006). The transient interaction of the PC chromo domain with trimethylated H3K27 can facilitate this looping action (Czermin et al., 2002). Unexpectedly, the known and presumptive PREs seem to be depleted of H3 methylation, probably because they are depleted of nucleosomes (Kahn et al., 2006). Prior to microarray approaches, the discovery of the specific binding of the PC chromo domain to methylated histones could suggest that methylation might recruit the PcG complexes (Czermin et al., 2002). This data can support the idea that if the PRE-binding proteins can recruit the PRC2 complex, the ensuing methylation would then recruit the PRC1 complex. But, the fact that the PRE is undermethylated demonstrates that PcG complexes might be recruited by the DNA binding proteins. How is the repression of transcription achieved by PcG function? Several *in vitro* experiments with purified and reconstituted PRC1 complexes led to the proposal that the PRC1 complex can inhibit chromatin remodeling and transcription (Shao et al., 1999; Francis et al., 2001; King et al., 2002; Levine et al., 2002). Also, such a reconstituted PRC1 complex was shown in vitro to cause compaction of a nucleosomal array and it was suggested that this could block transcription (Francis et al., 2004), although these findings are difficult to verify *in vivo*. On the contrary, Dellino et al.

(2004) found that the silencing did not exclude the binding of RNA polymerase II and some transcriptional factors, but it interfered with the initiation of RNA synthesis by POL II, using a transgene in which a *lacZ* reporter gene driven by the heat shock-inducible *hsp26* promoter was flanked with the well-established PRE from the *Ubx* gene as a model of PcG targets. Similar experiments with other promoters will be required to clarify these results. Moreover, another study found the presence of TBP and the elongation factor Spt5 at the inactive *Ubx* gene promoter, indicating that this transcription might be blocked even at a late step of transcriptional initiation (Papp and Muller, 2006). Another possibility is that repressive histone methylation at H3-K27, H3-K9, H4-K20 may directly or indirectly participate in preventing the deposition of histone marks associated with gene activation such as acetylation, ubiquitylation or trimethylation of H3K4 (Schwartz and Pirrotta, 2007). So far, the mechanism of transcriptional interference is still under debate.

Genome-wide PcG targets

Genome-wide microarray studies in *D. melanogaster* (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006) have shown that there is a large number of PcG target genes, which encode transcriptional regulators, as well as morphogens, receptors, signaling proteins associated with all of the main developmental pathways. Unexpectedly, the *polyhomeotic* gene and the *Psc-Su(z)2* site which must be active to assure the functioning of the PcG mechanism in the entire development turned out to be target sites of PcG. But, the *polyhomeotic* locus binds PC without a significant level of E(Z) and of H3K27 me3 (Schwartz et al., 2006), unlikely with other PcG

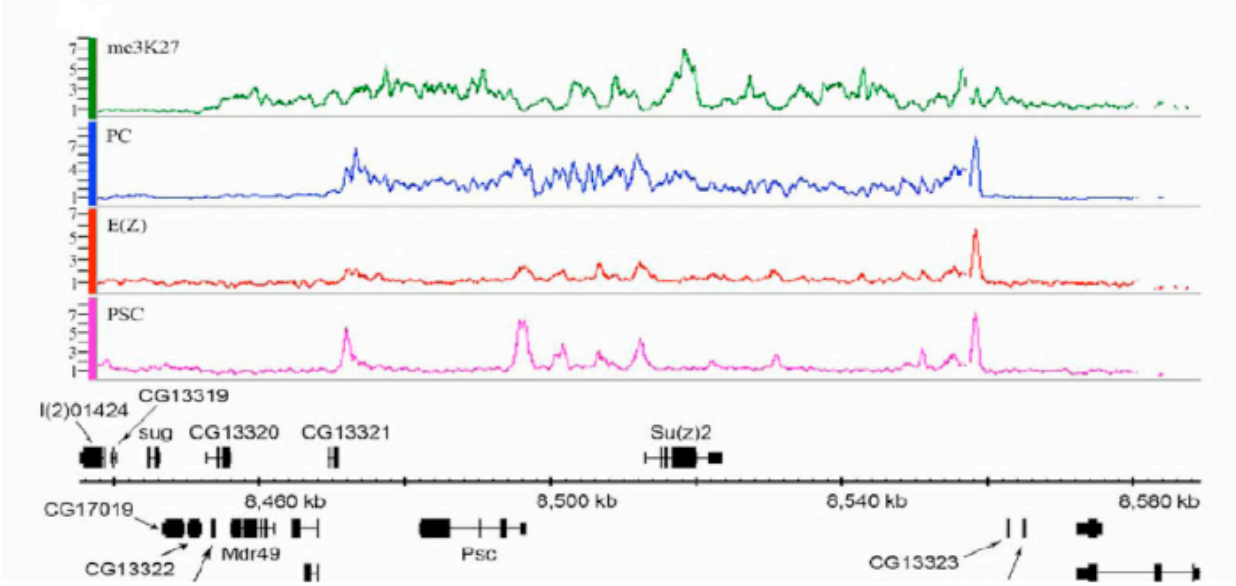


Figure 4. PcG proteins bind to *Psc* and *Su(z)2* genes, polycomb group genes

Microarray approaches with ChIP experiments (Schwartz et al., 2006) have shown that the *Psc-Su(z)2* region is well tri-methylated on H3K27 and binds PC, PSC, and E(Z) at multiple sites.

targets. In previous experiments with reporter gene constructs, a strong PRE identified in the *ph-p* gene appears unusual, because it requires at least 2kb of sequence, including the *ph* promoter rather than several hundred base pairs like usual PREs and does not work at long distance. Farvarque and Dura (1993) have shown that the expression of the *ph* gene is activated in the presence of *Psc* heterozygous mutation rather than silenced. Moreover, the transgenes containing the identified PRE has shown hyper repression even in the presence of *Pc* heterozygous mutation. (Bloyer et al., 2003). So, these results suggest that the *ph* PRE functions somewhat differently from typical PREs.

Another *PcG*-gene as a PcG target, *Psc-Su(z)2* region has shown multiple binding peaks of both PC, PSC and E(Z) and the domain of H3K27me3 (Fig.4). Prior to microarray approaches, Rastelli et al. (1993) showed that the polytene 49F region, the site of the *Psc* and *Su(z)2* genes, is apparently one of the strongest PcG binding sites on salivary chromosomes. Consistent with this observation, he found that in younger third instar larvae, salivary gland nuclei stain strongly with anti-PSC antibody but that, as the larvae matured and prepared for pupation, the stronger nuclear staining was extinguished in all-or-none fashion. But, in larvae homozygous for $E(z)^{S2}$, a temperature sensitive allele, all nuclei continue to stain strongly as in younger larvae (Rastelli et al., 1993), although these have proved difficult to reproduce. Later on, Ali and Bender (2004) found that *Psc* and *Su(z)2* are negatively regulated by PRC1 members PC, PH, and PSC by measuring the transcriptional profile, using subset of PcG mutations. Recently, genome-wide microarray studies strongly support that the *Psc-Su(z)2* region is one of PcG target sites as described above.

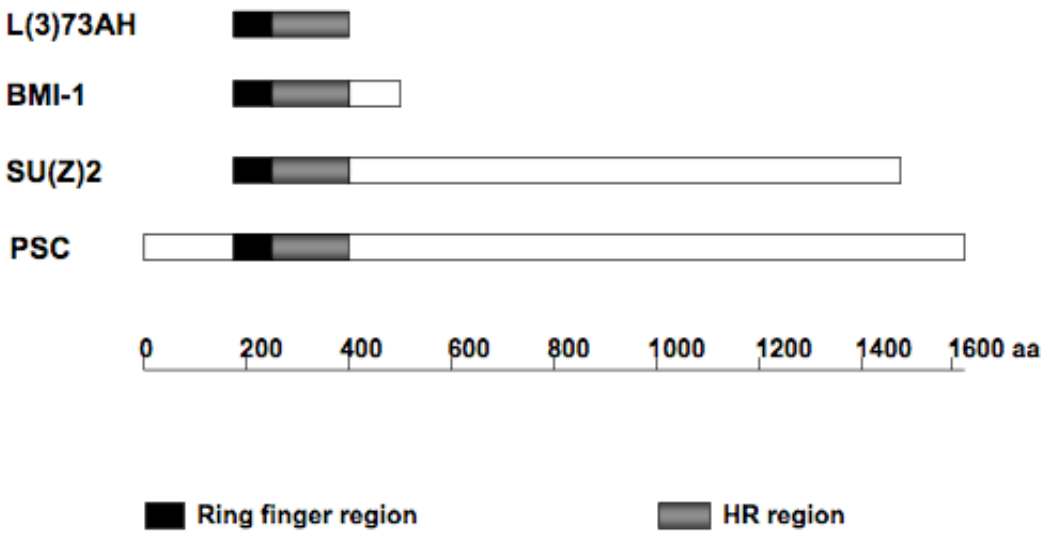


Figure 5. Comparison of the predicted amino acid sequences of L(3)73AH, BMI-1, SU(Z)2, and PSC

Ring finger region and HR (homology region) are common to all four proteins.

The PSC and SUZ2 proteins have large C-terminal regions that share little homology and PSC has also an N-terminal region (Irminger-Finger and Nothiger, 1995).

The PSC protein is composed of 1,603 amino acids and contains a region of 246 amino acids with sequence homology to the vertebrate proto-oncogene *bmi-1*, to *Suppressor 2 of zeste (Su(z)2)*, another PcG protein, and to the *Drosophila* gene *lethal (3)73Ah*, essential at the late pupal stage (Brunk et al., 1991; Van Lohuizen et al., 1991; Irminger-Finger and Nothiger, 1995) (Fig. 5). This homology region contains a C3HC4 RING finger motif that has often been found in E3 ubiquitin or SUMO E3 ligases, as well as a helix-loop-helix-loop-helix motif. *Psc* appears to share functional similarities with *Su(z)2* and lies adjacent to it in the *Su(z)2* complex (Sharp et al., 1994; Soto et al., 1995; Wu et al., 1995). The PSC and SUZ2 proteins have large C-terminal regions that share little homology and PSC has also an N-terminal region. These are absent in the RING proteins and in the mammalian homologues, which consist of little more than the homology region. The PSC protein accumulates in all somatic nuclei of early-stage embryos and then the expression is restricted only to the CNS at later stage (Martin and Adler, 1993). Kyba and Brock (1998) has shown that PSC plays a central role in that it interacts with both PC and PH, while these two do not interact directly with one another by yeast two hybrid studies. Recently, *in vitro* studies with reconstituted PRC1 complex suggest that the product of *Psc* gene might also play a central role for the inhibition of chromatin remodeling and transcription (Francis et al., 2001).

MATERIALS AND METHODS

Plasmid Construction

Most of fragments of interest were put into CaSpeR4-FRT-BH-*Ubx-lacZ* which has been originated from BHL4 construct (Poux et al., 2001) and modified by adding FRT sites. This construct has a *lacZ* reporter gene controlled by the *Ubx* gene promoter, a bx(I)b fragment of the BX embryonic enhancer (Qian et al., 1993), a 2212H1 imaginal disc enhancer (Poux et al., 1996) and a *white* gene as a marker. The putative PRE fragments into this construct were: a 4.1kb fragment (PD-4.1) which is 8kb downstream of *Psc*, a 4.2kb fragment (PP-4.2) which possesses the promoter, 1st exon, 1st intron of *Psc*, a 3.4kb fragment (PP-3.4) which possesses the promoter, 1st exon, 1st half of intron of *Psc*, a 5.0kb fragment (SP-5.0) which possesses the promoter, 1st exon of *Su(z)2,* and a 8.4kb fragment (SD-8.4) which is 35kb downstream of *Su(z)2*. To analyze an intergenic region between *Psc* and *Su(z)2*, a 4.3 kb and a 10kb fragments were inserted into CaSpeR-AUG-βgal construct (Thummel et al., 1988) under 2.3kb of *Psc* own promoter.

The FRT-PD-4.1-FRT-BH-*Ubxp-lacZ* construct: The 12kb genomic fragment which covers the end of the last exon and the downstream region of *Psc* gene was recovered from DS 06880 P1 clone by the *Not*I digestion and inserted in the *Not*I of the CaSpeR4 construct (Fig. 6). Then, the PD-4.1 fragment was generated by *Pst*I digestion from the 12kb- *Not*I fragment-CaSpeR4 construct and inserted in the *Pst*I site of the CaSpeR4-FRT-BH-*Ubx-lacZ* construct in both orientations. The orientations from these constructs were verified by nucleotide sequencing with using specific primers derived from flanked FRT sites (FWD: 5'-CGAGTACGCAAAGCTTGGCTG- 3').

The FRT-PP-3.4-FRT-BH-*Ubxp-lacZ* construct: The PP-3.4 fragment was recovered from λ #128 and λ #129 genomic clones that our lab possesses by *Xba*I and *Apa*I digestion and cloned into the *Xba*I and *Apa*I sites of the pBluescript KS^+ plasmid (Fig. 6). The PP-3.4 fragment was digested with *Not*I and *Kpn*I from the PP-3.4-pBluescript KS^+ construct and inserted into the *Not*I and *Kpn*I sites of the CaSpeR4-BH-*Ubx-lacZ* construct.

The FRT-PP-4.2-FRT-BH-*Ubxp-lacZ* construct: The half of the first intron of *Psc* gene was recovered by genomic PCR from DS 06880 P1 clone (Fig. 6), using the pair of primers: FWD 5'- AACTCCACTCGGCACTCTTCAC-3', BWD 5'-GGATTCCTTCTTTATGCCG-3'. This PCR product (0.8kb) was digested with *Apa*I and *Apo*I (blunted by Klenow) and inserted in the *Apa*I and *Sma*I sites of the pBluescript KS^+ plasmid. Then, the PP-3.4 fragment was digested with *Xba*I and *Apa*I from the PP-3.4-pBluescript KS^+ construct and the half of the first intron (0.8kb) was digested with *Apa*I and *Bam*HI from the PP-0.8-pBluescript KS^+ construct. Both fragments were inserted together into the *Xba*I and *Bam*HI of the pBluescript KS^+ plasmid. The PP-4.2 fragment was digested by *Xba*I and *Bam*HI from the PP-4.2- pBluescript KS^+ construct and inserted into the *Spe*I (compatible with *Xba*I site) and *Bam*HI sites of the CaSpeR4-FRT-BH-*Ubx-lacZ* construct.

The PP-2.3-*lacZ* construct: The first intron of *Psc* gene was removed by digestion with *Xca*I and *Apa*I (blunt ended by Klenow) and self-ligation from the PP-3.4-pBluescript KS^+ construct. Then, the PP-2.3-pBluescript KS^+ construct was generated. The 2.3kb promoter of *Psc* gene was digested with *Xba*I (blunt ended by Klenow) and *Kpn*I from the PP-2.3-pBluescript KS^+ construct and inserted into the *Eco*RI (blunt ended by

Klenow) and *Kpn*I sites of the CaSpeR-AUG-βgal construct.

The PP-6.6-*lacZ* construct: The PP-6.6kb fragment was digested from the PP-13.4-pBluescript KS$^+$ construct (see below) with *Eco*RI and *Xca*I and cloned into the *Eco*RI and *Kpn*I (blunt ended by Klenow) sites of the CaSpeR-AUG-βgal construct.

The PP-12.3-*lacZ* construct: The 10kb-intergenic fragment was recovered from λ #119 genomic clone that our lab possesses by *Xba*I digestion and cloned into the *Xba*I site of the pBluescript KS$^+$ plasmid (Fig. 6). The 10kb fragment was digested from this construct with *Xba*I and inserted into the *Xba*I site of the PP-3.4-pBluescript KS$^+$ construct. The 10kb fragment was digested from the PP-13.4-pBluescript KS$^+$ construct with *Xba*I and inserted into the *Xba*I site of the PP-2.3-pBluescript KS$^+$ construct. The 12.3kb fragment was digested with was digested with *Xba*I (blunt ended by Klenow) and *Kpn*I from this modified construct and inserted into the *Eco*RI (blunt ended by Klenow) and *Kpn*I sites of the CaSpeR-AUG-βgal construct.

The FRT-SP-5.0-FRT-BH-*Ubxp-lacZ* construct: The SP-5.0 fragment was recovered by genomic PCR with AC007472 (BACR30D19) clone (BDGP) (Fig. 6), using this pair of primers: FWD 5'-CGGTTGGGTTTCTAGTGACC-3', BWD 5'-ACACGCTCACACGACTGCAAC-3'. This long genomic PCR could be performed with Elongase Amplification system (Invitrogen) according to manufacturer instructions. This fragment was digested with *Spe*I and *Pst*I and inserted in the site of the *Spe*I and *Pst*I of the CaSpeR4-FRT-BH-*Ubx-lacZ* construct.

The FRT-SD-8.4-FRT-BH-*Ubxp-lacZ* construct: The 10kb genomic fragment which harbors the SD-8.4kb fragment was recovered by genomic PCR with AC007472

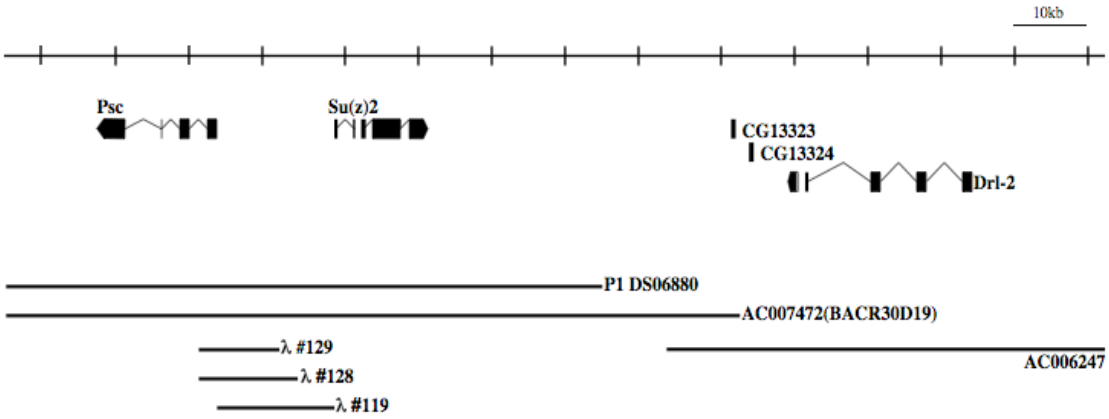


Figure 6. Genomic clones in *Psc-Su(z)2* locus

To make transgenic constructs, all fragments were recovered from λ genomic clones that our lab possessed, P1 clone and BAC clones from BDGP by genomic PCR and enzyme digestion.

(BACR30D19) clone (BDGP) (Fig. 6), using this pair of primers: FWD 5'-AAGTCTGCCCATTGTGCCACGATG-3', BWD 5'-TGAGGCTACAGATTCGGATAG-3'. This long genomic PCR could be performed with Elongase Amplification system (Invitrogen) according to manufacturer instructions. This fragment was digested with *Bam*H1 and inserted in the *Bam*H1site of the CaSpeR4-FRT-BH-*Ubx-lacZ* construct and the CaSpeR4 construct in both orientations. The orientations from these constructs were verified by nucleotide sequencing with using specific primers derived from flanked FRT sites (FWD: 5'-CGAGTACGCAAAGCTTGGCTG- 3').

Generation of transgenic lines

All constructs as described above were transformed to the host fly; $Df(1)w^{67c22}$ in which the white gene is partially deleted, causing complete lack of eye pigmentation (Pirrotta et al., 1983). The mutant stocks used for analysis of genetic interactions are: $Pc^{3}/TM6$; $Su(z)2^{1.b8}$ */GFP CyO*. Pc^{3} is protein-null mutation for *Pc*. $Su(z)2^{1.b8}$ is deletion of both *Psc* and *Su(z)2*. To excise the analyzed fragments, the FPF line was crossed with flies carrying a heat shock-inducible FRT transposase on X chromosome. The progeny were heat shocked for 1hr at 37°C on 5 successive days during larval and pupal growth. In the following generation, F1 adults were crossed with balancers and established as homozygotes. Some excisions could be selected for a change in eye color. Otherwise, the excision was verified by genomic PCR, using specific primers derived from flanked FRT sites (FWD: 5'-CGAGTACGCAAAGCTTGGCTG- 3', BWD: 5'-CGAGGTCGACGATAAGCTTG-3').

To know the insertion sites of transposons, genomic DNAs were isolated from flies for each transgenic line, according to the BDGP protocol. The resulting DNAs were digested with *Sau*3AI, ligated and used for inverse PCR with primers appropriate for the type of P element according to the BDGP protocol (Pry4 (FWD) 5'-CAATCATATCGCTGTCTCACTCA-3', Pry1(BWD) 5'-AGCATGTCCGTGGGGTTTGAA-3'). The sequences flanking P element were identified, using the same primers.

