# HOMEOTIC GENE SILENCING IN *DROSOPHILA*

by

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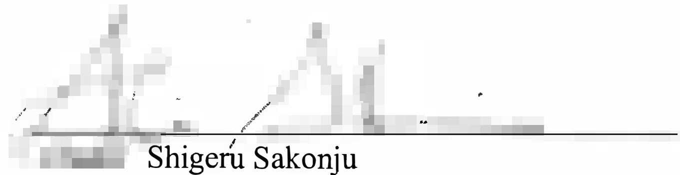
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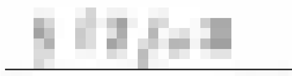
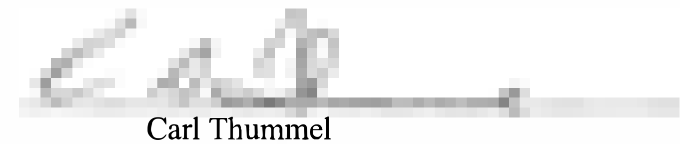
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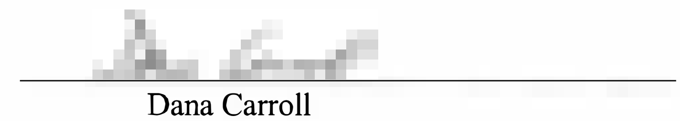
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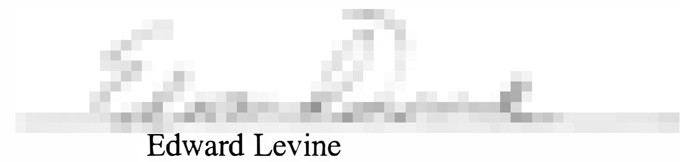
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## ABSTRACT

Homeotic genes specify segment identity in *Drosophila*. Their pattern of expression is set early in the embryo by the transient expression of the segmentation genes. Later in development, when the products of the segmentation genes are no longer present, the Polycomb Group of genes (PcG) silences the homeotic genes within the appropriate segments. In an effort to study how *PcG* genes mediate silencing of homeotic genes, I carried out a screen for genetic interactions between a mutation in the *Polycomb* locus ($Pc^3$) and a series of mutations in genes that affect heterochromatic Position effect variegation (PEV). The idea that PcG silencing shares molecular mechanisms with PEV has existed for a long time, but no one has reported a detailed study in the literature.

We observed that several PEV modifiers interacted strongly with $Pc^3$. One such gene is *Su(var)2-10*, also called *dPIAS*. We set out to characterize *Su(var)2-10* genetically, biochemically and *in vivo* in an effort to understand how it might control PcG silencing. The *Su(var)2-10* gene product, bears a high sequence identity to mammalian PIAS proteins, some of which have been reported to function as E3 enzymes in a protein modification pathway called SUMOylation. Thus we assessed whether SUMOylation contributes to PcG

silencing and to PEV, and whether the SU(VAR)2-10 protein is a SUMO E3 enzyme. In our effort to study interacting partners of SU(VAR)2-10, we identified the GAGA factor. We established that GAGA factor is a substrate for SUMOylation and that this modification has biological significance for GAGA function in PEV.

This dissertation is dedicated to the greatest teacher I have ever had, who always inspired me to work hard and to never ever give up- my grandmother Zlatka Petkova Dimova

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# CHAPTER 1

# INTRODUCTION

Homeotic genes specify segment identity in *Drosophila*. Their pattern of expression is set early in the embryo by the transient expression of the segmentation genes. Later in development, Polycomb Group of genes (PcG) silence the homeotic genes within the segments, as initially specified by the segmentation gene products. Although PcG genes were initially identified as repressors of the homeotic genes, they are implicated in a variety of developmental processes, such as cell proliferation (Martinez and Cavalli, 2006), stem cell identity and cancer (Jones and Baylin, 2007; Sparmann and van Lohuizen, 2006), genomic imprinting in plants and mammals (Delaval and Feil, 2004; Guitton and Berger, 2005), and X-inactivation (Heard, 2005; Yang and Kuroda, 2007). This extensive biological significance of PcG genes underlies the task of elucidating their mechanisms of action and determining how Polycomb proteins silence homeotic genes. These studies provide insight into how PcG-mediated processes are executed during development.

## *Drosophila* homeotic genes

Polycomb proteins were first discovered as repressors of homeotic genes that specify segmental determination in *Drosophila* (Garcia-Bellido, 1975; Lewis, 1978) (Figure 1.1). It has been shown that the homeotic proteins are continuously required in determined cells and in their descendants. Homeotic gene expression is regulated at two steps. During early embryogenesis, locally expressed proteins encoded by the segmentation genes repress homeotic gene transcription by binding to their *cis*-regulatory sequences (Bienz and Muller, 1995; Busturia and Bienz, 1993; Paro and Zink, 1993) and preventing, through short-range competition, the binding of activators, such as the pair-rule gene products (Bienz and Muller, 1995; Qian et al., 1993). The products of the segmentation genes decay by 5-7 h of development and afterwards the maintenance of homeotic gene expression requires proteins encoded by two groups of genes: the Polycomb group (PcG) and the trithorax group (trxG) of genes. The Polycomb group of proteins represses homeotic gene expression outside the initially specified domains, while the trithorax proteins were initially identified as suppressors of the Polycomb silencers (Kennison, 1995). Mutations in any of the PcG genes leads to multiple homeotic transformations resulting from derepression of the homeotic genes outside their specified segments (Simon et al., 1992). The PcG/trxG regulatory system is evolutionarily conserved from *Drosophila* to mammals and it has been reported to regulate many other