Staining of embryos and larval tissues

Embryos were fixed, stained and mounted by the methods of LAWRENCE and JOHNSTON (1989). The rabbit anti-β-galactosidase antibody was preobsorbed against 0~14 hours embryos overnight at 1:10 dilution and used at a further dilution of 1:250 for embryos. Secondary biotinylated goat anti-rabbit antibody and Vectastain ABC-HRP kit (Vector Labs) were used to reveal the antibody complexes. Imaginal discs from third instar larvae were dissected, fixed with 1% glutaraldehyde in 50mM NaCacodylate pH 7.0, washed and stained with 0.2% X-gal in staining solution (100mM $NaHPO_4$ pH7.0; 150mM NaCl; 1mM $MgCl_2$; 5mM $K_3[Fe(III)(CN)_6]$; 5mM $K_4[Fe(II)(CN)_6]$) to reveal the β-galactosidase activity. The stained preparations were mounted in 80% glycerol and microscopy was carried out with a Zeiss and the images photographed and treated with Adobe Photoshop. The eyes' colors were photographed with a camera mounted on Zeiss microscope using flies raised at 25°C.

In situ hybridization (embryos & cells)

Sense or antisense digoxigenin labeled RNA was transcribed with T7 or SP6 RNA polymerase from linearized pSPT18 or pSPT19 plasmids (Roche) containing the following fragments. For *Psc* we used the 1.018kb digested with *Bam*HI and *Pst*I in C-terminal region of cDNA. For *Su(z)2*, we used the 1.015kb digested with *Hind*III and *Bam*HI in N-terminal region of cDNA. Transcriptions were performed from about 1ug plasmid DNA in 20ul reactions containing 2ul of 10X NTP labeling mixture, 2ul of transcription buffer, 1ul of RNase inhibitor, and 2ul of T7 or SP6 RNA polymerase (Roche). The reactions were incubated for 2hr at 37°C and stopped by adding 2ul of 0.2M EDTA pH 8.0.

Overnight aged embryos (0~16hrs) were collected and dechorionated by incubation in 3~2.5% Na-hypochlorite solution for 3min at RT. The prepared embryos were fixed with 3.7% Formaldehyde for 30 minutes at 37°C under vigorous shaking. After washes several times, embryos were re-fixed with the same condition and treated with 50ug/ml Proteinase K for 3~6 minuites on rotator and the reaction was stopped with glycine (2mg/ml) on ice. The samples were re-fixed as above, washed, and prehybridized in a hybridization buffer (50% deionized formamide; 5X SSC; 100ug/ml Hering sperm DNA; 50ug/ml Heparin; 0.1% Tween 20) for 1 ~ 4hours at 55°C. Hybridization was performed with the same solution containing digoxigenin-labeled probe (10ng/ul) overnight at 55°C. The treated samples were washed with hybridization solution and 1:1 hybridization/PBT solution for 20min each at 55°C, followed by several washes in PBT for 1hr at RT. Afterwards, the samples were incubated for 1hr at RT in anti-DIG antibody (Roche), diluted 1:2,000 in PBT. After several washes in PBT, the samples were rinsed with

staining buffer (100mM NaCl; 50mM $MgCl_2$; 100mM Tris-HCl pH 9.5; 0.1% Tween 20) and developed in NBT/BCIP solution. When color was satisfactory, the samples were washed in PBT and mounted in 80% glycerol. Microscopy was carried out with a Zeiss and the images photographed and treated with Adobe Photoshop.

Generation of deletion mutants

For P element mobilization, the N1363 P-element which is inserted in *Psc-Su(z)2* locus (2R;49E) was used. As a transposase source, w^+; P(Δ2-3) CyO/l(lethal) males were mated to N1363 females. The P (Δ2-3) element provides constitutively active transposase to mobilize other P-elements but cannot itself transposase. To establish precise or imprecise excision lines, w^-; Sco/CyORoi stock was used as balancer. To isolate imprecise excisions out of 194 candidates, the extensive genomic PCR was carried out with the isolated genomic DNA from 194 candidates, using the two kinds of primer pairs (1. FWD 5'-ACATTGCTCGTGAGCAAATTC-3', BWD 5'- CAAATCCGACCAATAGCAATG-3': the amplicon which is 50bp distant in the left side of N1363 insertion. 2. FWD 5'-TACAGTCGTGCTTGACCG-3', BWD 5'-GGTTTGCTTTCTTCCCT-3': the amplicon which is 266bp distant in the right side of N1363 insertion.)

To do mapping of deletion lines, the isolated genomic DNA was digested with *Bam*HI and then separated by electrophoresis in 0.8% agarose gel. Genomic southern blotting was done, according to the methods described in Sambrook, Fritsch and Maniatis (1989), and fixed by a UV stratalinker (Stratagene). The membrane was then prehybridized in a hybridization solution (5XSSC; 1% blocking buffer; 0.1% N-Lauroylsarcosine; 0.02% SDS). Hybridization was performed with the same solution containing probe: 2.5kb

*EcoR*I fragment which was generated from the CaSpeR4-SD-8.4 construct for the left side of the N1363 insertion and 3.7kb *EcoR*I fragment which was generated from the CaSpeR4-SD-8.4 clone for right side which was labeled with DIG-High prime labeling mixture (Roche), according to manufacturer instructions. After washed, the membrane was incubated in a solution containing anti-digoxigenin Fab fragments conjugated to alkaline phosphatase and then equilibrated with detection buffer (0.1M Tris-HCl pH9.5; 0.1M NaCl) according to manufacturer instructions. The membrane was incubated with CDP-Star working solution (Roche) and exposed to a Kodak X-omat AR X-ray film. To know the precise breakpoint of deletions, genomic PCR was carried out with several sets of primer pairs and nucleotide sequencing was performed with the same primer. The list of primers is shown in Table 2.

Reverse Transcriptase-PCR and qPCR

Total RNA was isolated from deletion lines; A27 and B34, from the original transposon insertion (N1363), and from the *w67* control at three different embryonic stages; 4~8hrs, 10~14hrs, and 16~22hrs, using TRIzol Reagent (Invitrogen) according to the manufacturer instructions. After DNase I treatment, 5ug of total RNA was used for random primed synthesis of the first cDNA strand with First Strand cDNA Synthesis Kit (Amersham) according to manufacturer instructions. In parallel, the control reaction was run simultaneously without reverse transcriptase. After heat-inactivation of reverse transcriptase at 65°C, the resulted cDNA was purified with QIAquick PCR Purification Kit and eluted in 100ul of elution buffer (Qiagen) according to the manufacturer instructions. 0.05-5ul of resulted cDNA solution were used for cDNA quantification with

Region amplified	**Primer name**	**Sequnce 5'--3'**
close to 3.7kb-*EcoRI* fragment probe and A27 deletion area	A27 FWD	AGCACACATTTTGGTCGC
	A27 BWD	TTGCCCCCCAAAAAGTCACG
close to 2.5kb-*EcoRI* fragment probe and B34 deletion area	nest B34-F1	TACTGTCCTTCGGCACCTTC
	nest B34-F2	CGCATTTCGGTTTCATCG
	nest B34-F3	TTCAAAACGCCCCCATTCG
	nest B34-F4	GCCAATCCAAAATACTCGC
	nest B34-F5	ACCTTGACTTTCTGCCGTC
close to 3.7kb-*EcoRI* fragment probe and B34 deletion area	B34-B1	TCTGTGTGCTGTGTGCTAAC

Table 2. The list of primers for mapping of deletions

Region amplified	**Primer name**	**Sequence 5'--3'**
rp49	RP49FW	GAAGAAGCGCACCAAG**G**ACT
	RP49REV	AACGCGGTTCTGCATGAGCA
Psc	PSCEX1.1	TCCATTGACCATTCGCACAG
	PSCEX1.2	TTTCACCTTGATGGGTTTCAG
Su(z)2	Su(z)2 FWD-4	TCCAAGGAGCCAAAGGATGC
	Su(z)2 BWD-4	ATGTGCGATTCAGTGCCTCG
CG13323	CG13323F-2	GATTCTACAACATCTCCGCC
	CG13323B-2	TCACCTGACCACGAAGATTC
Drl-2	Drl-2F-2	GTTATGAACTGCTGCTGGCA
	Drl-2B-2	ACTCATTGAGGTCCATCCCA

Table 3. The list of primers for the analysis of mRNA expression

real-time PCR. The quantification was carried out with real-time PCR in the same way as described for quantification of ChIP products (Schwartz et al., 2006) except that serial dilutions of genomic DNA from *w67* fly were used to make standard curve. The amount of cDNA for genes of interest; *Psc, Su(z)2, CG13323-CG13324,* and *Drl-2* in the given preparation was normalized by the amount of *RpL32 (rp49)* cNDA. The list of primers for real-time PCR is shown in Table 3.

Chromatin Immunoprecipitation and qPCR

Overnight aged embryos (0~16hrs) from the line of interest; *w67*, N1363 transposon, A27 and B34 deletion lines, PP4.2(1M-2M) transposon and Δ PP4.2 (1M-2M) excision line were collected and dechorionated by incubation in 3~2.5% Na-hypochlorite solution for 3min. at RT. Approximately 250mg embryos were crosslinked in 10ml of freshly made 1.8% formaldehyde in X-linking buffer (25mM HEPES pH 7.6; 0.5mM EDTA pH 8.0; 0.25mM EGTA pH 8.0; 50mM NaCl) and incubated for 20minutes at room temperature on a rocking platform (400 rpm). The reaction was stopped by addition of glycine pH 7.0 to a final concentration of 0.125M. Embryos were washed with Washing buffer A (10mM HEPES pH 7.6; 10mM EDTA pH 8.0; 0.5mM EGTA pH 8.0; 0.25% Triton X100) and B (10mM HEPES pH 7.6; 100mM NaCl; 1mM EDTA pH 8.0; 0.5mM EGTA pH 8.0; 0.01% Triton X100) at 4°C and then sedimented. To make a soluble chromatin preparation, the crosslinked embryos were resuspended in 5ml of RIPA buffer (140mM NaCl; 10mM Tris-HCl pH 8.0; 1mM EDTA; 1% Triton X100; 0.1% SDS; 0.1% DOC). The sample was subjected to sonication with a Branson 250 sonifier equipped with

microtip with 6x 30sec bursts. The resulting sample was incubated for 10min on rotating wheel at 4°C and cleared by 5min centrifugation at maximum speed, divided in 500ul aliquots and stored at -80°C.

The 500ul of lysate prepared as above was precleared by incubation for 1hr at 4°C with Protein A-Sepharose beads (Sigma). The cleared lysate was incubated with 5ul of the anti-me3K27 antibody (1ug/ul; Abcam) for 15hrs at 4°C. The antibody complexes were precipitated by incubation with Protein A-Sepharose beads (Sigma) for 3h at 4°C. The beads were washed five times with 1ml RIPA, once with 1ml LiCl buffer (250mM LiCl; 10mM Tris-HCl pH8.0; 1mM EDTA; 0.5% NP-40; 0.5% sodium deoxycholate), twice with 1ml TE (10mM Tris-HCl pH8.0; 1mM EDTA) and pelleted by spinning for 30sec at 4°C. The beads were resuspended in 100ul TE with 50ug/ml of RNAse A and incubated for 30min at 37°C. The proteins were digested overnight with 0.5mg/ml of proteinase K and 0.5% of SDS at 37°C. To be reverse-crosslinked, the samples were incubated at 65°C for 6hrs and then extracted with phenol-chloroform. The extracted DNA solution is precipitated by addition of EtOH, 30ul of 3M NaAc pH5.0, and 2ul of glycogen (20mg/ml). Immunoprecipitated DNA was dissolved in 150ul water for Real-Time PCR analysis. PCR reactions were performed by mixing 5ul of total 150ul of immunoprecipitated DNA prepared as above with 10ul of 2xSYBR Green PCR Master Mix (ABgene), 100nM of corresponding primers, 100nM of ROX as a reference dye and pure water to 20ul. All primers were annealed at 55°C. The reaction was carried out in 96-well plates with the Mx3000P instrument (Stratagene). The quantification was carried out with real-time PCR in the same way as described in Schwartz et al., (2006). The list of primers for real-time PCR is shown in Table 4.

Region amplified	**Primer name**	**Sequence 5'--3'**
Ubx FM6	FM6a.1	TTGCGAGTGCTTTTCCATATC
	FM6a.2	AAACAACAAACAACCGATGCC
umbl	ubml1.1	CGCTGGATTGTGTATAGGAG
	umbl1.2	TCCTCGCATGTGAGAGTAAC
3'end of A27 deletion	A27-3'F	CGTAATCGCAATGGACGGC
	A27-3'B	TGTCGCCCGAGGGATATGT
5'end of B34 deletion	B34nest F5	ACCTTGACTTTCTGCCGTC
	B34-5'B	CACGAATGGGGGCGTTTTG
CG13323	CG13323F-2	GATTCTACAACATCTCCGCC
	CG13323B-2	TCACCTGACCACGAAGATTC
Drl-2	Drl-2F-2	GTTATGAACTGCTGCTGGCA
	Drl-2B-2	ACTCATTGAGGTCCATCCCA
lacZ (LZ)	5'-lacZ	CCAGCGAATACCTGTTCCG
	3'-lacZ	CACACTGAGGTTTTCCGCC
mini-white (W)	wp1	AGTCAGCGCTGTTTGCCTC
	wp2	CCTCTTGGCCCATTGCCG
galectin (gal-1)	CG11372F1	TGACGGCAATAATACTCCTGGC
	CG11372B1	TGACACCTGACGCAACCAAAGC
galectin (gal-2)	gal F2	TTATTGCCGCGAGCGTCAG
	gal B2	ATAAGCACAACCGCTAAGCCAC

Table 4. The list of primers for real time PCR

Climbing measurement

To analyze climbing activity, ten seven days old males from each line; w67, N1363, A27, B34 were placed into a vial that was divided into 3 different levels. The climbing rate of these flies was measured at the following time intervals, 10s, 20s, and 2min. after striking the tube on the table. To examine this phenotype, dependent on aging, 5 vials of each type containing each 10 males from *w67* and both of deletion lines; A27, B34 were used (3 types x 5 vials/type x 10 flies/vial = 150 males). The flies were maintained at 25 °C, and transferred every 3 days to a new vial to provide fresh food to the flies. The flies were observed for their ability to climb to the top of the vial every 3 days (from 6 to 21 days of age). The vials were gently shaken and set on the table to observe the activity of the flies after 5 seconds.

CHAPTER I

The characterization of multiple PcG binding sites in *Psc-Su(z)2* locus

INTRODUCTION

Genomic analysis of Polycomb binding sites and the distribution of H3K27-trimethylation reveals that the *Psc-Su(z)2* region possesses specific sites of PcG binding as well as abundant H3K27 modification (Schwartz et al., 2006). PC, PSC, and E(Z) colocalize at several distinct sites in this domain, suggesting that these sites are putative PRE elements. Based on distinct peaks, best identified in the PSC distribution, the location of the putative PRE elements is as follows: 8kb downstream of *Psc* (PD), the promoter regions for *Psc* (PP) and *Su(z)2* (SP), and 35kb downstream of *Su(z)2* (SD) (Fig. 7A). Paradoxically, the *Psc* gene must be active to assure the functioning of the PcG mechanism and the presence of PcG complexes has been detected by immunoprecipitation. Therefore, the mechanism appears in some way different from the all-or-none silencing paradigm derived from PcG regulation of homeotic genes. I propose the downregulation paradigm from the PcG regulation of the *Psc-Su(z)2* genes. So, in this study, I would like to understand how PcG mechanisms can downregulate rather than silence a target gene. To analyze whether all of the putative PRE elements in the *Psc-Su(z)2* locus are functional PREs, DNA fragments containing each putative PRE, flanked by FRT sites, were cloned into a reporter gene construct (Poux et al., 2001). This construct has a *lacZ* reporter gene controlled by the *Ubx* gene promoter, a *Ubx* embryonic enhancer (bx), a *Ubx* imaginal disc enhancer (H1) and a *white* gene controlled by the *white* gene promoter as a marker (Fig. 7B). This construct has been used in previous studies of PRE function because the *Ubx* promoter and enhancers are well known as representative PcG targets. The embryonic *Ubx* enhancer is repressed by Hunchback

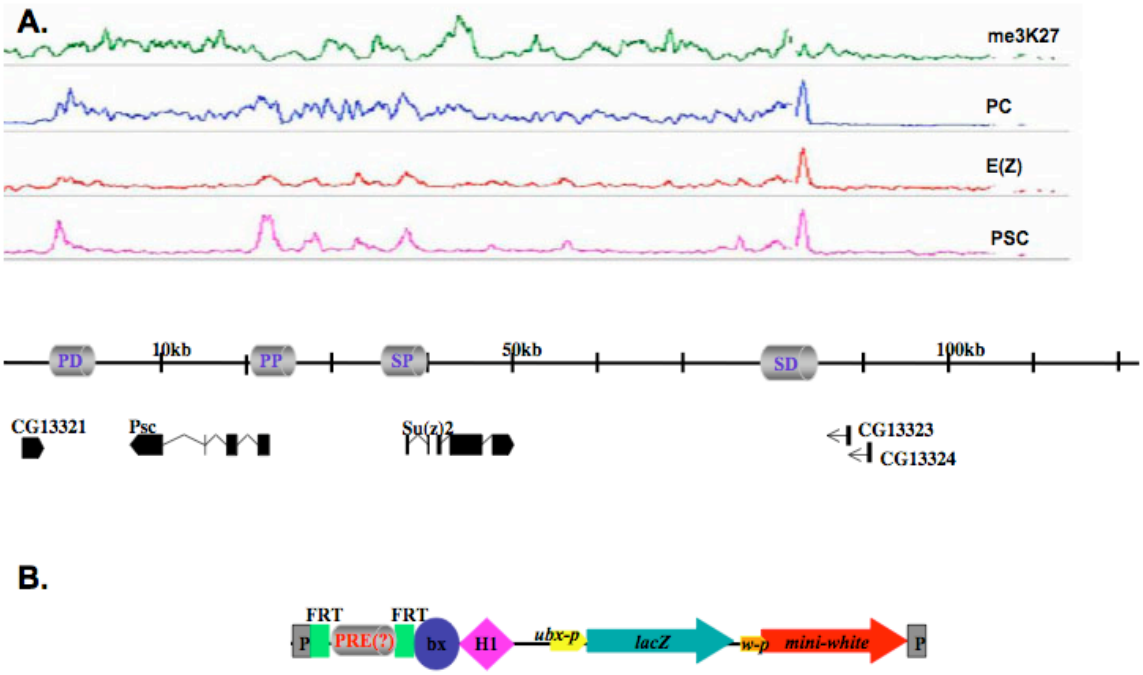


Figure 7. The localization of putative PREs and transgenic construct

(A) The gray boxes indicate the fragments containing putative PRE elements, which are showing significant binding peaks for PC, PSC, and E(Z) (Schwartz et al., 2006). The tested fragments are indicated as PD: 8kb downstream of *Psc* gene, PP: a promoter region of *Psc* gene, SP: a promoter region of *Su(z)2* gene, SD: 30kb downstream of *Su(z)2* gene.

(B) Each putative PRE fragment, flanked by FRT sites (green rectangles), is put into the construct which has a *lacZ* reporter gene (dark green arrow) controlled by the *Ubx* gene promoter (yellow), a *Ubx*-bx embryonic enhancer (blue circle), an H1 imaginal disc enhancer (pink diamond) and a *white* gene controlled by its own promoter (orange) as a marker.

anterior to parasegment 6, while activating the *Ubx* promoter in even numbered parasegmants 6, 8, 10 and part of 12 in posterior part (Qian et al., 1991, 1993). When Hunchback goes away, we should get derepression of the *lacZ* reporter gene in all segments. If there is PRE activity, the repression will be maintained in the anterior region to parasegment 6 in late embryonic stage. Imaginal disc enhancers are active only at later stages in the head, wing and haltere discs (Pirrotta et al., 1995). If there is PRE activity, the repression that is established in the embryo will be maintained in the anterior region to parasegment 6; that is, in the eye-antenna disc, in the wing disc and in the anterior half of the haltere disc. The H1 imaginal disc enhancer would then be active preferentially in PS6 (the posterior half of the haltere disc) (Fig. 8). To examine whether some regulatory effect is derived from the inserted fragment, it can be excised by crossing to a strain expressing the FLP recombinase under control of the heat shock promoter (Fig. 7B). So, several putative PRE fragments in the *Psc-Su(z)2* locus have been characterized with this transgenic context.

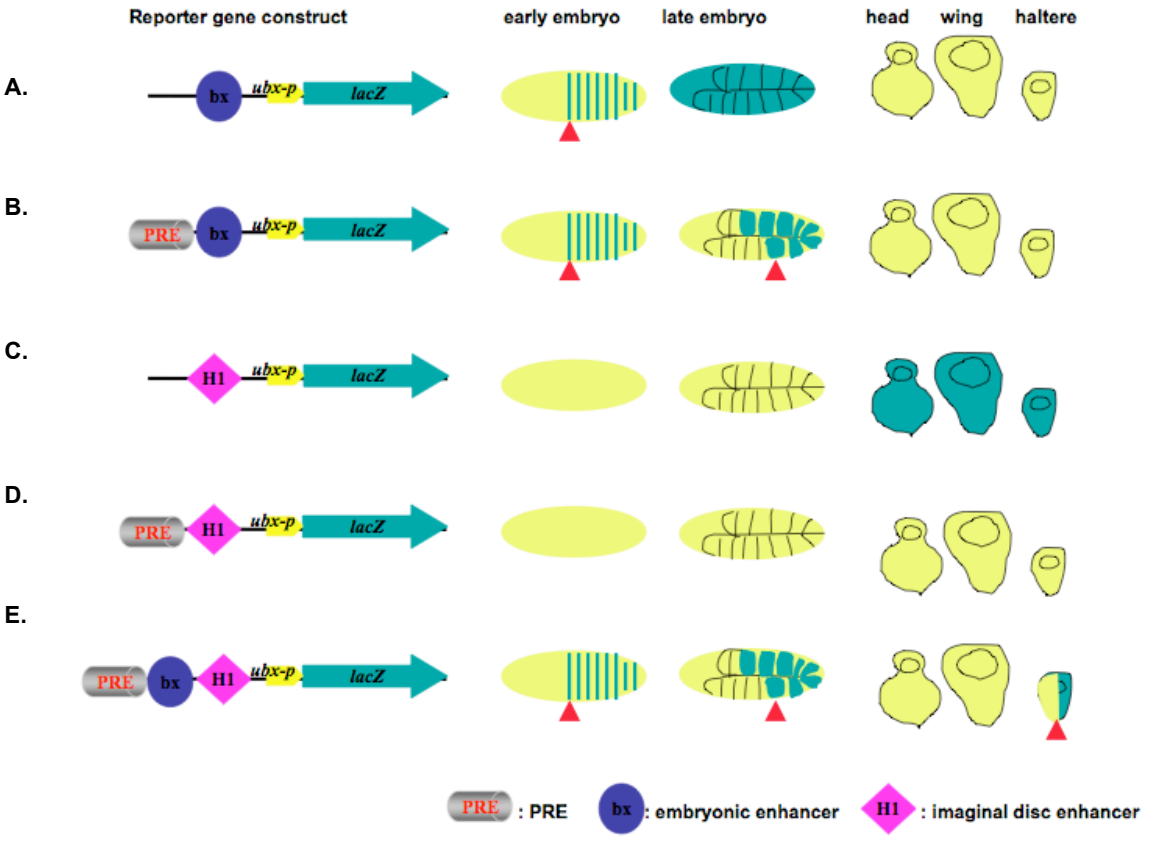

Reporter gene construct
early embryo
late embryo
head
wing
haltere
A.
B.
C.
D.
E.
bx
ubx-p
lacZ
PRE
H1
: PRE
: embryonic enhancer
: imaginal disc enhancer

Figure 8. Regulation of the expression of *Ubx* gene

The reporter gene construct which has the *Ubx* promoter, the *Ubx* embryoninc enhancer, Polycomb response element (PRE) and imaginal disc enhancer can show the appropriate expression of *Ubx* gene. The embryonic enhancer activates the *Ubx* promoter in even-numbered parasegments in posterior part but is repressed by Hunchback in the anterior to parasegment 6. When the early repressors disappear, the reporter gene is expressed in all segments (A). If the PRE is added to this enhancer, repression is maintained in the anterior region (B). In the head, wing and haltere discs, the reported gene can be activated by imaginal disc enhancers (C). If the PRE is added to this enhancer, the expression will be in "off" state that is established in the embryo (D). If all three elements are combined, the expression pattern which is established by the early enhancer would be maintained repression anterior to parasegment 6 utill post-embryonic stage by the PRE activity (E). The red arrow indicates posterior to parasegment 6 (Schwartz and Pirrotta, 2007).

RESULTS

The putative SD PRE has PRE activity.

All independent lines that have the SD-8.4 fragment in this construct showed variegated expression patterns of the *lacZ* reporter gene in wing imaginal disc from 3rd instar larvae, when stained to reveal β-galactosidase activity, indicating repression. SD-F1-M1, SD-M7-M4, and SD-F9-M1 are representative independent lines. All-or-none-fashion of silencing can cause variegated expression of genes affected, because relatively stable heritance of repressive state may be established in some embryonic cells, but not others, and maintained in their cellular progeny. A transposon insertion named N1355 which has the same construct containing the *Ubx-lacZ* reporter gene controlled by the same *Ubx* embryonic enhancer and imaginal disc enhancer with no inserted fragment (Poux et al, 2001) showed uniform expression of the *lacZ* reporter gene in wing imaginal disc, indicating the enhancers might not produce variegation (Fig. 9B). To test if the variegated expression was due to mosaic repression by PcG genes, these transgenic lines were crossed with $Pc^3/TM6$ and $Su(z)2^{1.b8}/CyOGFP$. Pc^3 is protein-null mutation for *Pc*. $Su(z)2^{1.b8}$ is deletion of both *Psc* and *Su(z)2* genes. The variegated pattern of *lacZ* reporter gene in imaginal disc became uniform in either Pc^3 or $Su(z)2^{1.b8}$ heterozygous background, indicating derepression (Fig. 9B). The degree of repression or derepression is variable, depending on the insertion site, because the genomic context may influence the assembly of PcG complex (Pirrotta, 1997). In fact, the excision lines were not able to be generated by FRT/FLP system. Nevertheless, all of 10 independent transgenic lines showed variegated expression patterns of the *lacZ* reporter gene in wing imaginal disc,

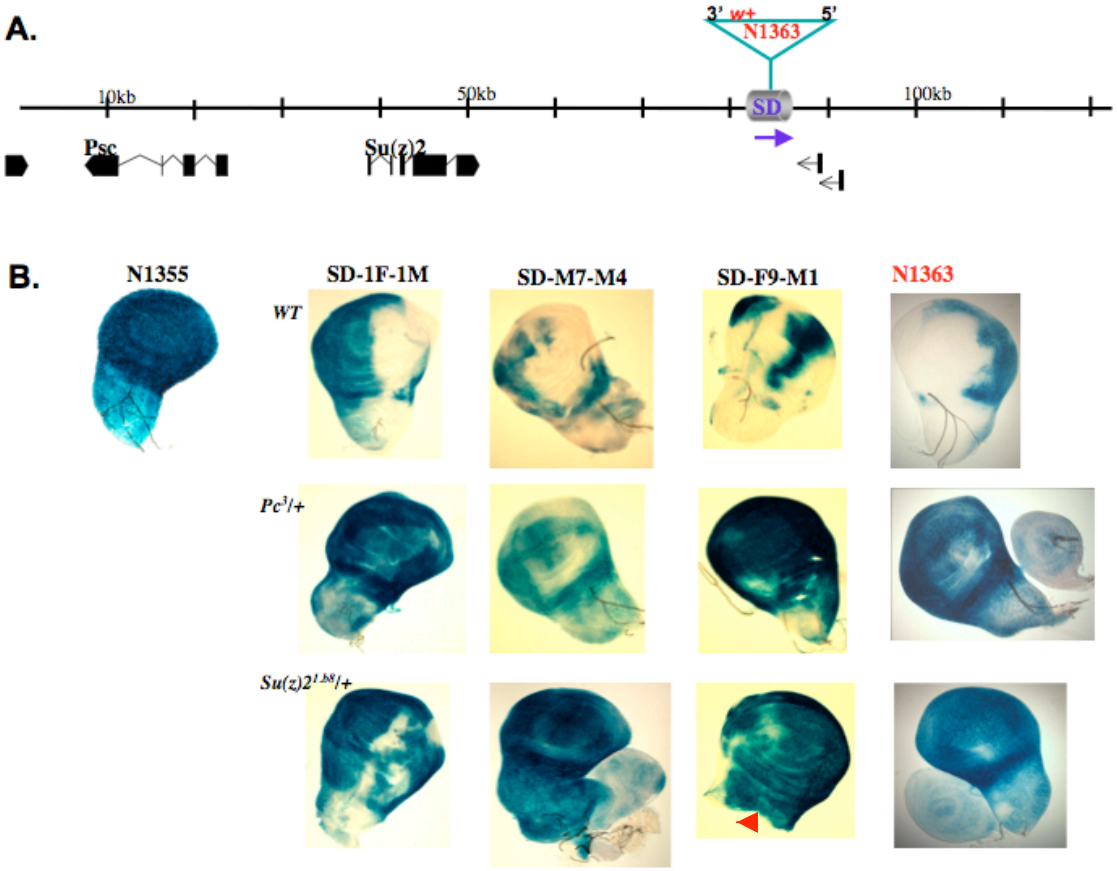

A.
3' w+ 5'
N1363
10kb
50kb
SD
100kb
Psc
Su(z)2
B.
N1355
SD-1F-1M
SD-M7-M4
SD-F9-M1
N1363
WT
Pc3/+
Su(z)21.58/+

Figure 9. The SD PRE shows a real PRE activity.

(A) SD is a 8.4kb-fragment which is located in approximately 30kb downstream from *Su(z)2* gene. N1363 is the random insertion of a control construct into the SD region. The transcriptional orientation of the reporter gene of N1363 line was opposite orientation relative to that of *Su(z)2* gene. Blue arrow indicates that the orientation of the SD fragment is the same with that of reporter gene in the reporter gene construct. **(B)** N1355 is the transposon insertion of a control construct which does not have any regulatory elements. All of independent lines that have the SD-8.4 fragment display variegated *lacZ* expression and became derepressed in *PcG* heterozygous background. SD-F1-M1, SD-M7-M4, and SD-F9-M1 are representatives among lines.
The insertion named N1363 behaves in the way similar to SD transposons.
The red colored arrowhead indicates the breakage of disc during preparation.

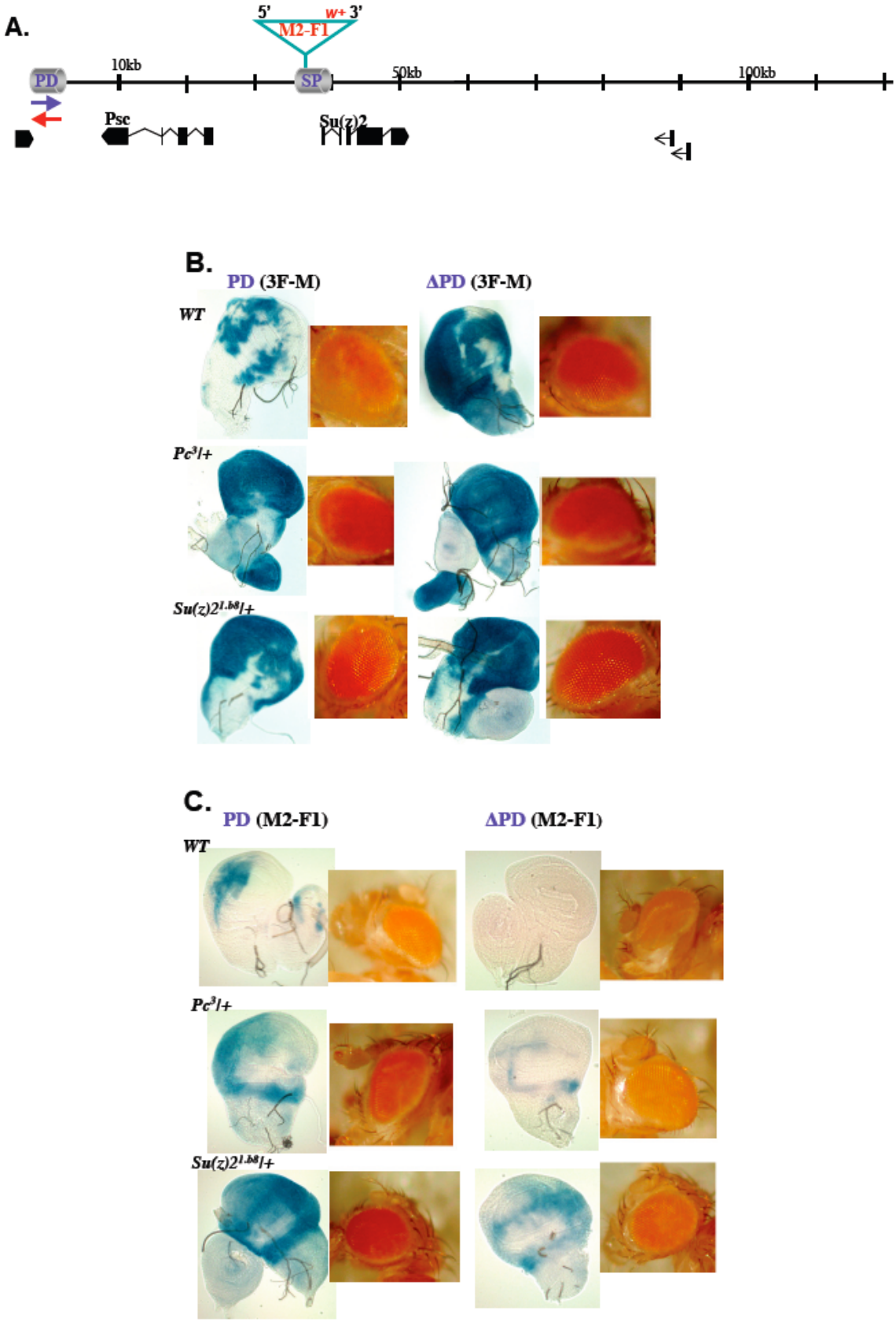
dependent on *PcG* dosage. So, I conclude that that the SD putative PRE seems to act a functional PRE. Additionally, in earlier work using a very similar construct containing the *Ubx-lacZ* reporter gene controlled by the same *Ubx* embryonic enhancer and imaginal disc enhancer (Poux et al., 2001), a transposon insertion named N1363 (Fig. 9A) had been recovered that mapped very close to the *Psc* locus. The *lacZ* expression produced by this transposon showed a typically silenced *Ubx*-like pattern in imaginal disc that becomes derepressed in *PcG* mutants. Since this construct contains no PRE, the maintenance of repression anterior to parasegment 6 suggests the presence of a PRE near insertion site. By inverse PCR, the insertion turned out to be located 35kb downstream from *Su(z)2* gene, within a sequence containing the SD PcG-binding peak. So, this repression might be caused by the endogenous SD region, residing in the *Psc-Su(z)2* locus. Recently, Chetverina et al. (2008) reported that the *mini-white* used as a reporter gene might have itself carried insulator-like activity associated with its 3'end, on the basis of several reporter gene constructs. In fact, N1363 transposon is inserted in the SD region so that: 1) the transcriptional orientation of the reporter gene was opposite orientation relative to that of *Su(z)2* gene (Fig. 9A); 2) the N1363 transposon bisected the SD region into two halves which will be called proximal and distal parts. If the insulator in 3'end of *mini-white* gene is active, the repressive effect derived from the proximal part of SD region would be blocked. Then, the repression of the reporter gene might be caused by the distal part of SD region. Most of all, these transgenic studies strongly suggest that the SD region behaves like a typical PRE.

This SD-8.4 might contain a boundary element or insulator to block silencing, because, according to the microarray data (Fig. 7A), the methylation domain terminates and the

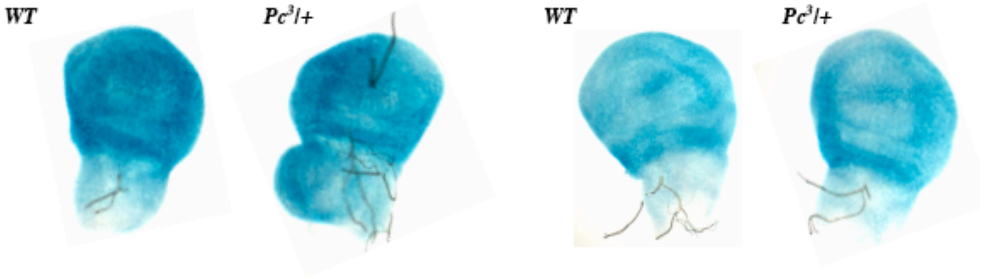
extended binding peak of PC also disappears immediately outside of the SD region (Kahn et al., 2006). But, all transgenic lines presented above contain the SD region placed into a reporter gene construct in the same orientation relative to the reporter gene. All independent lines showed variegated expression patterns of the *lacZ* reporter gene in wing imaginal disc from 3rd instar larvae, depending on *PcG* dosage like typical PRE. If this region contained a classical boundary element, the SD segment would block PRE/promoter communication of the reporter gene, resulting in no silencing of the *lacZ* gene. So, I did not see any effects suggestive of the presence of an insulator.

The PD region behaves like a PRE or an anti-silencer.

The PD-4.1 fragment, which is located in 8kb downstream of *Psc* gene, was put into the same reporter construct (Fig. 10A). Among 11 independent lines, three lines show the repressive effects in the expression of both reporter genes, *lacZ* and *mini-white*. One line, named 3F-M, showed an expression pattern typical of PRE regulation. It showed variegated *lacZ* expression in wing imaginal disc and weak expression of *mini-white* in adult eyes. In either Pc^{3} or $Su(z)2^{1.b8}$ heterozygous background, the expression of *lacZ* and *mini-white* genes became uniform. To examine whether this effect is derived from the PD-4.1 fragment, this fragment was excised by crossing to a strain expressing the FLP recombinase under control of the heat shock promoter. The ΔPD (3F-M) line in which the fragment was excised was identified by genomic PCR, because the excision produced too little change of eye color to be distinguished. This excised line was derepressed in both


A.
5'
w+
3'
M2-F1
PD
10kb
SP
50kb
100kb
Psc
Su(z)2
B.
PD (3F-M)
ΔPD (3F-M)
WT
Pc3/+
Su(z)21.b8/+
C.
PD (M2-F1)
ΔPD (M2-F1)
WT
Pc3/+
Su(z)21.b8/+

D. PD (M5-F1) ΔPD (M5-F1)



E. PD (M3-M2)

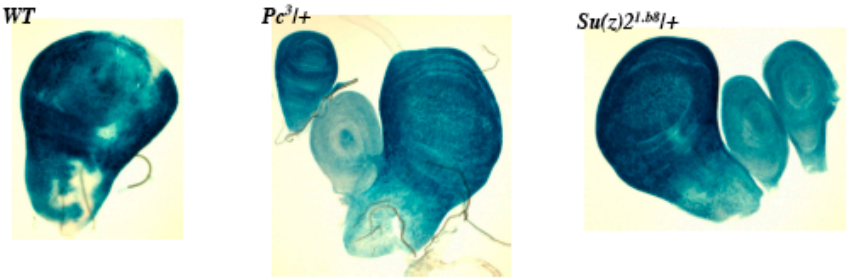


Figure 10. The PD region behaves as like PRE or an anti-silencer.

(A) PD is a 4.1kb-fragment which is located in 8kb downstream of *Psc* gene. This PD-4.1 fragment was examined in both orientations (blue: the same orientation relative to that of reporter gene, red: the opposite orientation relative to that of reporter gene). **(B)** One of transgenic line, called PD-3F-M line, that have the PD-4.1 fragment displays variegated *lacZ* expression and became derepressed in PcG mutant background. When this fragment is excised by flipase, the ΔPD-3F-M line is derepressed in both *lacZ* and *white* expression. **(C)** The other line, called PD-M2-F1, shows variegated *lacZ* expression and became derepressed in PcG mutant background. When this fragment is excised by flipase, it causes stronger repression of the *lacZ* and *white* gene. This line turns out to be inserted in SP (a promoter of *Su(z)2*) region). **(D)** Six lines, like M5-F1 line shown above, show that the *lacZ* reporter gene is uniformly expressed in imaginal discs and the expression pattern is insensitive to *Pc* mutation. When this fragment is excised by flipase, the expression of *lacZ* becomes weaker. **(E)** In two of lines, like M3-M2 line shown above, the expression of *lacZ* reporter gene is weakly variegated and became uniform in PcG mutant background.

lacZ and *mini-white* expression, although some cells of the wing disc remained silenced (Fig. 10B). Inverse PCR showed that this transposon is inserted nearby the promoter of the *dsf* gene on 2L chromosome. Recently, ChIP/chip analysis in *Drosophila* embryos has shown that the *dsf* region contains H3 K27 trimethylation and low but significant binding of PcG proteins (Negre et al., 2006). This would explain why some repression persisted even in the absence of the PD-4.1 fragment, presumably produced from flanking genomic context. So, I conclude that the PD region behaves as a typical PRE and may produce stronger repressive effect in this genomic context. The other line, M2-F1, represents a more complicated case. It showed variegated *lacZ* expression in imaginal discs and weak expression of *mini-white* in adult eyes. The expression of the *lacZ* reporter gene became derepressed in the presence of *PcG* heterozygous mutations. Surprisingly, the excision of PD-4.1 fragment by FRT-FLP system in this line caused stronger repression of the *lacZ* and *mini-white* genes (Fig. 10C). The excision of PD-4.1 fragment did not disrupt the sequences of the imaginal enhancers in the transposon (data not shown). This repression is also sensitive to the dosage of *PcG* genes. Surprisingly, from inverse PCR, this transposon turned out to be inserted in the promoter region of the *Su(z)2* gene (SP), which according to the ChIP/chip results, is significantly enriched in PC, PSC, and E(Z) (Fig.7A). So, this stronger repression could be caused by the endogenous PREs like SP region or other putative PREs, residing in the *Psc-Su(z)2* locus. In fact, the SP region which includes the 5kb-promoter region of *Su(z)2* gene was constructed into the same plasmid, but the generation of transgenic lines has failed. Nonetheless, the result from M2-F1 line is indicating that the *Psc-Su(z)2* locus is functionally regulated by PcG silencing mechanism. Most probably, in the endogenous situation, the promoters of *Psc*

and *Su(z)2* genes might remain partly active despite the binding of PcG proteins and the H3K27 trimethylation. So, if the transposon containing the PD-4.1 fragment showed more expression of reporter genes than the excision line, this fragment might be considered to act as an anti silencer in this genomic context. Most of all known or presumptive PRE also bind TRX, which functions as anti-repressors antagonistically to the PcG proteins rather than typical activators (Papp and Muller, 2006). The same DNA sequences can behave as a PRE or a TRE, depending on early events that set the epigenetic state of the gene. To clarify if the PD-4.1 fragment behaves also as a TRE in this genomic context, the expression of the reporter gene must be examined in the *trx* mutant background.

In fact, M2-F1 transposon is inserted in the same orientation relative to *Su(z)2* gene (Fig. 10A). If the insulator in 3'end of *mini-white* gene is functioning (Chetverina et al., 2008), the repressive effect may be derived from the proximal part of SP region, because the silencing from the distal part of SP region would be prohibited.

In other genomic locations, the PD-4.1 fragment shows variable effects. Six other independent lines, like PD-M5-F1 line show that the *lacZ* reporter gene is uniformly expressed in imaginal discs. The excision of PD-4.1 fragment by FRT-FLP system in these lines caused weaker expression of the *lacZ*. But, the expression from non-excised and excised lines is insensitive to the dosage of *PcG* genes (Fig. 10D). So, in this genomic context, the PD-4.1 fragment may play a role to stimulate the expression of the reporter gene. Two of these lines displayed weak variegation and this expression became derepressed in *PcG* heterozygous mutant background (Fig. 10E). So, in this context, the PD-4.1 fragment seems to behave as a weak PRE. Probably, certain insertion sites may

be resistant to repression because these transposons are integrated near or into genes that are actively transcribed. So, these transgenic studies suggest that the PD-4.1 fragment may have dual functions as either PRE or anti-silencer (TRE), dependent on chromosomal environment.

In addition, this PD-4.1 fragment was tested in both orientations to determine if it might contain a boundary element to block silencing, because the microarray data (Fig. 7A) shows that the binding peaks of PC drop immediately outside of PD-4.1 region and the methylation domain is decreased. If this segment includes a boundary element, the PD region placed into a reporter gene construct, in the opposite orientation relative to the reporter gene would not show the repressive effect on reporter genes. But, three out of ten lines with this orientation showed repressive effect. So, I did not see any effects suggestive of the presence of an insulator (data not shown).

The PP region behaves as downregulation module.

As described above, line M2-F1 in which the PD-4.1transposon is inserted in the promoter region of the *Su(z)2* gene showed strong silencing due to the flanking sequences. But, how can *Psc* and *Su(z)2* genes remain active to assure PcG mechanism in endogenous situation, despite the binding of PcG proteins and the H3K27-trimethylation? To address this question, the PP-4.2 fragment that contains the promoter, the 1st exon and the 1st intron of *Psc* gene was put into the same reporter construct (Fig. 11A). All independent lines, like PP 4.2 (1M-2M) line, exhibit very weak and confined expression of the *lacZ* in imaginal discs and very weak expression of *mini-white* in adult eyes. Importantly, these repressive effects are not sensitive to *PcG* dosage. The excision of PP-

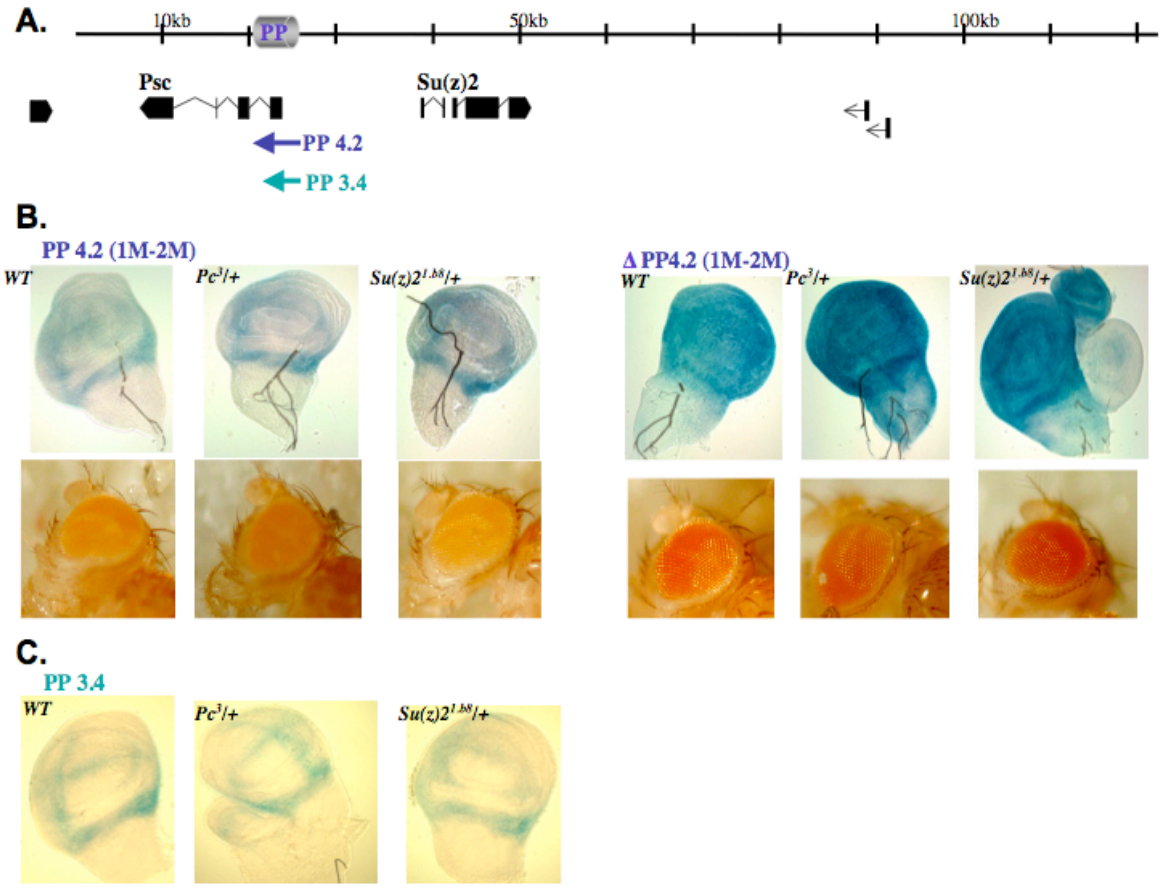


Figure 11. The PP region behaves as down-regulation module.

(A) PP-4.2 is a 4.2kb-fragment which has a promoter, 1st exon and 1st intron of *Psc* gene. PP-3.4 is a 3.4kb-fragment which has a promoter, 1st exon and half of the 1st intron of *Psc* gene.

(B) All of lines, like 1M-2M line shown above, exhibit very weak expression of the *lacZ* gene in imaginal disc and *mini-white* gene in adult eye. These expression patterns would not change in *PcG* mutant background. When this fragment is excised by flipase, both reporter genes express strongly.

(C) The line which has PP-3.4 fragment shows the same effect with PP-4.2 line.

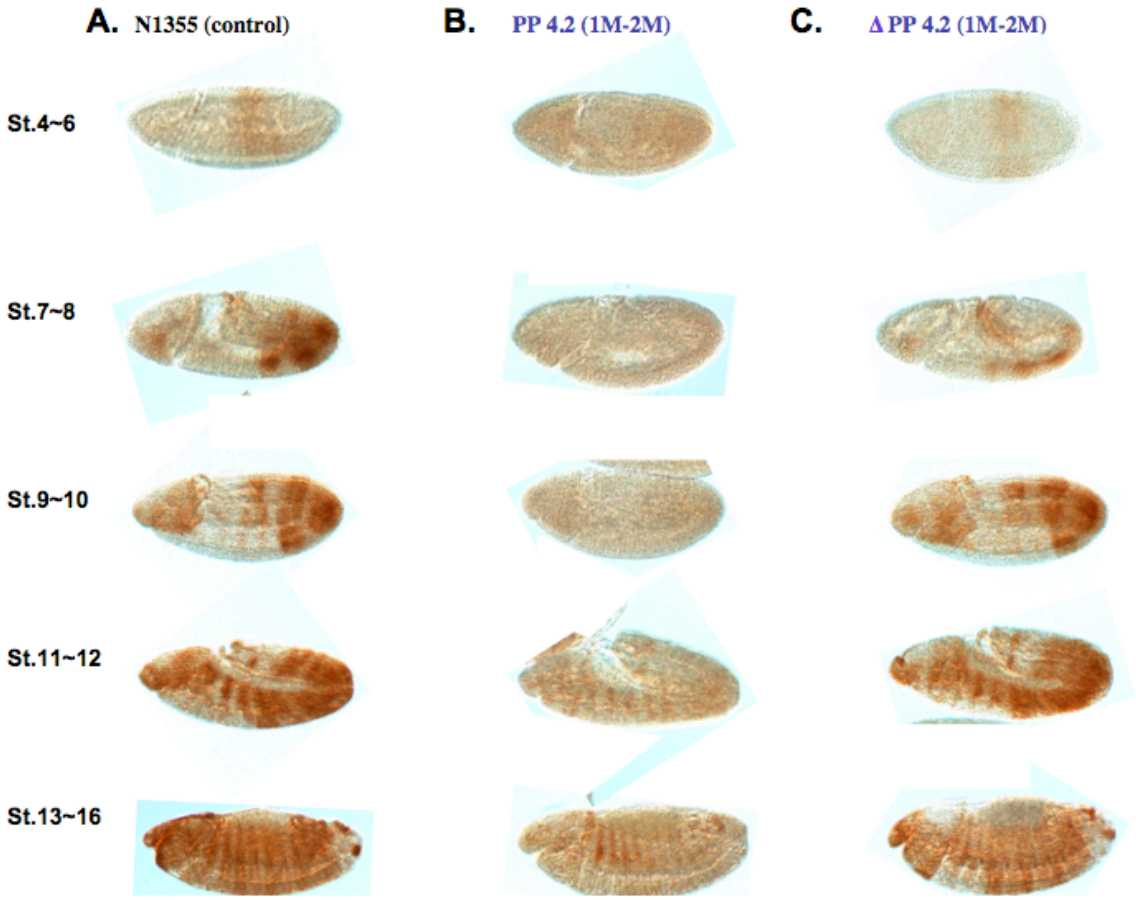
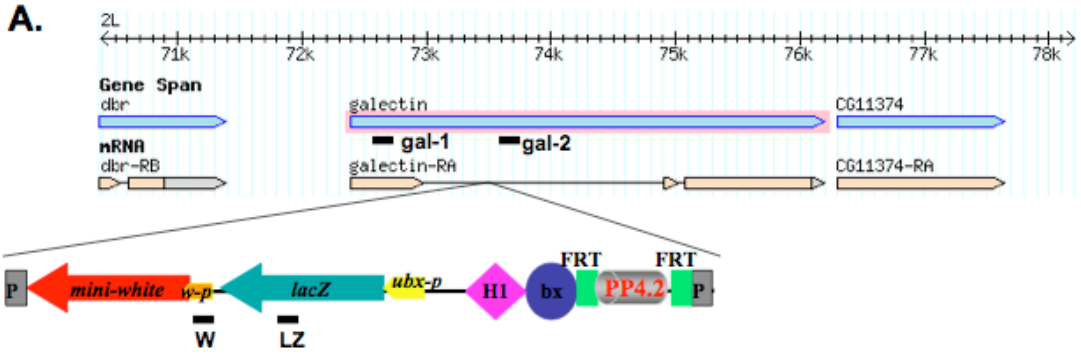
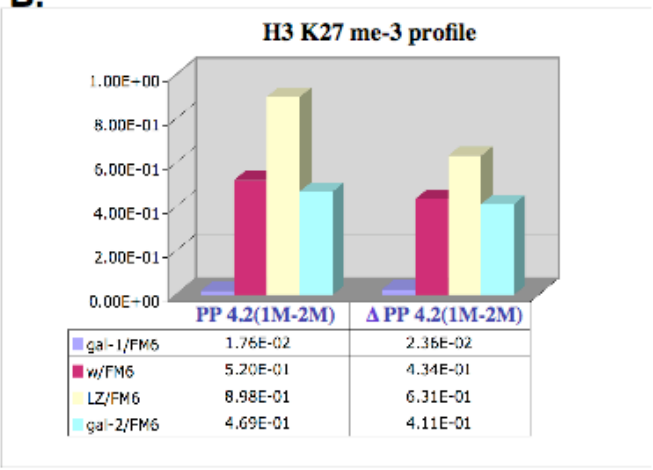


Figure 12. The PP region behaves as a down-regulation module in embryonic stage. Embyos of flies carrying the transposons are stained with anti-*β*-galactosidase antibody. **(A)** N1355 is the random insertion of a control construct which does not have any regulatory elements. In early stages, it shows a specific segmental pattern in posterior part. At the end of germ band extension, expression appears in all parasegments. **(B)** The PP-4.2 line shows very weak expression of the *lacZ* reporter gene in entire embryonic stage. **(C)** When the PP-4.2 fragment is excised by flipase, the expression pattern of the *lacZ* reporter gene is recovered like N1355 control line.

4.2 fragment by FRT/FLP system results in increased expression of both reporter genes (Fig. 11B). Also, a slightly shorter fragment, the PP-3.4-fragment lacking half of 1st intron gives the same effect (Fig. 11C). The *lacZ* expression has been determined also in embryonic stages by immunostaining with anti-*β*-galactosidase antibody. The control line, N1355, containing the same vector with no inserted fragment, displays specific parasegmental pattern during early embryogenesis: stage 4-10, because the bx embryonic enhancer activates the *Ubx* promoter in even-numbered parasegments but is repressed by Hunchback in the anterior half part of the embryo. In the later embryo, it shows ectopic expression of *lacZ* in anterior segments, because the construct lacks a PRE (Fig. 12A). But, the PP 4.2 (1M-2M) line showed very weak expression without any specific pattern at all stages (Fig. 12B). This repressive effect, like that in larval stages, was not sensitive to *PcG* dosage (data not shown). So, the specific module in this PP-4.2 fragment somehow seems to interfere with enhancer activity and down-regulates the expression of reporter gene in entire development. When the PP-4.2 fragment was excised by FRT/FLP system, the parasegmental expression of *lacZ* is recovered, resembling that of the N1355 control line (Fig. 12C). Nonetheless, the promoter region of *Psc* and *Su(z)2* genes might have PRE, because this region has significant binding peaks for PcG proteins and H3K27me3. It is possible that this presumptive PRE in the promoter region might be too weak to initiate PcG silencing mechanism alone and would need to cooperate with other PREs. To clarify if this down-regulation module is independent to PcG mechanism, the expression of reporter gene needs to be examined under homozygous *PcG* mutant background. As an alternative way to address this question, I performed chromatin immunoprecipitation to determine the H3K27 tri-methylation profile of the reporter genes


A.
2L
71k
72k
73k
74k
75k
76k
77k
78k
Gene Span
dbr
galectin
CG11374
gal-1
gal-2
mRNA
dbr-RB
galectin-RA
CG11374-RA
P
mini-white
w-p
lacZ
ubx-p
H1
bx
FRT
PP4.2
FRT
P
W
LZ

B.


H3 K27 me-3 profile
1.00E+00
8.00E-01
6.00E-01
4.00E-01
2.00E-01
0.00E+00
PP 4.2(1M-2M)
Δ PP 4.2(1M-2M)
gal-1/FM6 1.76E-02 2.36E-02
w/FM6 5.20E-01 4.34E-01
LZ/FM6 8.98E-01 6.31E-01
gal-2/FM6 4.69E-01 4.11E-01

C.

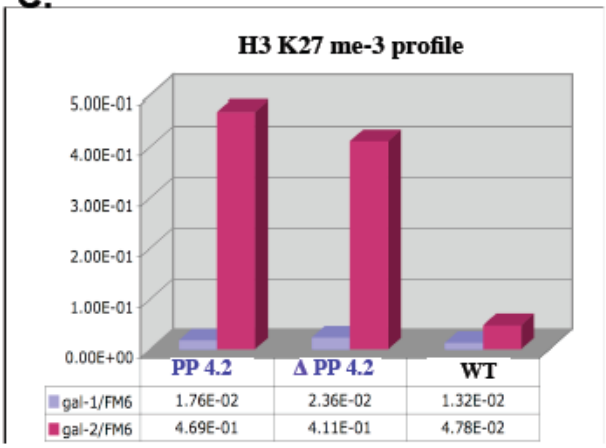

H3 K27 me-3 profile
5.00E-01
4.00E-01
3.00E-01
2.00E-01
1.00E-01
0.00E+00
PP 4.2
Δ PP 4.2
WT
gal-1/FM6 1.76E-02 2.36E-02 1.32E-02
gal-2/FM6 4.69E-01 4.11E-01 4.78E-02

Figure 13. Both PP-4.2 line and Δ PP-4.2 line show the high enrichment of H3K27me3.

Overnight aged embryos from PP-4.2 line, Δ PP-4.2 line, and wild type are subjected to chromatin-immunoprecipitation with anti H3K27me3 antibody.

(A) The PP-4.2-1M-2M transposon is located on the 1st intron of *galectin* gene on 2L. The map is derived from BDGP. The positions of DNA fragments amplified for quantification of ChIP results are indicated as black lines. The amplicons are named as gal-1, W, LZ, and gal-2.

(B) The precipitation of DNA fragments with anti H3K27-me3 was quantified by Real-Time PCR and the precipitation values are normalized to those of FM6 amplicon from the *bxd*-PRE. The distribution of H3K27-me3 along the transposon and flanking regions in embryos of the PP-4.2 line and the Δ PP-4.2 line is shown.

(C) The distribution of H3K27-me3 along the transposon and flanking regions in embryos of the PP-4.2 line, the Δ PP-4.2 line, and wild type is shown.

of both the PP 4.2 (1M-2M) line and the Δ PP 4.2 (1M-2M) line (Fig. 13). To this end, the chromatin isolated from overnight embryos (0-16h) of both transgenic lines and wild type was immunoprecipitated with anti H3K27me3 antibody or mock IP without antibody as a negative control (data not shown). DNA isolated from these IPs was amplified using both positive and negative control primer sets as well as primers from the region of interest. FM6 region in *Ubx-PRE* core as a known positive control is highly precipitated with this antibody in all lines, but the *umbl* region which is used as a negative control is much less precipitated (data not shown) (Schwartz et al., 2006). Then, the level of H3K27me3 was determined in the flanking regions, and in the *lacZ* gene and *mini-white* gene of the transposon, using quantitative Real Time PCR. All absolute values from amplicons were normalized to that from FM6 region. This transposon turned out to be inserted in the 1st intron of the *galectin* gene (Fig. 13A). On the gal-1amplicon which is 700bp distant from this insertion site, both the PP 4.2 (1M-2M) line and the ΔPP 4.2 (1M-2M) line showed very low enrichment of methylation. But, surprisingly, both lines showed high enrichment of H3K27me3 on the *lacZ* transcript and significant enrichment on *mini-white* gene promoter. This significant enrichment of H3K27me3 extended to the other flanking site, gal2 which is 60 bp distant from the insertion site (Fig. 13B). This methylation is presumably derived from flanking region. To clarify this, H3K27me3 ChIP was carried out with chromatin from wild type embryos and measured with amplicons gal1 and gal2. Unlike the transposon insertion line, the wild type host showed very low methylation on both sites (Fig. 13C). This suggests that the transposon insertion may interfere with transcriptional activity and low transcription seems to produce an enrichment of H3K27me3 by unknown mechanism. So, in this genomic

context, I could not determine if this downregulation manner is related to PcG silencing mechanism. These results need to be confirmed with other independent lines, where the transoposon is inserted in more neutral chromatin environments.

To clarify this down-regulation pattern in the promoter region of *Psc* gene, it is worthwhile to analyze this region in a more natural genomic context. So, I tested the promoter region of *Psc* gene with another construct which has the lacZ reporter gene driven by *Psc* own promoter with no enhancers and *mini-white* gene as a marker (Fig. 14A). The upstream region was also added to this construct. Transgenic lines which have DNA fragments corresponding to half (4.3kb) or most (10kb) of the intergenic region between *Psc* and *Su(z)2* placed in front of the *Psc* own promoter and *lacZ* gene showed weak expression of the *lacZ* reporter gene in imaginal discs, independent of *PcG* genes dosage (Fig. 14). But, some of the lines containing the 2.3kb promoter fragment of *Psc* showed repression of the *lacZ* gene rather than derepression in the presence of a Pc^{3} heterozygous mutation, while it would not change in the presence of $Su(z)2^{1.b8}$ heterozygous mutation (Fig. 14B). This effect has been reported also in another target gene of PcG, *polyhomeotic* (Fauvarque and Dura, 1993; Bloyer et al., 2003). The transgenes containing the *ph*-PRE have shown hyper-repression in the presence of *Polycomb* mutation. So, these results suggest that the promoter region of *Psc* gene might have a down-regulation module that seems to behave differently from a usual PRE. It remains to be elucidated whether this effect is associated with PcG mechanism or not.

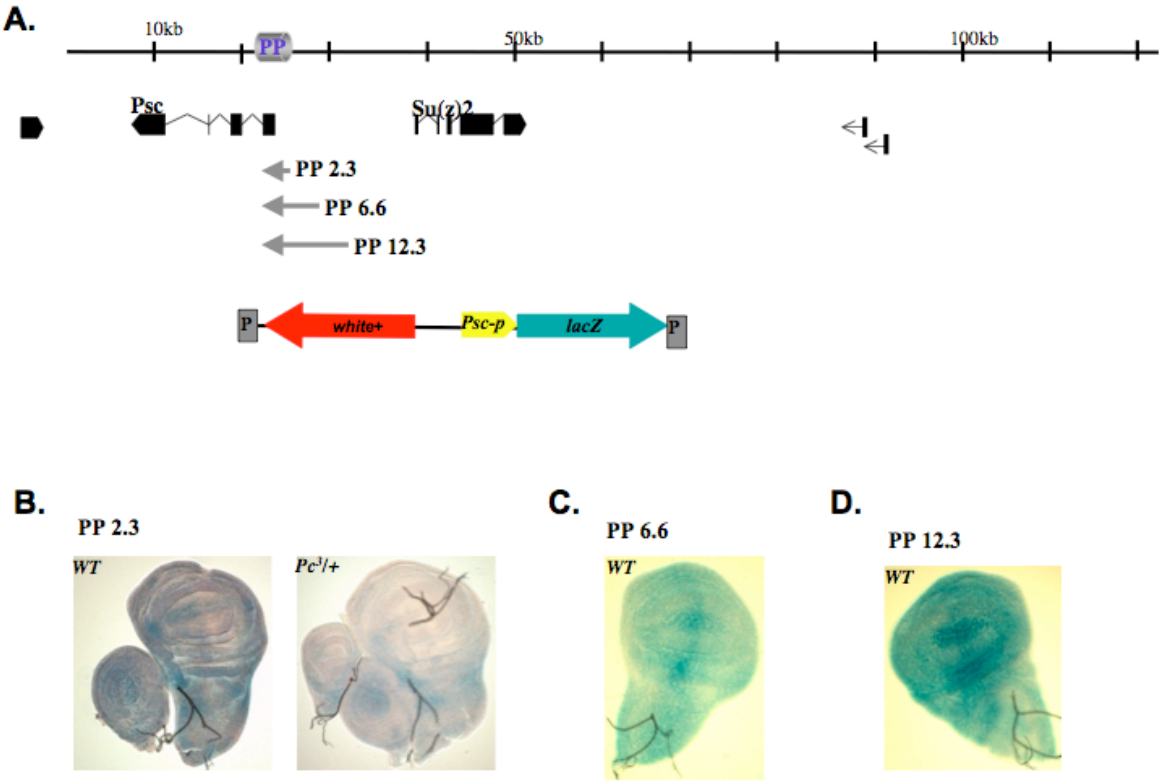


Figure 14. Intergenic regions between *Psc-Su(z)2* genes show downregulation under *Psc* own promoter.

(A) The gray arrows indicate the fragments containing a half 4.3kb (PP 6.6) of intergenic region, most 10kb (PP 12.3) of intergenic region under 2.3kb promoter (PP 2.3)of *Psc* gene. Each fragment is put into the construct which has a *lacZ* reporter gene (dark green arrow) controlled by the *Psc* gene promoter (yellow) and a *white* gene as a marker. **(B)** The PP-2.3 transposon display very weak expression of *lacZ* reporter gene. Under *polycomb* heterozygous mutant background, it shows much weaker expression. The PP-6.6 **(C)** and the PP-12.3 **(D)** transposons show weak expression of *lacZ* reporter gene.

CONCLUSION

I have identified if the presumptive PREs that are predicted from the microarray data are functional PREs by reporter gene assay. There are at least two functional PREs in *Psc-Su(z)2* locus. SD PRE which is located downstream of *Su(z)2* gene behaves as a typical PRE and another, PD PRE which is located downstream of *Psc* gene seems to behave as a PRE and anti-silencer depending on chromosomal context. One of transposon which is inserted in the vicinity of *Su(z)2* promoter confirmed that the intergenic region between *Psc-Su(z)2* genes is functionally regulated by PcG mechanism. Additional regulation module like a down-regulation module is found in the vicinity of *Psc* promoter. Transgenic studies cannot support the presence of boundary elements beyond the PD region and the SD region, but it still remains to be elucidated with other reporter gene construct. These observations from transgenic studies may demonstrate that several different kinds of regulatory elements might be cooperative to modulate the expression of *Psc-Su(z)2* expression, despite binding PcG.

DISCUSSION

The characterization of multiple PcG binding sites in *Psc-Su(z)2* locus

The major questions in this work concern understanding how PcG mechanisms down-regulate rather than silence the *Psc-Su(z)2* genes through the putative PREs in this locus. The ChIP on chip analysis, using sg4 *Drosophila* embryonic cell line has shown that the *Psc-Su(z)2* region, containing two *PcG* genes, binds both PC, PSC and E(Z) at multiple sites, while the distribution of trimethylated H3K27 extends over the entire transcriptional unit, the promoter, the upstream and the downstream region. It strongly suggests that the *Psc-Su(z)2* region is one of PcG target sites. But, this gene must be active to ensure the functioning of the PcG mechanism and the presence of protein has been detected by immunoprecipitation and immunostaining (data not shown) in sg4 cells. Therefore, the PcG mechanism must be in some way different from the all-or-none paradigm from PcG regulation of *homeotic* genes in this locus. So, how do PcG proteins regulate the expression of *Psc* and *Su(z)2* genes? How can the expression of *Psc* and *Su(z)2* genes remain active under PcG mechanism? How do those putative PcG binding sites behave? Are they acting as PRE or other modules? Do all putative PREs which were found in microarray contribute to regulate the expression of *Psc* and *Su(z)2* genes *in vivo*? To address these questions, firstly, we analyzed whether those putative PRE fragments are functional *in vivo* by transgenic studies, because the microarray has been carried out on sg4 cell line. As expected, the results presented here show that the putative PRE (SD) which contains the biggest binding peak of PcG proteins turned out to act as a typical PRE, using transgenic lines. All 10 independent lines showed the variegated expression

of the *lacZ* reporter gene, dependent on *PcG* dosage, but the expression of the *mini-white* gene was not influenced by the SD region in any independent lines. Some PRE sites can act in long distance from the promoter region of target gene. But, the SD PRE does not seem to be able to act on the *mini-white* gene which is ~9kb distant from it in the transposon construct. Nevertheless, the SD PRE appears to be strong enough to be unaffected by flanking genomic context and is able to initiate PcG silencing in all ten genomic insertion sites obtained. But, the assembly of PcG proteins seems to be influenced by genomic context, at least to some extent, because the degree of derepression was stronger in the presence of *Pc* heterozygous mutation than *Psc-Su(z)2* heterozygous mutation or vice versa, depending on insertion sites. Additionally, the N1363 transgenic line strongly suggests that the SD region behaves as a typical PRE *in vivo*. If the insulator in 3'end of *mini-white* gene is active (Chetverina et al., 2008), the repressive effect derived from the proximal part of SD region would be blocked, because this N1363 transgene is inserted in opposite orientation relative to *Su(z)2* gene. Then, the repression of the reporter gene might be caused by the distal part of SD region. In fact, the distal part of SD region harbors the highest binding peak of PcG proteins. The SD-8.4kb fragment might contain more than one PRE, because there is low but significant binding peak of PC, E(Z) and PSC beside the highest peak.

Also, it is possible that this region may harbor an insulator, because the domain of methylation drop off and the binding peak of PC also disappears immediately outside of the SD region (Kahn et al., 2006). But, none of transgenic lines which have this SD region showed signs of an insulator activity that could block the communication between the SD PRE and the *Ubx* promoter, since in all lines there was strong repression of the

Ubx-lacZ gene. But, we cannot exclude the possibility of the existence of insulator in this SD region, because the enhancers may interfere with the appropriate function of insulator in this transgenic construct or the insulator might be stage-specific. It would be helpful to examine the effect of this SD region in a construct containing a *mini-white* gene with no enhancers. The other possibility is that the insulator in the SD region may require cooperative action with other presumptive insulator near the PD region in which the methylation domain subsides and the binding peak of PC also drops off immediately outside of the PD region (Kahn et al., 2006).

Another putative PRE (PD), located 8kb downstream from *Psc* gene, has shown various effects, depending on genomic context. Most probably, the PD region is enough flexible or weak to be affected by transcriptional state from nearby genes. Depending on insertion sites, the PD fragment behaves as PRE and anti-silencer or TRE (Trithorax response element). Both 3F-M and M2-F1 lines which exhibited PRE activity are inserted nearby other PRE containing sites. It may be explained by a homing effect, which PRE-containing transposon tends to be inserted in the vicinity of other PRE containing chromosomal sites (Farvarque and Dura, 1993). It has been observed that PRE-containing transposons often show a dramatic enhancement of silencing, called pairing-sensitive repression, when the fly is homozygous for transposon insertion. In fact, the 3F-M line showed stronger repression of the *lacZ* reporter gene, when they are homozygous than heterozygous (data not shown). Also, in this context, the PD-4.1 PRE could act in long distance to repress the *mini-white* gene, unlike the case of the SD PRE. On the contrary, the other line named M2-F1, which is inserted in a promoter region of *Su(z)2* gene (SP)

behaves as an anti-silencer to inhibit PcG silencing, because after excision of the PD region repression was stronger than in the non-excised line. Certainly, this M2-F1 line should be examined in the presence of *trx* mutations to know if the PD-4.1 region has both PRE and TRE activity. Nonetheless, the expression of *lacZ* in some cells of wing imaginal disc are still repressed even in the presence of the PD region. Probably, this anti-silencer or TRE may be relatively stable to compete with PRE in some cells, but not others. Most of all, this observation from the M2-F1 line strongly suggests that the putative SP region may be a functional PRE *in vivo* and the *Psc-Su(z)2* locus proves to be functionally regulated by PcG mechanism, corresponding to the ChIP on chip data. If there is an insulator-like module in the 3'end of *mini-white* (Chetverina et al., 2008) in M2-F1 line, this module could block the PRE activity from the distal part of SP region, because this transgene is inserted in the same orientation relative to *Su(z)2* gene. Also, it is possible that this region may harbor an insulator, because the binding peaks of PC protein drop off immediately and the domain of methylation subsided near outside of PD region. But, transgenic lines which have the inverted PD region still show repression of the *lacZ* reporter gene. So, this argues against the presence of an insulator in the PD region that could block the communication between the PD PRE and the *Ubx* promoter. But, we cannot exclude the possibilities of the existence of insulator in this PD region. The illustration of reason is the same with the case of the SD region.

But, despite the presence of strong functional PREs in the *Psc-Su(z)2* region, how can the *Psc* and *Su(z)2* genes remain active to assure PcG mechanism? To address this question, I tested transgenic constructs containing the promoter region of *Psc* gene. Firstly, all independent lines which have a promoter, the 1st exon and either the 1st intron of *Psc* or

the half of the 1st intron (PP-4.2) display down-regulation pattern in reporter gene assay during embryonic and larval stages, but this pattern was insensitive to *PcG* dosage. Probably, this module might interfere with the enhancer activity and down-regulate the expression of reporter genes. Since the specific pattern mediated by the *bx* embryonic enhancer disappeared in this transgenic line, this module must be functional from early embryonic stages, when this enhancer is activated. So, the PP-4.2 region seems to behave differently from a typical PRE, whose function sets in during gastrulation. Nonetheless, the promoter regions of *Psc* and *Su(z)2* genes might have a PRE, because these regions have significant binding peaks for PcG proteins and H3K27me3. It is possible that this presumptive PRE in the promoter region might be too weak to initiate PcG silencing mechanism alone and would need to cooperate with other PREs. This may explain why ΔM2-F1 transposon which is inserted in the promoter of *Su(z)2* showed strong PcG silencing effect in endogenous context. To clarify if this downregulation module is independent of PcG mechanisms, the expression of the reporter gene needs to be examined under homozygous *PcG* mutant background. As an alternative way to address this question, chromatin IP have been performed to measure the change of H3K27me3 profile in the *lacZ* and the exogenous *mini-white* genes with both PP-4.2-1M-2M and ΔPP-4.2-1M-2M lines. Paradoxically, both lines showed high enrichment of H3K27me3 in reporter genes and nearby flanking region. It is possible that the transposon insertion may interfere with transcriptional activity and low transcription might produce an enrichment of H3K27me3 by unknown mechanism. But, how can the reporter genes express actively in ΔPP-4.2-1M-2M line even in the highly methylated context? This is quite an extraordinary case on the basis of current paradigm. It might be relevant to check

the profile of H3K4me3 which is an active transcriptional mark. If the same region can show the high enrichment of H3K4me3 as well as H3K27me3, the transcription is not necessarily silenced. Perhaps, the ratio between the levels of H3K4me3 and H3K27me3 might determine the activity of transcription. It remains to be understood. So, these results need to be confirmed with other independent lines, where the transposon is inserted in more neutral chromatin environment. Another possible explanation for down-regulation is enhancer sharing. In fact, the *bx* embryonic enhancer and H1-imaginal disc enhancer are placed between the *Ubx* promoter and the PP-4.2 region. This PP-4.2 fragment contains also the *Psc* own promoter as well as 1st exon and 1st intron, although the order is the opposite orientation relative to reporter genes. Since these enhancers can act on both promoters, enhancer sharing might cause less transcriptional activity of reporter gene driven by *Ubx* promoter. To clarify this downregulation pattern of the PP-4.2 region, it is worthwhile to analyze this region in a more natural genomic context. To this end, I tested this region is another construct which has the lacZ reporter gene driven by *Psc* own promoter with no enhancers and using the *mini-white* gene as a marker. The upstream region was also added to this construct. The transgenic lines which have half (4.3kb) or most (10kb) of the intergenic region showed weak expression pattern of *lacZ* reporter gene under *Psc* own promoter. Also, some of the lines which have only 2.3kb *Psc* own promoter showed weak expression. These observations suggest that the promoter region of the *Psc* gene harbors down-regulation module, which behaves differently from a typical PRE and seems to be independent of PcG regulation. Moreover, some of the lines in this series, containing only the 2.3kb *Psc* promoter fragment showed very unusual repression in the presence of a *Pc* heterozygous mutation

rather than derepression. So, in this context, the PC bound on the promoter region seems to behave as an activator, instead of a repressor, but other constructs which have additional upstream region did not show any sensitivity to the *PcG* dosage. The similar effect has been reported in another target gene of PcG, *polyhomeotic* (Fauvarque and Dura, 1993; Bloyer et al., 2003). The *polyhomeotic* gene is also a member of the *PcG* genes so as to be active to ensure the PcG mechanism in entire development. In experiments with reporter gene constructs, an identified PRE in the *ph-p* (*polyhomeotic-proximal)* appears unusual, because it requires at least 2kb of sequence containing the *ph* promoter rather than several hundred base pairs like usual PRE. Also, the *Psc* gene is required for activation of *ph* gene, while other *PcG* genes negatively regulate the *ph* gene. Moreover, the transgenes containing the identified PRE has shown hyper-repression in the presence of a *Pc* heterozygous mutation, while derepression under other *PcG* mutations. Recently, the genome-wide microarray studies (Schwartz et al., 2006) have also shown the *ph* locus binds PC without a significant level of E(Z) and of H3K27me3 unlikely with other PcG targets. So, these results suggest that the *ph* PRE functions somewhat differently from a usual PRE. These data demonstrate that both the *Psc-Su(z)2* locus and the *ph* gene might be modulated by PcG mechanism instead of being silenced to remain active. The unusual PRE-like modules in their promoter regions might contribute to attenuate the PcG silencing in these locus.

On the basis of transgenic studies, I characterized several putative PRE fragments in *Psc-Su(z)2* locus. These observations suggest the following. The PD region acts as PRE or anti-silencer, depending on insertion site, and the SD region behaves as typical PRE.

And, the insertion of transposons in the SD region and in the SP region strongly support the idea that *Psc* and *Su(z)2* genes are functionally regulated by PcG mechanism. There is a down-regulation module in the vicinity of the promoter of *Psc* gene, which is acting by unknown mechanism. So, these different modules might cooperate to modulate the expression of *Psc* and *Su(z)2* genes, despite the binding of PcG proteins on several sites. It is possible that the down-regulation module might attenuate the PcG silencing to make *Psc* and *Su(z)2* genes be active partially.

CHAPTER II

The function of SD region in *Psc-Su(z)2* locus

INTRODUCTION

From chapter I, I conclude that several different regulatory fragments might cooperate to modulate the expression of *Psc* and *Su(z)2* genes. Among them, the SD region might be the major PRE to initiate PcG silencing and cooperate with other relatively weak PREs to spread PcG silencing and trimethylation of H3K37 in entire *Psc-Su(z)2* locus, because the SD region has shown the highest binding peak of PcG proteins, on the basis of microarray data and it presented strong PRE activity, according to the transgenic studies. The SD PRE seems to regulate the expression of *Psc* and *Su(z)2* genes even in far distance, because the methylation domain does not extend into the distal genes and the binding peaks of other PcG proteins disappear completely outside of the SD region. The SD PRE might cooperate with other putative PREs to regulate the *Psc-Su(z)2* locus, but not influence into distal genes. So, to understand whether the expression of *Psc-Su(z)2* genes is functionally regulated by newly identified PREs, firstly, I generated small deletion lines in the SD region, using the N1363-P element. I expected that the loss of the SD PRE might influence the expression level of *Psc* and *Su(z)2* genes, because the PcG binding profile and H3K27me3 domain would be changed in entire locus, according to the cooperativity hypothesis.

RESULTS

P element mobilization to make a small deletion in SD PRE

To generate small deletions, the N1363-P element, which is located in the middle of SD PRE, was mobilized by crossing to a stock that expresses transposase (Fig. 15A). To identify imprecise excisions out of 192 candidate lines, genomic PCR was carried out with the isolated genomic DNA from 192 candidates, using two kinds of primer pairs: one pair producing an amplicon which is 50bp distant from the left side of N1363 insertion and one pair producing an amplicon which is 266bp distant from the right side of N1363 insertion. Out of 192 candidates, 184 lines turned out to be precise excisions that gave the expected size of products with both sets of primers. Two deletion lines (A27 and B34) did not give any products with either set of primers, indicating that they are imprecise excisions (data not shown). Two other lines (C6 and C43) could generate the product with the primer pairs on the left side of N1363 insertion, but could not generate the product with the primer pairs on the right side. This suggests that these lines have lost some sequences from the right side of N1363 insertion. To map these small deletions, the genomic DNA was isolated from imprecise excision lines (A27, B34, C6 and C43), *w67* as control, N1363 original transposon, and the B46 line, which is one of the precise excisions (Fig. 16). It was digested with *Bam*HI and hybridized by DIG-High prime labeled probes: 2.5kb *EcoR*I fragment for the left side of the N1363 insertion and 3.7kb *EcoR*I fragment for right side. As expected, both probes hybridized to a 8.3kb fragment in *w67* but in the N1363 line, the left probe revealed a band of approximately 18kb and

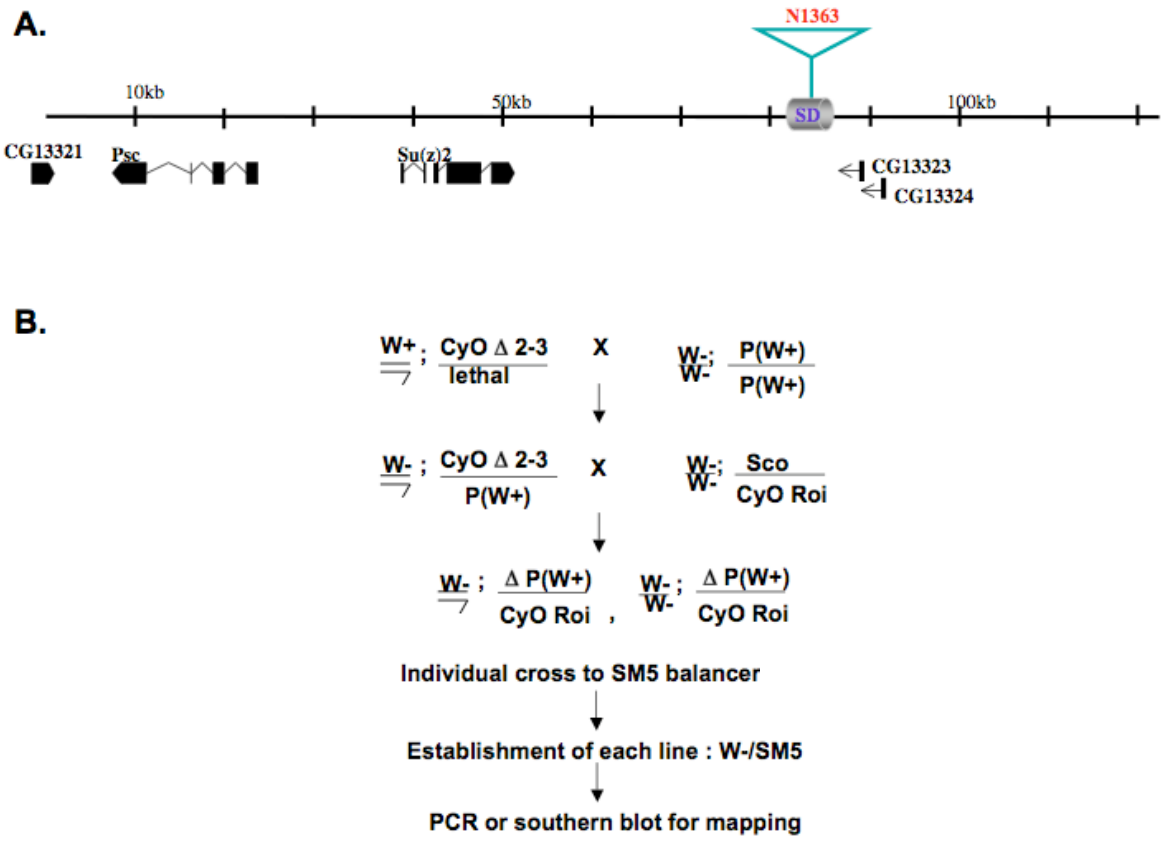


Figure 15. P element mobilization to make a small deletion

(A) The imprecise excision is generated with the N1363 line which is located in the middle of SD PRE.

(B) P-element, N1363, can be mobilized by a cross a stock that expresses transposase, w^{+}; P(Δ2-3) CyO/l. Either precise or imprecise excision lines are crossed with w^{-}; Sco/CyORoi. The genomic PCR and southern blot are carried out to isolate imprecise excision lines out of 192 candidates.

the right probe a fragment of 3.5kb, because there is one more *Bam*HI site inside of 5'end of transposon construct. In the case of the A27 deletion line, the size of the band was reduced to 5~6kb and the intensity was much weaker with the right probe than with the left probe. Presumably, the A27 deletion line has lost some flanking sequences from the right side. In the case of the B34 deletion line, the size of the band was also reduced to 4~5kb and the intensity was almost the same with both of probes (Fig. 16B,C). Probably, the B34 deletion line has lost some flanking sequences from both sides. Two other lines (C6 and C43) showed slightly smaller size of the band with both probes relative to that from either *w67* or the B46 precise excision line. According to the data from genomic PCR, they might have lost a little sequence from the right side. As expected, the B46 line gave the same size of band with both probes as *w67*. To map more precisely the breakpoints of the A27 and B34 deletion lines, the genomic DNA from imprecise excision lines (A27 and B34), *w67* as control, N1363 original transposon, and the A 42 line which is one of precise excisions (Fig. 17) was digested with *EcoR*I and hybridized by DIG-High prime labeled probes: 2.8kb-fragment (digested by *Bam*HI and *Xba*I) for the left side of the N1363 insertion and 2.3kb- fragment (digested by *Bgl*II and *Bam*HI) for right side. As expected, the left probe hybridized to 2.8kb and 2.5kb fragments in *w67* and the N1363 line. The A27 deletion line also gave the 2.8kb and the 2.5kb bands like *w67* with this probe. As expected from the previous Southern blot, the A27 deletion line has lost the fragment from the right side of the N1363 insertion. But, the B34 deletion line produces the 2.8kb band and a faint ~ 3kb band in place of 2.5kb band (Fig. 17B). Most probably, part of the 2.5kb fragment is deleted and combined with flanking sequences. The right probe hybridized to 3.7kb and 2.6kb fragments in *w67*, but N1363

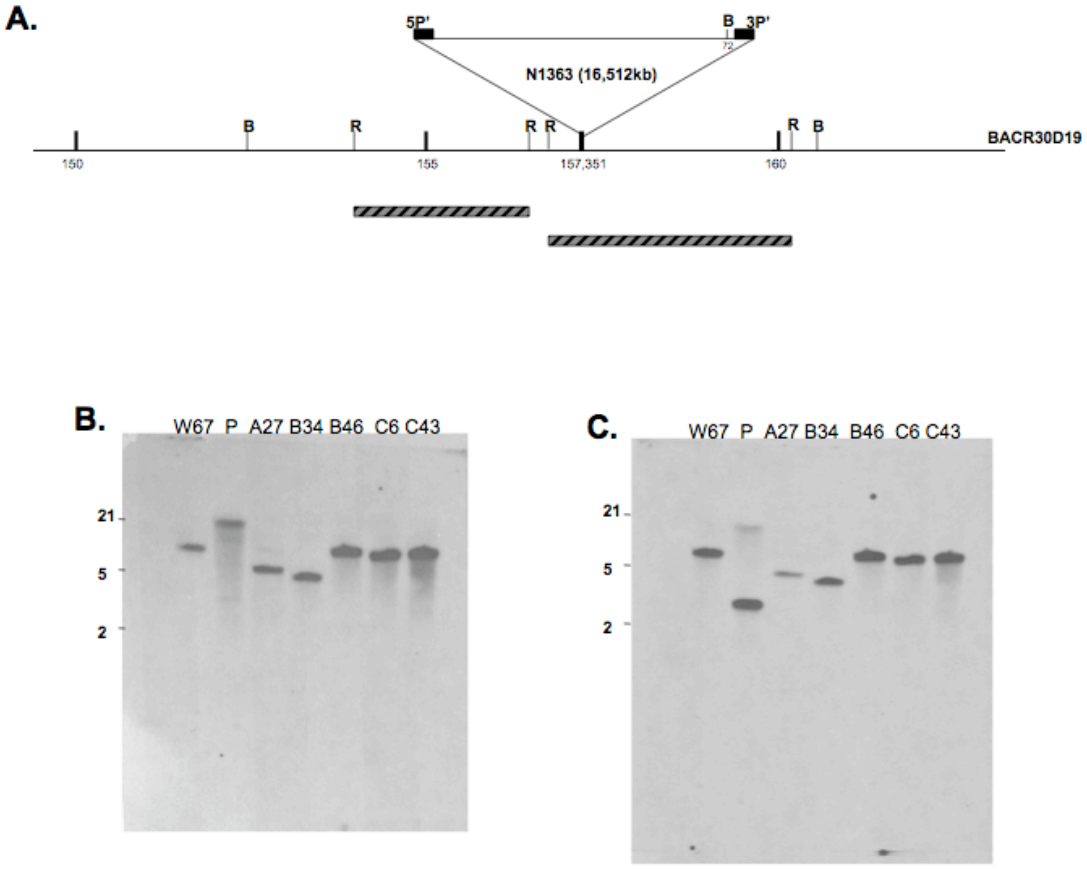


Figure 16. Genomic Southern blot I

(A) The location of N1363 line is marked on the map of BACR30D19 clone from BDGP. Sites for the following endonucleases are indicated: *Bam*HI (B) and *Eco*RI (R).

The gray-colored lines indicate the probes that are used for genomic southern blot: 2.5kb *Eco*RI fragment (left) and 3.7kb *Eco*RI fragment (right).

(B) Genomic DNA is isolated from w67, P (N1363 line), and excision lines: A27, B34, B46, C6, and C43. It is digested with *Bam*HI and labeled with DIG labeled-2.5kb *Eco*RI fragment. **(C)** The same blot is deprobed and labeled with with DIG labeled-3.7kb *Eco*RI fragment.

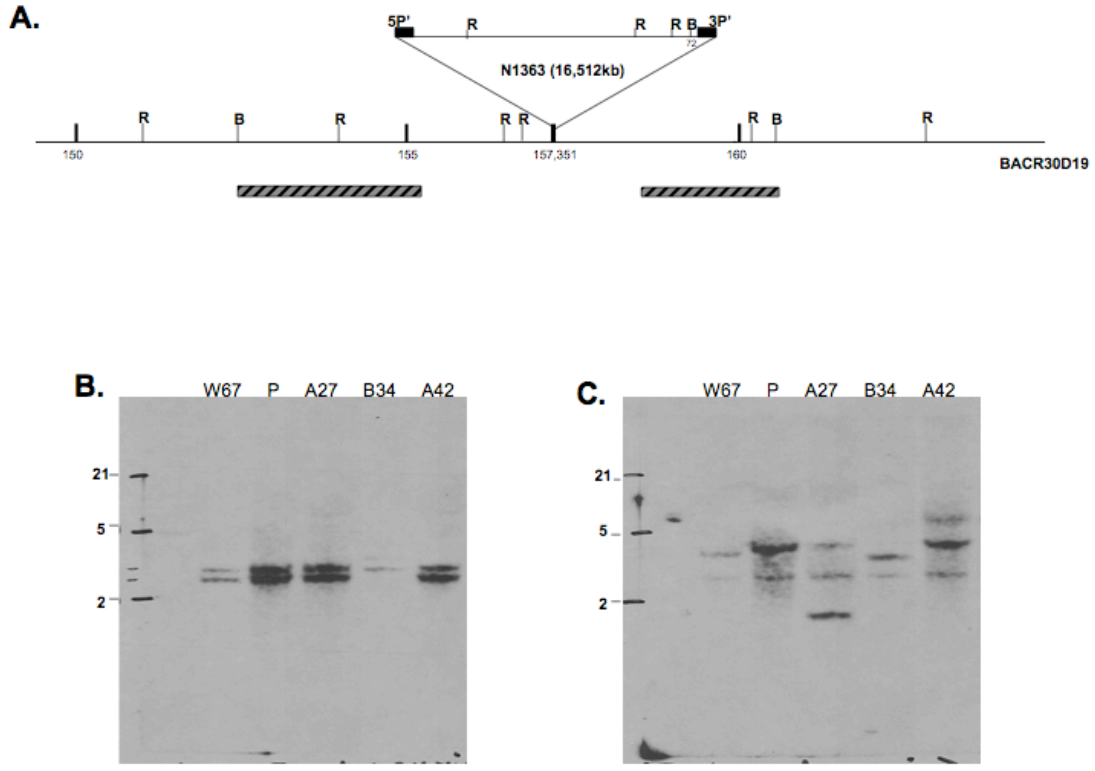


Figure 17. Genomic Southern blot II

(A) The location of N1363 line is marked on the map of BACR30D19 clone from BDGP. Sites for the following endonucleases are indicated: *Bam*HI (B) and *Eco*RI (R).

The gray-colored lines indicate the probes that are used for genomic southern blot: 2.8kb-fragment (digested by *Bam*HI and *Xba*I) for the left side of the N1363 insertion and 2.3kb- fragment (digested by *Bgl*II and *Bam*HI) for right side.

(B) Genomic DNA is isolated from w67, P (N1363 line), and excision lines: A27, B34, and A42. It is digested with *Eco*RI and labeled with DIG labeled-2.8kb-fragment.

(C) The same blot is deprobed and labeled with with DIG labeled-2.3kb- fragment.

line has shown 2.6kb and little bit larger band than 3.7kb, because there is one *Eco*RI site near to 3'P from the transposon. And, the intensity of the 3.7kb band was stronger than that of the 2.6kb band, because the right probe covered more portion of the 3.7kb fragment than the 2.6kb fragment. In the case the A27 deletion line, the size of the 3.7kb band was reduced to ~1.3 kb and the size of the 2.6 kb band was not changed. Again, in the case of the A27 deletion line, a part of the 3.7kb fragment is deleted and the size reduced to ~1.3kb. In the case of B34 line, the size of the 3.5kb band was reduced to ~3kb and 2.6kb band. Most probably, part of the 3.7kb fragment was deleted and combined with flanking sequences (Fig. 17C).

On the basis of the Southern blot, genomic PCR and sequencing were carried out to determine the precise breakpoints of deletions (Fig. 18A). In the case of the A27 deletion line, since the 3.7kb *Eco*RI-fragment in the right side of the N1363 insertion was disrupted and the size was reduced to ~1.3kb, genomic PCR was performed with primer sets close to 5'end and 3'end of the 3.7kb *Eco*RI-fragment (Fig. 18B). As expected, a ~1.3kb band was produced. Sequencing of this product with the same FWD primer showed that the A27 deletion line lost 2.49kb from the right side of the N1363 insertion. In the case of the B34 deletion line, since the 2.6kb *Eco*RI-fragment in the left side and the 3.7kb *Eco*RI-fragment in the right side of the N1363 insertion were disrupted, genomic PCR was performed with primer sets close to 5'end of the 2.6kb fragment and 3'end of the 3.7kb fragment. A fragment of ~3.5kb was produced from this PCR (Fig. 18B). Sequencing of this product with a nested primer showed that the B34 deletion line lost 3.2kb, part from one side and part from the other side of the N1363 insertion. Both deletions lost most of the sequences that bind E(Z), PC and PSC (Fig. 18A).

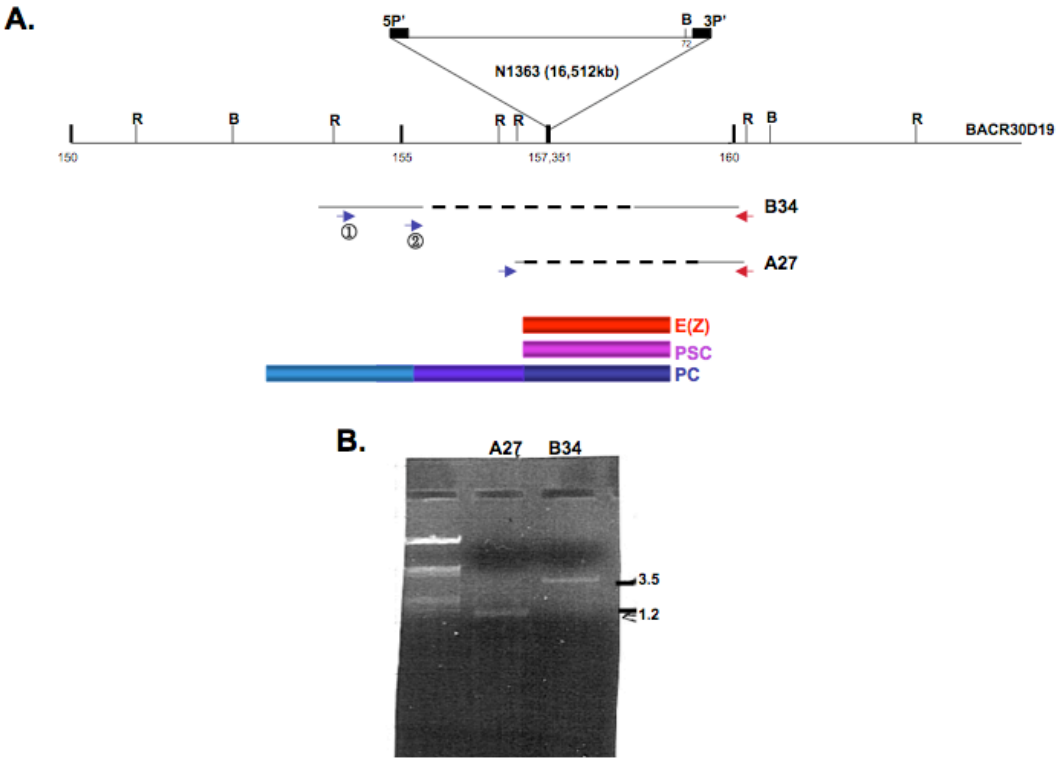

A.
5P'
B
3P'
72
N1363 (16,512kb)
R
B
R
R
R
R
B
R
BACR30D19
150
155
157,351
160
B34
①
②
A27
E(Z)
PSC
PC
B.
A27
B34
3.5
1.2

Figure 18. The precise mapping with PCR and Sequencing

(A) The location of N1363 line is marked on the map of BACR30D19 clone from BDGP. Sites for the following endonucleases are indicated: *Bam*HI (B) and *Eco*RI (R). The blue arrow implied the site of FWD primers for genomic PCR and sequencing. The red arrow indicate the site of BWD primers for genomic PCR and sequencing. Two dashed lines in A27 and B34 lines present deleted regions, which loose most of sequences that bind PcG proteins. Each colored bar represents the area of binding peak for E(Z) (red), PSC (purple), and PC (blue), according to microarray profile (Fig. 7).

(B) Genomic DNA is isolated from excision lines (A27 and B34) and amplified by genomic PCR with primer sets: for A27 line (A27 FWD and A27 BWD, see M & M) and for B34 line (nest B34-F2 ① and B34-B1, see M & M). For microsequencing, A27-FWD primer was used for A27 line and nest B34-F5 primer ② was used for B34 line.

Analysis of *Psc-Su(z)2* and *CG13323-CG13324* expression pattern in the deletion lines

To check whether transcriptional levels of *Psc-Su(z)2* genes are changed by the deletion of the SD region, a Reverse Transcriptase-PCR was done with RNA extracted from the two deletion lines, from the original transposon insertion line and from the *w67* control at embryonic stages (Fig. 20). In parallel, a control reaction was run simultaneously without reverse transcriptase. On the basis of embryonic transcriptional profiles of *Psc* and *Su(z)2* (microarray analysis of expression profiles during Drosophila embryonic development, kindly provided by *Affymetrix*, Fig. 19 and published in Manak et al., 2006). I analyzed three different embryonic stages: 4-8hrs in which *Psc* is highly expressed, 10-14hrs in which both of *Psc-Su(z)2* genes are well expressed and 16-22hrs when *Su(z)2* is significantly expressed, while the expression of *Psc* is decreased. The level of expression of *Psc-Su(z)2* genes was quantified by real time PCR and the value was normalized to the amount of the ribosomal protein gene *rp49*. The level of *Psc* and *Su(z)2* expression is increased by two to three fold in the A27 deletion line compared to that of wild type, solely in 16~22hrs embryos, while in other stages, there is no significant difference among lines (Fig. 20B, C). These data showed that loss of one putative PRE could derepress by two to three fold the expression of *Psc-Su(z)2* genes at late embryonic stages. Perhaps, the increased levels of *Psc-Su(z)2* gene products when the SD PRE is deleted might be self-regulating because of increased by binding to the other putative PREs in the *Psc-Su(z)2* locus. Alternatively, RT PCR might fail to detect a more significant tissue specific difference, because the input RNA represents the entire cell population of the embryo. To test this possibility, in situ-hybridization with RNA probes

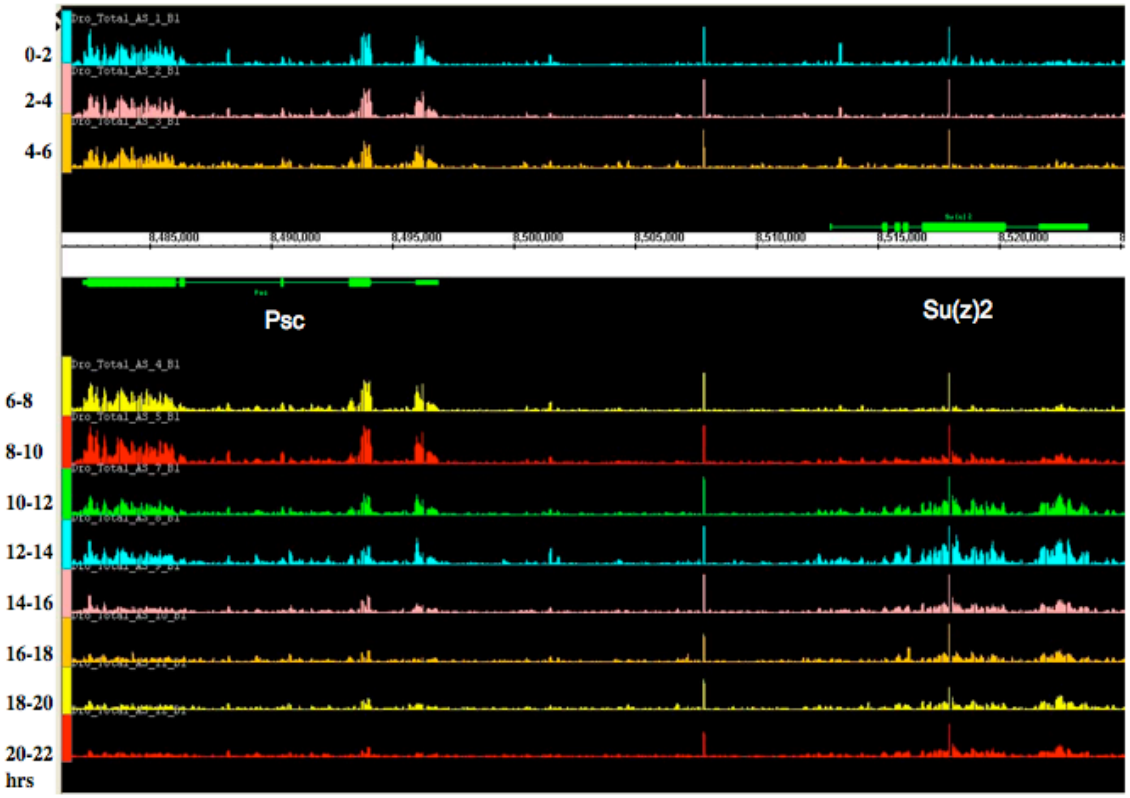
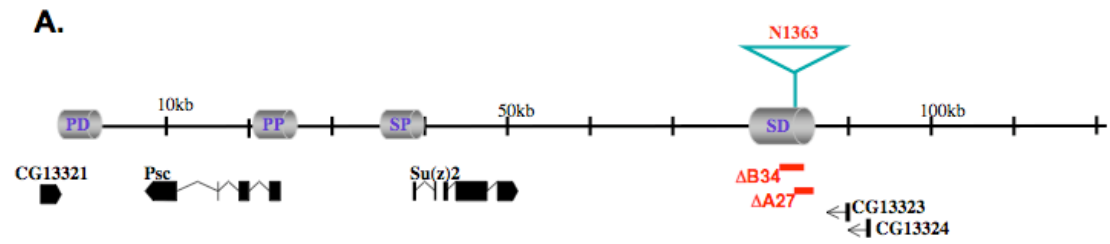
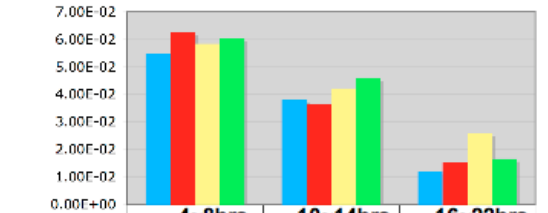


Figure 19. *Psc-Su(z)2* expression profile

The transcriptional levels of *Psc* and *Su(z)2* genes are shown during embryonic stages (0~22hrs). This data is provided from *Affymetrix* (Manak et al., 2006).

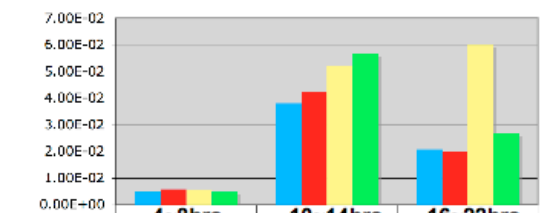


B.



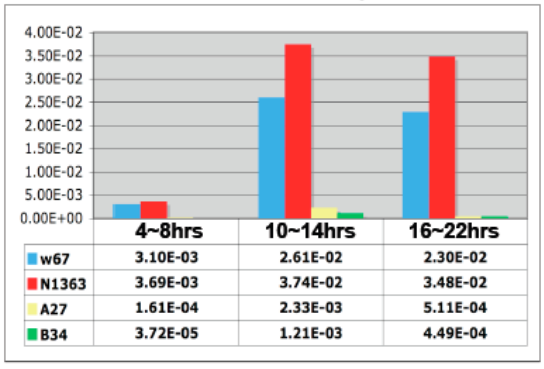
	4~8hrs	10~14hrs	16~22hrs
w67	5.49E-02	3.80E-02	1.21E-02
N1363 line	6.25E-02	3.65E-02	1.56E-02
A27	5.84E-02	4.21E-02	2.60E-02
B34	6.03E-02	4.60E-02	1.68E-02

C.



	4~8hrs	10~14hrs	16~22hrs
w67	4.80E-03	3.81E-02	2.09E-02
N1363	5.79E-03	4.23E-02	1.99E-02
A27	5.53E-03	5.22E-02	6.00E-02
B34	4.81E-03	5.69E-02	2.69E-02

D.



	4~8hrs	10~14hrs	16~22hrs
w67	3.10E-03	2.61E-02	2.30E-02
N1363	3.69E-03	3.74E-02	3.48E-02
A27	1.61E-04	2.33E-03	5.11E-04
B34	3.72E-05	1.21E-03	4.49E-04

Figure 20. Analysis of *Psc-Su(z)2* and *CG13323-CG13324* expression pattern with deletion lines

(A) This diagram is showing the insertion site of N1363 and the map of both of deletion-lines, A27 and B34.

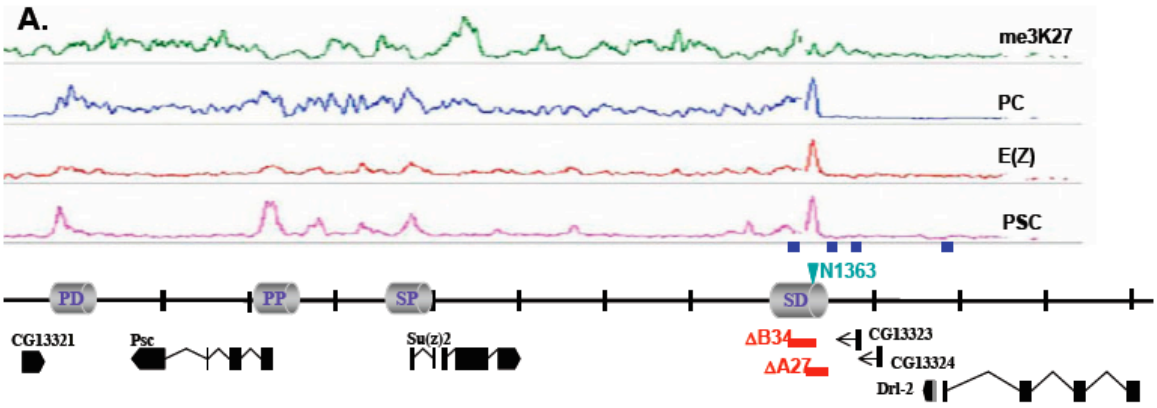
(B) A Reverse Transcriptase-PCR is done with RNAs from both of deletion lines, A27 (yellow) and B34 (green), from the original transposon insertion line, N1363 (red) and from the *w67* control (blue) at three different stages: 4-8hrs, 10-14hrs, and 16-22hrs. The quantification is carried out with real time- PCR and the amount of cDNA is normalized by the amount of *RpL32 (rp49)* cDNA. This graph is showing the *Psc* expression pattern among lines. **(C)** This graph is showing the *Su(z)2* expression pattern among lines. **(D)** This graph is showing the level of expression for *CG13323* and *CG13324*, which are distal to the outside of the SD PRE in *Psc-Su(z)2* locus.

The data from three independent experiments are averaged.

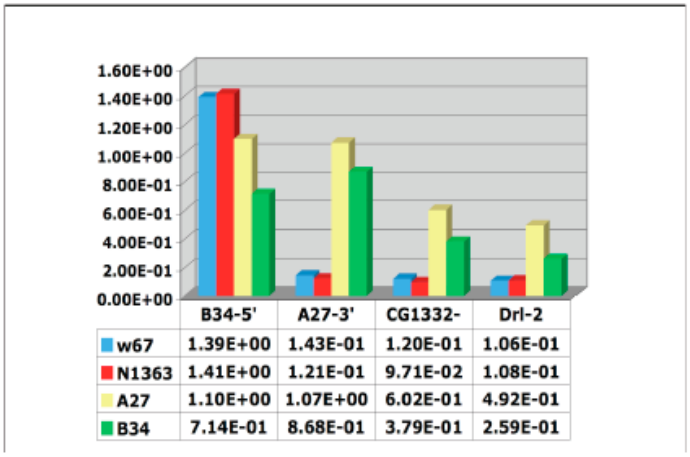
for *Psc* or *Su(z)2* was performed, but I could not detect any significant difference among the lines (data not shown). Also, we checked the level of expression for *CG13323* and *CG13324*, two small transcription units immediately distal to the SD PRE in *Psc-Su(z)2* locus. Unexpectedly, the mRNA levels of both *CG13323* and *CG13324* decreased 20 to 50 fold in deletion mutants compared to those of wild type at all stages (Fig. 20D). These two genes are almost identical and their predicted protein products consist of 112 amino acids, but their function is unknown. Why would deletion of the SD PRE region result in the loss of expression of the genes immediately outside of the *Psc-Su(z)2* locus? There are two possible explanations for this phenomenon. One possibility is that the excision of the P-element (N1363) in the SD PRE removes an enhancer necessary for the proper expression of the *CG13323* and *CG13324* genes. Another explanation for this phenomenon is that the excised fragment contained a DNA boundary element, separating the two genetic regions and protecting the two genes from repression by the SD PRE. The loss of the boundary would allow spreading of chromatin silencing into the adjacent region, downregulating the expression of the *CG13323* and *CG13324* genes.

Analysis of the profile of H3K27me3 in deletions

To address these questions, I analyzed the H3K27-trimethylation profile in the deletion lines, using chromatin IP performed on 0~14hrs old embryos from each line (Fig. 21). Chromatin isolated from embryos for all lines was immunoprecipitated with the anti H3K27me3 antibody or mock IP without antibody as a negative control. DNA isolated from these IPs was amplified using both positive and negative control primer sets as well as primers from the region of interest. The FM6 region in *UBX*- PRE core as a known



B.



	B34-5'	A27-3'	CG1332-	Drl-2
w67	1.39E+00	1.43E-01	1.20E-01	1.06E-01
N1363	1.41E+00	1.21E-01	9.71E-02	1.08E-01
A27	1.10E+00	1.07E+00	6.02E-01	4.92E-01
B34	7.14E-01	8.68E-01	3.79E-01	2.59E-01

Figure 21. Analysis of the profile of H3K27me3 in deletions

(A) Microarray approaches with ChIP experiments (Schwartz et al., 2006) is showing that H3K27me3 domain is decreased behind the SD PRE.

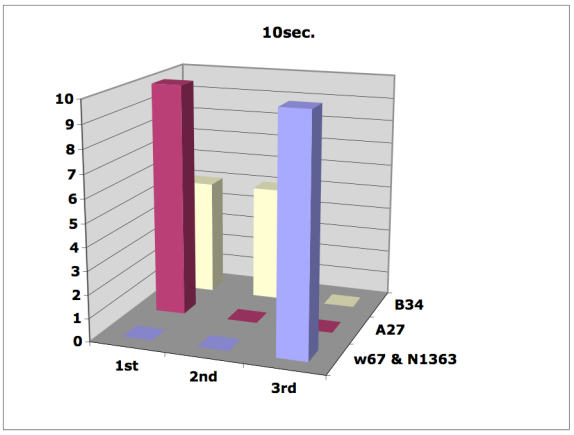
(B) Overnight aged embryos from *w67*, N1363, A27 and B34 lines are subjected to chromatin-immunoprecipitation with anti H3K27me3 antibody. The precipitation of DNA fragments with anti H3K27-me3 was quantified by Real-Time PCR and the precipitation values are normalized to those of FM6 amplicon from the *bxd*-PRE. The distribution of H3K27me3 along the end of deletion in embryos of four lines is shown. The positions of DNA fragments amplified for quantification of ChIP results are indicated as blue lines (A). The amplicons are named as B34-5', A27-3', CG1332-, and Drl-2 from left to right.

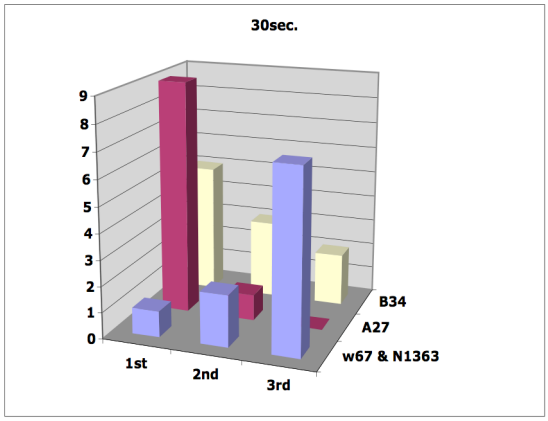
positive control is highly precipitated with this antibody in all lines, but *umbl* region which is used as a negative control is much less precipitated (data not shown) (Schwartz et al., 2006). Then, the profile of H3K27-trimethylation was monitored at the border of the deletions and all absolute values from the amplicons were normalized to that from the FM6 region. At the 5'end of the B34 deletion line, all the fly stocks show high enrichment of H3K27-trimethylation with almost the same level as the positive control. But, at the 3'end of the A27 deletion line, both of *w67* and N1363 show that the level of H3K27-trimethylation is certainly low, while in both deletion lines, H3K27-trimethylation continues at a high level. On the transcriptional units of genes *CG13323-CG13324* and *Drl-2*, the H3K27-trimethylation gradually decreases in the deletion lines, though still more significantly enriched than in *w67* and N1363 (Fig. 21B). Analysis of the amplicons from the region surrounding the SD PRE from wild type, the original P element insertion (N1363), and the two deletion lines A27 and B34 suggests the following. There is a clear demarcation in H3K27-trimethylation levels in wild type and the original P element insertion line (N1363) between the *Psc-Su(z)2* locus and the distal region. In the deletion mutants, H3K27-trimethylation extends into the adjacent region. What prevents the spreading of H3K27-trimethylation and silencing from the *Psc-Su(z)2* locus to the adjacent genes in wild type flies? It does not appear to be a boundary element that acts to block this spreading. Based on my transgenic studies (Fig. 9B), the SD region placed into a reporter gene construct, in the same orientation relative to the reporter gene, acts as a PRE element. If this region contained a classical boundary element, the SD segment would block PRE/promoter communication of the reporter gene, resulting in no silencing of the *lacZ* gene. So, it is possible that the removed fragment harbors the

enhancer for *CG13323-CG13324* genes and the inactive transcriptional state admits the spreading of PcG silencing.

Analysis of climbing activity

While examining the phenotype of the deletion lines, I noticed that the adult males homozygous deletion showed reduced climbing activity, compared to wild type. This phenotype is stronger in the A27 deletion line than in the B34 deletion line. To examine this phenotype, 10 seven days-old males from each line were placed into a vial that was divided into 3 different levels. The climbing rate of these flies was measured at the following time intervals, 10s, 20s, and 2min. after striking the tube on the table. All males from *w67* and original transposon (N1363) climbed up immediately in 10s and started to move down again in 30 sec. Males from the B34 deletion line climbed up very slowly and most of them could reach the highest level of tube in 2min. Males from the A27 deletion line could not climb again after being knocked down and their movements remained limited to the bottom of the tube at 2min. (Fig. 22). Then, to examine the climbing activity as a function of age, three lines of flies; *w67*, A27, and B34 were tested at different ages (Fig. 23). 5 vials of each line containing 10 flies each were used and the flies were tested for their ability to climb to the top of the vial every 3 days from 6 to 21 days of age. The vials were gently shaken and set on the table and the activity of the flies was observed after 5 seconds. The number of flies that stayed at the bottom of the vial was recorded. The probability "P" is the ratio the number of flies that climbed away from the bottom to the total number of flies. As expected, males from *w67* climbed up very rapidly in 5sec. from day6 to day21, independent on aging. In the case of males from the





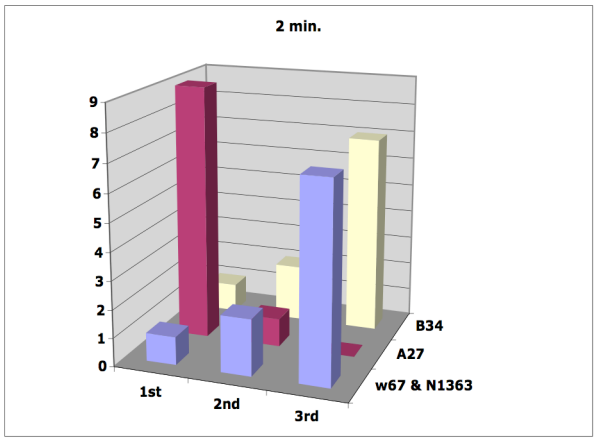


Figure 22. Analysis of climbing phenotype

To examine this phenotype, ten seven days old males from each line were placed into a vial that was divided into 3 different levels (1^{st}, 2^{nd}, 3^{rd}). The climbing rate of these flies was measured at the following time intervals, 10s, 30s, and 2min. after striking the tube on the table.

(X axis: the level of tube, Y axis: the number of flies, Z axis: the name of fly lines)

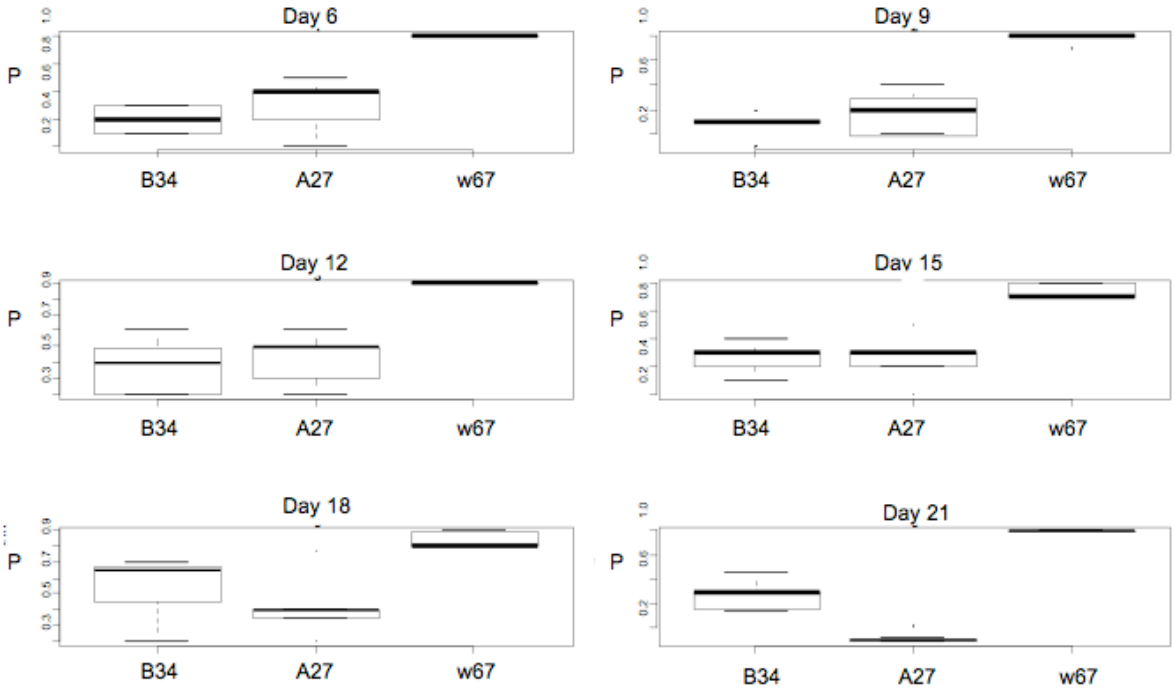


Figure 23. Analysis of climbing activity on aging

To measure their climbing activity, three types of flies; W67, A27, and B34 were tested. 5 vials of each type containing 10 flies each were used (3 types x 5 vials/type x 10 flies/vial = 150 flies). The flies were observed for their ability to climb to the top of the vial every 3 days (from 6 to 21 days of age). The vials were gently shaken and set on the table to observe the activity of the flies after 5 seconds. The number of flies that stayed at the bottom of the vial was recorded. The probability "P" is the ratio the number of flies on the top to the total number of flies.

B34 deletion line, some of them could climb up from day 6 to day 21, but slower than *w67*. Also, few males from the A27 deletion line could climb up from day 6 to day 18, but slower than *w67*. On day 21, all of males failed to climb. The case of females has shown the similar results (data not shown). So, from this analysis, I conclude that the activity of the A27 line seems to depend on aging, because the climbing ability is decreased as time goes on, but the B34 line is also less active than *w67*, independent of aging. The A27 deletion line seems to show more severe effects on climbing than the B34 deletion line. Most probably, the additional sequences removed from the A27 deletion line aggravate the phenotype relative to that of the B34 deletion line. Which gene in *Psc-Su(z)2* locus could cause this phenotype? This phenotype might be related to the reduced level of *CG13323-CG13324* but conclusive tests remain be carried out.

CONCLUSION

Deletion of SD PRE increases slightly the expression level of *Psc* and *Su(z)2* genes. This result may suppose that other putative PREs can compensate to function properly even without SD PRE or, it might support our hypothesis that the expression of *Psc* and *Su(z)2* genes is modulated under PcG mechanism rather silenced. On the contrary, the expression of *CG13323* and *CG13324* genes in the A27 and B34 deletion lines is silenced. Compared to wild type, the adult flies showed reduced climbing activity, suggesting that *CG13323* and *CG13324* genes may be involved in climbing phenotype. Both deletion lines showed H3K27me3 spreading into adjacent genes outside of the SD PRE from the ChIP experiment. The fragment may harbor an enhancer necessary for the proper expression of *CG13323* and *CG13324* genes.

DISCUSSION

The function of SD PRE in *Psc-Su(z)2* region

To understand whether the expression of *Psc* and *Su(z)2* genes is functionally regulated by the newly identified PREs, firstly, we generated small deletion lines in the SD region, using the N1363-P element. Because the SD region has the highest binding peak of PcG proteins and behaves as a strong PRE in the transgenic studies, we expected that the loss of SD PRE might influence the level of expression of *Psc* and *Su(z)2* genes. RT- PCR experiments show that the A27 deletion line seem to derepress slightly the expression of *Psc* and *Su(z)2* genes only by 2~3 fold at late embryonic stages, while the B34 deletion line does not show any significant difference. Most probably, the A27 deletion line might have lost more functional sites for PcG silencing than the B34 deletion line. But, RT PCR would fail to show a tissue-specific difference, because the input RNA represents all cells in the embryo. So, in situ-hybridization with RNA probe for *Psc* and *Su(z)2* genes was performed to clarify this result, but we could not detect any significant difference in the tissue specificity of expression. Perhaps the increased expression of *Psc* can partially compensate by binding to the other putative PREs in the *Psc-Su(z)2* locus or the other putative PREs are redundant to regulate the normal expression of *Psc-Su(z)2*.

Enhancer or insulator beyond SD PRE

Although the level of expression of *Psc* and *Su(z)2* genes does not change significantly in either deletion, we could detect a strong change of expression of *CG13323-CG13324* genes, which are located just beyond the SD region. Using RT-PCR, the level of

expression of these transcripts in either deletion decreased dramatically by 20 to 50 fold during entire embryonic stages. It is possible that the mutants might have lost an enhancer for *CG13323* and *CG13324* or a boundary element that would block the H3K27-trimethylation and the silencing effect. Chromatin IP with anti H3K27-me3 antibody has shown that in the two deletions, H3K27-trimethylation spreads into the flanking genes by 10kb further. If these deletions remove the enhancer for *CG13323* and *CG13324* genes, lack of transcription may allow the methylation to spread onto those genes, because the methylation domains might spread until they encounter 'active' chromatin, characterized by histone acetylation or methylation of H3 K4, marks typical of transcriptionally active genes (Schwartz et al., 2006). Alternatively, the deletions remove an insulator that blocks PcG silencing, resulting in extended methylation and silencing of both genes. But, all transgenic lines presented above contain the SD region placed into a reporter gene construct in the same orientation relative to the reporter gene. All independent lines showed variegated expression patterns of the *lacZ* reporter gene in wing imaginal disc from 3rd instar larvae, depending on *PcG* dosage as like typical PRE. If this region contained a classical boundary element, the SD segment would block PRE/promoter communication of the reporter gene, resulting in no silencing of the *lacZ* gene. So, I did not see any effects suggestive of the presence of an insulator. We cannot exclude the possibility that this putative insulator is too weak to block PcG silencing by itself in the context of the transgene. To clarify this question, it will be worthwhile to put the fragment that contains both presumptive PRE and presumptive insulator in both orientations into a plain *white+* reporter gene construct without any other regulatory elements, which might interfere. As an alternative way, the level of expression *CG13323-*

CG13324 genes need to be determined in the presence of *PcG* mutations. If the *CG13323-CG13324* genes are repressed by the spread of H3K27me3, they should be derepressed in *PcG* mutations. Otherwise, if they have lost an enhancer, the level of expression of these genes would not changed in *PcG* mutations.

But, how can the H3K27me3 be highly enriched and even spread further into adjacent genes, despite of the loss of the SD PRE in deletion lines? On the basis of microarray data, small binding peaks have been detected in proximal region of the SD region (Fig. 21). It is possible that these putative PREs can compensate to keep the domain of methylation as normal state, even without the SD PRE. So, the deletion line that looses more regions might show the decrease of the PcG binding and the methylation profile in this locus.

The function of the unknown genes; *CG13323-CG13324, Drl-2*

Compared to wild type, the climbing activity is reduced in either of the two deletions, in which the level of expression for *CG13323* and *CG13324* decreased by 20 to 50 fold. These two small genes are almost identical and their protein products of unknown function, consist of 112 amino acids. The loss of expression might suggest that these genes may be involved in climbing phenotype. To address this question, a transgene could be generated possessing the genomic DNA of *CG13323* and a presumptive enhancer part. This transgene could be used to examine whether the climbing defect of deletion lines can be rescued. In addition, the A27 deletion line exhibits a stronger climbing defect phenotype and more enrichment of H3K27-me3 on *Drl-2* as well as *CG13323-CG13324*, relative to B34 deletion line. It may suggest that A27 deletes a

greater portion of enhancer or boundary element for adjacent genes. Another possibility is that the climbing defect might be caused by abnormal expression of *Drl-2* gene, because trimethylation of H3K27 is extended significantly also into *Drl-2* gene. Though the quantitative RT PCR did not show a significant difference of expression level for *Drl-2* between two deletion lines (data not shown), in situ hybridization might show the tissue specific difference. We suspect that the increased level of methylation inhibits the expression of one or both of these genes in a portion of the CNS responsible for fly locomotion or some aspect of the behavior. The gene *Drl-2* is thought to be involved in central nervous system development (personal communication, Santschi, 2002).

REFERENCES

1. Ali JY and Bender W. (2004) Cross-regulation among *Polycomb* group genes in *Drosophila melanogaster*. Mol. Cell. Biol. 24: 7737-7747

2. Beisel C, Imhof A, Greene J, Kremmer E and Sauer F. (2002) Histone metylation by the *Drosophila* epigenetic transcriptional regulator Ash1. Nature 419: 857-862.

3. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, and Lander ES. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125: 315-326

4. Beuchle D, Struhl G, and Muller J. (2001) Polycomb group proteins and heritable silencing of Drosophila Hox genes. Development 128: 993-1004

5. Bloyer S, Cavalli G, Brock HW, and Dura JM. (2003) Identification and characterization of *polyhomeotic* PREs and TREs. Dev. Biol. 261: 426-442

6. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, and Jaenisch R. (2006) Polyomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441: 349-353

7. Bracken AP, Dietrich N, Pasini D, Hansen KH, and Helin K. (2006) Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev. 20: 1123-1136

8. Brown JL, Grau DJ, Devido SK, and Kassis JA. (2005) An Sp1/KLF binding site is important for the activity of a Polycomb group response element from the *Drosophila engrailed* gene. Nucleic Acids Res. 33: 5181-5189

9. Brown JL, Mucci D, Whiteley M, Dirksen ML, and Kassis JA. (1998) The Drosophila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. Mol. Cell. 1: 1057-1064

10. Brunk BP, Martin EC, and Adler PN. (1991) *Drosophila* genes *Posterior Sex Combs* and *Suppressor two of zeste* encode proteins with homology to the murine *bmi-1* oncogene. Nature 353: 351-353

11. Bryd KN and Shearn A. (2003) ASH1, a *Drosophila* trithorax group protein, is required for methylation of lysine 4 residues on histone H3. Proc. Natl. Acad. Sci. 100: 11535-11540

12. Cao, R, Tsukada Y, and Zhang Y. (2005) Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol. Cell. 20: 845-854

13. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, and Zhang Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298: 1039-1043

14. Chan CS, Rastelli L, and Pirrotta V. (1994) A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression. EMBO J. 13(11): 2553-2564

15. Chetverina D, Savitskaya E, Maksimenko O, Melnikova L, Zaytseva O, Parshikov A, Galkin AV, and Georgiev P. (2008) Red flag on the white reporter: a versatile insulator abuts the white gene in Drosophila and is omnipresent in mini-white constructs. Nucleic Acids Res. 36(3): 929-37

16. Chinwalla V, Jane EP, and Harte PJ. (1995) The Drosophila trithorax protein binds to specific chromosomal sites and is co-localized with Polycomb at many sites. EMBO J. 14(9): 2056-2065

17. Comet I, Savitskaya E, Schuettengruber B, Nègre N, Lavrov S, Parshikov A, Juge F, Gracheva E, Georgiev P, and Cavalli G. (2006) PRE-mediated bypass of two Su(Hw) insulators targets PcG proteins to a downstream promoter. Dev. Cell 11: 117-124

18. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, and Pirrotta V. (2002) *Drosophila* Enhancer of Zeste/ESC complexes have a histone H3 methyltranserase activity that marks chromosomal Polycomb sites. Cell 111: 185-196

19. DeCamillis M, Cheng NS, Pierre D, and Brock HW. (1992) The polyhomeotic gene of Drosophila encodes a chromatin protein that shares polytene chromosome-binding sites with Polycomb. Genes Dev. 6(2): 223-232

20. Déjardin J, Rappailles A, Cuvier O, Grimaud C, Decoville M, Locker D, and Cavalli G. Recruitment of *Drosophila* Polycomb proteins to chromatin by DSP1. (2005) Nature 434: 533-538.

21. Dellino GI, Schwartz YB, Farkas G, McCabe D, Elgin SC, and Pirrotta V. (2004) Polycomb silencing blocks transcription initiation. Mol. Cell 13: 887-893

22. de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, Nesterova TB, Silva J, Otte AP, Vidal M, Koseki H, and Brockdorff N. (2004) Polycomb group proteins RING1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev. Cell 7: 663-676

23. Ebert A, Schotta G, Lein S, Kubicek S, Krauss V, Jenuwein T, and Reuter G. (2004) Su(var) genes regulate the balance between euchromatin and heterochromatin in *Drosophila*. (2004) Genes Dev. 18: 2973-2983
24. Faucheux M, Roignant JY, Netter S, Charollais J, Antoniewski C, and Théodore L. (2003) batman Interacts with polycomb and trithorax group genes and encodes a BTB/POZ protein that is included in a complex containing GAGA factor. Mol Cell Biol. 23(4):1181-1195

25. Fauvarque MO and Dura JM (1993) *polyhomeotic* regulatory sequences induce developmental regulator-dependent variegation and targeted P-element insertions in *Drosophila*. Genes Dev. 7: 1508-1520

26. Fauvarque MO, Zuber V, and Dura JM. (1995) Regulation of *polyhomeotic* transcription may involve local changes in chromatin activity in *Drosophila*. Mec. Dev. 52: 343-355

27. Fischle W, Wang Y, Jacobs SA, Kim Y, Allis CD, and Khorasanizadeh S. (2003) Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomain. Genes Dev. 17: 1870-1881

28. Francis NJ, Kingston RE, and Woodcock CL. (2004) Chromatin compaction by a Polycomb group protein complex. Science 306: 1574-1577

29. Francis NJ, Saurin AJ, Shao Z, and Kingston RE. (2001) Reconstitution of a functional core Polycomb repressive complex. Mol. Cell 8: 545-556

30. Franke A, DeCamillis M, Zink D, Cheng N, Brock HW, and Paro R. (1992) Polycomb and polyhomeotic are constituents of a multimeric protein complex in chromatin of Drosophila melanogaster. EMBO J. 11(8): 2941-2950

31. Gindhart JG Jr and Kaufman TC. (1995) Identification of Polycomb and trithorax group responsive elements in the regulatory region of the Drosophila homeotic gene Sex combs reduced. Genetics 139(2): 797-814.

32. Grimaud C, Bantignies F, Pal-Bhadra M, Ghana P, Bhadra U, and Cavalli G. (2006) RNAi components are required for nuclear clustering of polycomb response elements. Cell. 124: 957-971

33. Grimaud C, Nègre N, and Cavalli G. (2006) From genetics to epigenetics: the tale of Polycomb and trithorax group genes. Chromosome research 14: 363-375.

34. Hodgson JW, Argiropoulos B, and Brock HW. (2001) Site-specific recognition of a 70-base-pair element containing d(GA)(n) repeats mediates bithoraxoid Polycomb group response element-dependent silencing. Mol. Cell. Biol. 21: 4528-4543

35. Horad B, Tatout C, Poux S, and Pirrotta V. (2000) Structure of a Polycomb response element and *in vitro* binding of Polycomb group complexes containing GAGA factor. Mol. Cell. Biol. 20: 3187-3197.

36. Irminger-Finger, I. and Nothiger, R. (1995) The *Drosophila melanogaster* gene *lethal(3)73Ah* encodes a ring finger protein homologous to the oncoproteins MEL-18 and BMI-1. Gene 163: 203-208

37. Iwama A, Oguro H, Negishi M, Kato Y, and Nakauchia H. (2005) Epigenetic regulation of hematopoietic stem cell self-renewal by polycomb group genes. Int. J. Hematol. 81: 294-300

38. Iwama A, Oguro H, Negishi M, Kato Y, Morita Y, Tsukui H, Ema H, Kamijo T, Katoh-Fukui Y, Koseki H, van Lohuizen M, and Nakauchi H. (2004) Enhanced self -renewal of hemotopoetic stem cells mediated by the polycomb gene product Bmi-1. Immunity. 21: 843-851

39. Jürgens G (1985) A group of genes controlling the spatial expression of the bithorax complex in *Drosophila*. Nature 316: 153-155.

40. Kahn TG, Schwartz YB, Dellino GI, and Pirrotta V. (2006) Polycomb complexes and the propagation of the methylation mark at the *Drosophila Ubx* gene. J. Biol. Chem. 281: 29064-29075

41. Kassis JA. (1994) Unusual properties of regulatory DNA from the *Drosophila engrailed* gene: three "pairing-sensitive" sites within a 1.6kb region. Genetics 136: 1025-1038

42. Ketel CS, Andersen EF, Vargas ML, Suh J, Strome S, and Simon JA. (2005) Subunit contributions to histone methyltransferase activities of fly and worm Polycomb group compexes. Mol. Cell. Biol. 25: 6857-6868

43. King IF, Emmons RB, Francis NJ, Wild B, Müller J, Kingston RE, and Wu CT (2005) Analysis of a Polycomb group protein defines regions that link repressive activity on nucleosomal templates to *in vivo* function. Mol. Cell. Biol. 25: 6578-6591

44. King IF, Francis NJ, and Kingston RE. (2002) Native and recombinant Polycomb complexes establish a selective block to template accessibility to repress transcription *in vitro*. Mol. Cell. Biol. 22: 7919-7928

45. Klymenko T and Müller J. (2004) The histone methyltransferases Trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins. EMBO Rep. 5: 373-377

46. Klymenko T, Papp B, Fischle W, Köcher T, Schelder M, Fritsch C, Wild B, Wilm M, and Müller J. (2006) A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. Genes Dev. 20: 1110-1122

47. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, and Reinberg D. (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. 16: 2893-2905

48. Kyba M and Brock HW. (1998) The *Drosophila* Polycomb group protein Psc contacts ph and Pc through specific conserved domains. Mol. Cell. Biol 18: 2712-2720

49. Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, and Young RA. (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125: 301-313

50. Lessard J, Schumacher A, Thorsteinsdottir U, van Lohuizen M, Magnuson T, and Sauvageau G. (1999) Functional antagonism of the Polycomb-group genes eed and Bmi1 in hemopoetic cell proliferation. Genes. Dev. 13: 2691-2703

51. Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P, Van Lohuizen M, and Marino S. (2004) Bmi1 is essential for cerebellar development and is overexpressed in human medullobalstomas. Nature 428: 337-341

52. Levine SS, Weiss A, Erdjument-Bromage H, Shao Z, Tempst P, and Kingston RE. (2002) The core of the Polycomb repressive complex is compositionally and functionally conserved in flies and humans. Mol. Cell. Biol. 22: 6070-6078

53. Lewis EB (1951) Pseudoallelism and gene evolution. Cold Spring Harbor Symp Quant Biol 16: 159-174

54. Lewis EB (1978) A gene complex controlling segmentation in *Drosophila*. Nature 276: 565-570.

55. Li Z, Cao R, Myers MP, Zhang Y and Xu RM. (2006) Structure of a Bmi-1-RING1B Polycomb group ubiquitin ligase complex. J. Biol. Chem. 281: 20643-20649

56. Mallin DR, Myung JS, Patton JS, and Geyer PK. (1998) Polycomb group repression is blocked by the *Drosophila* suppressor of Hairy-wing insulator. Genetics 148: 331-339

57. Manak JR, Dike S, Sementchenko V, Kapranov P, Biemar F, Long J, Cheng J, Bell I, Ghosh S, Piccolboni A, and Gingeras TR. (2006) Biological function of unannotated transcription during the early development of Drosophila melanogaster. Nat Genet. 38(10): 1101-1102

58. Martin CE and Adler PN. (1993) The *Polycomb* group gene *Posterior Sex Combs* encodes a chromosomal protein. Development 117: 641-655

59. Müller J and Bienz M. (1991) Long range repression conferring boundaries of Ultrabithorax expression in the Drosophila embryo. EMBO J.10: 3147-3155

60. Müller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, and Simon JA (2002) Histone methyltransferase activity of a *Drosophila* Polycomb group repressor complex. Cell 111: 197-208

61. Muravyova E, Golovnin A, Gracheva E, Parshikov A, Belenkaya T, Pirrotta V, and Georgiev P. (2001) Loss of insulator activity by paired Su(Hw) chromatin insulators. Science 291: 495-498

62. Nègre N, Hennetin J, Sun LV, Lavrov S, Bellis M, White KP, and Cavalli G. Chromosomal distribution of PcG proteins during *Drosophila* development. PLoS Biol. 4: e170

63. Papoulas O, Beek SJ, Moseley SL, McCallum CM, Sarte M, Shearn A, and Tamkun JW. (1998) The *Drosophila* trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. Development 125: 3955-3966

64. Papp B and Muller J. (2006) Histone methylation and the maintenance of transcriptional ON and OFF states by TrxG and PcG proteins. Genes Dev. 20: 2041-2054

65. Petruk S, Sedkov Y, Riley KM, Hodgson J, Schweisguth F, Hirose S, Jaynes JB, Brock HW, and Mazo A. (2006) Transcription of bxd Noncoding RNAs promoted by Trithorax represses Ubx in cis by transcriptional interference. Cell 127: 1209-1221

66. Petruk S, Sedkov Y, Smith S, Tillib S, Kraevski V, Nakamura T, Canaani E, Croce CM, and Mazo A. (2001) Trithorax and dCBP acting in a complex to maintain expression of a homeotic gene. Science 294: 1331-1334

67. Pirrotta V (1997) PcG complexes and chromatin silencing. Curr. Opin. Genet. Dev 7: 249-258

68. Pirrotta V and Rastelli L. (1994) White gene expression, repressive chromatin domains and homeotic gene regulation in Drosophila. Bioessays 16(8): 549-556

69. Polo SE and Almouzni G. (2006) Chromatin assembly: a basic recipe with various flavours. Curr. Opin. Genet. Dev. 16: 104-111

70. Poux S, Horard B, Sigrist CJ, and Pirrotta V. (2002) The Drosophila Trithorax protein is a coactivator required to prevent re-establishment of Polycomb silencing. Development 129: 2483-2493.

71. Poux S, Kostic C, and Pirrotta V. (1996) Hunchback-independent silencing of late Ubx enhancers by a Polycomb Group Response Element. EMBO J. 2;15(17):4713-4722.

72. Rastelli L, Chan CS, and Pirrotta V. (1993) Related chromosome binding sites of zeste, suppressor of zeste and Polycomb group proteins in Drosophila and their dependence on Enhancer of zeste function. EMBO J. 12: 1513-1522

73. Sanchez T, Gou D, Kremmer E, and Sauer F. (2006) Noncoding RNAs of Trithorax Response Elements recruit Drosophila Ash1 to Ultrabithorax. Science 311: 1118-1123

74. Saurin AJ, Shao Z, Erdjument-Bromage H, Tempst P, and Kingston RE. (2001) A *Drosophila* Polycomb group complex includes Zeste and dTAFII proteins. Nature 412: 655-660

75. Schmit S and Paro R. (2006) RNA at the steering wheel. Genome Biol. 7: 218

76. Shao Z, Raible F, Mollaaghababa R, Guyon JR, Wu CT, Bender W, and Kingston RE. (1999) Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell 98: 37-46

77. Sharp EJ, Martin EC, and Adler PN. (1994) Directed overexpression of suppressor 2 of zeste and Posterior Sex Combs results in bristle abnormalities in *Drosophila melanogaster*. Dev. Biol. 161: 379-392

78. Sigrist CJ and Pirrotta V. (1997) Chromatin insulator elements block the silencing of a target gene by the *Drosophila* Polycomb response element (PRE) but allows *trans*
interactions between PREs on different chromosomes. Genetics 147: 209-221

79. Simon J (1995) Locking in stable states of gene expression: transcriptional control during *Drosophila* development. Curr. Opin. Cell. Biol 7: 376-385.

80. Smith ST, Petruk S, Sedkov Y, Cho E, Tillib S, Canaani E, and Mazo A. (2004) Modulation of heat shock gene expression by the TAC1 chromatin modifying complex. Nat. Cell. Biol. 6: 162-167

81. Schwartz YB and Pirrotta V. (2007) Polycomb silencing mechanisms and the management of genomic programs. Nature Rev Genet Jan;8(1): 9-22

82. Schwartz YB, Kahn TG, Nix DA, Li XY, Bourgon R, Biggin M, and Pirrotta V. (2006) Genome-wide analysis of Polycomb targets in *Drosophila melanogaster*. Nature Genet. 38: 700-705

83. Soto MC, Chou TB, and Bender W. (1995) Comparison of germline mosaics of genes in the Polycomb group of *Drosophila melanogaster*. Genetics 140: 231-243

84. Tillib S, Petruk S, Sedkov Y, Kuzin A, Fujioka M, Goto T, and Mazo, A. (1999) Trithorax-and Polycomb group response elements within an Ultrabithorax transcription maintenance unit consists of closely situated but separable sequences. Mol. Cell. Biol.19: 5189-5202

85. Tolhuis B, de Wit E, Muijrers I, Teunissen H, Talhout W, van Steensel B, and van Lohuizen M. (2006) Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in *Drosophila melanogaster*. Nature Genet. 38: 694-699

86. van Lohuizen M, Frasch M, Weintjens E, and Berns A. (1991) Sequence similarity between the mammalian *bmi-1* proto-oncogene and the Drosophila regulatory genes *Psc* and *Su(z)2*. Nature 353: 353-355

87. Wang H, Wang L, Erdjument-Bromage H, Vidal M, Tempst P, Jones RS, and Zhang Y. (2004) Role of histone H2A ubiquitination in Polycomb silencing. Nature 431: 873-878

88. Wu CT and Howe M. (1995) A genetic analysis of the Suppressor 2 of zeste complex of *Drosophila melanogaster*. Genetics 140: 139-181

89. Zink D and Paro R. (1995) Drosophila Polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. EMBO J. 14 (22): 5660-5671

CURRICULUM VITA

SUNG-YEON PARK

EDUCATION:

Ph.D. 2002-2008 Rutgers University Piscataway, NJ
Biochemistry (transferred from University of Geneva, Zoology, 2004)

M.S. 1997-1999 Kwangju Institute of Science and Technology, Korea
Department of Life Science

B.S. 1993-1997 Ewha womans' University, Korea
Department of Biological Science

SCIENTIFIC PUBLICATION:

Alexander Y. Konev, Martin Tribus, **Sung Yeon Park**, Valerie Podhraski, Chin Yan Lim, Alexander V. Emelyanov, Elena Vershilova, Vincenzo Pirrotta, James T. Kadonaga, Alexandra Lusser, and Dmitry V. Fyodorov **(2007)** CHD1 motor protein is required for deposition of histone variant H3.3 into chromatin in vivo. ***Science*** **317: 1087-1090**.