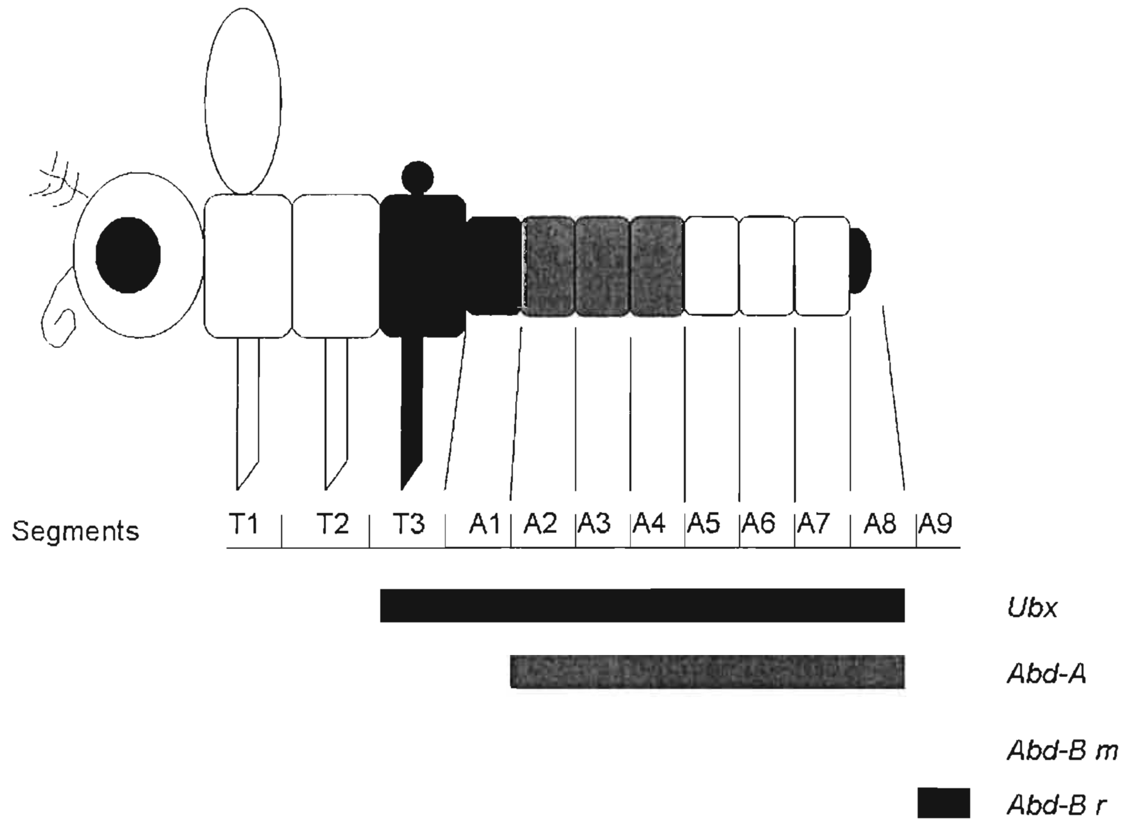


**Figure 1.1 Homeotic genes specify segment identity in *Drosophila*** Shown is the expression of some homeotic genes along the body axis in adult fly.

target genes, but its best-understood role is in the regulation of the homeotic genes. My research focuses on how the PcG silences homeotic genes.

## Silencing in euchromatin: Polycomb group-mediated silencing of homeotic genes

Thus far, 18 PcG genes have been identified and most of the proteins that they encode have been characterized (Ringrose and Paro, 2004). The PcG proteins do not share domains or structural motifs, but they form a family based on a common function. They associate biochemically in two distinct multimeric complexes ESC-E(Z) and PRC1 (Figure 1.2). The PRC1 core components are Polycomb (*Pc*), Polyhomeotic (*PH*) and Posterior sex combs (*PSC*). The ESC-E(Z) core components are Extra sex comb (*Esc*) and Enhancer of Zeste (*E(z)*). Not much is known about the activities of the PcG complexes, although chromatin-associated modification activities have been described for both. Several lines of evidence suggest that some ESC-E(Z) complexes have histone deacetylase activity and/or methyltransferase activity. RPD3 from ESC-E(Z) is a histone deacetylase (Tie et al., 2001) while E(Z) has been reported to methylate histone H3, mostly at K27 (Czermin et al., 2002; Muller et al., 2002). PRC1 has been shown to block chromatin remodeling by trxG proteins (Shao et al., 1999) and also to induce compaction of chromatin in defined nucleosomal arrays (Francis et al., 2004).

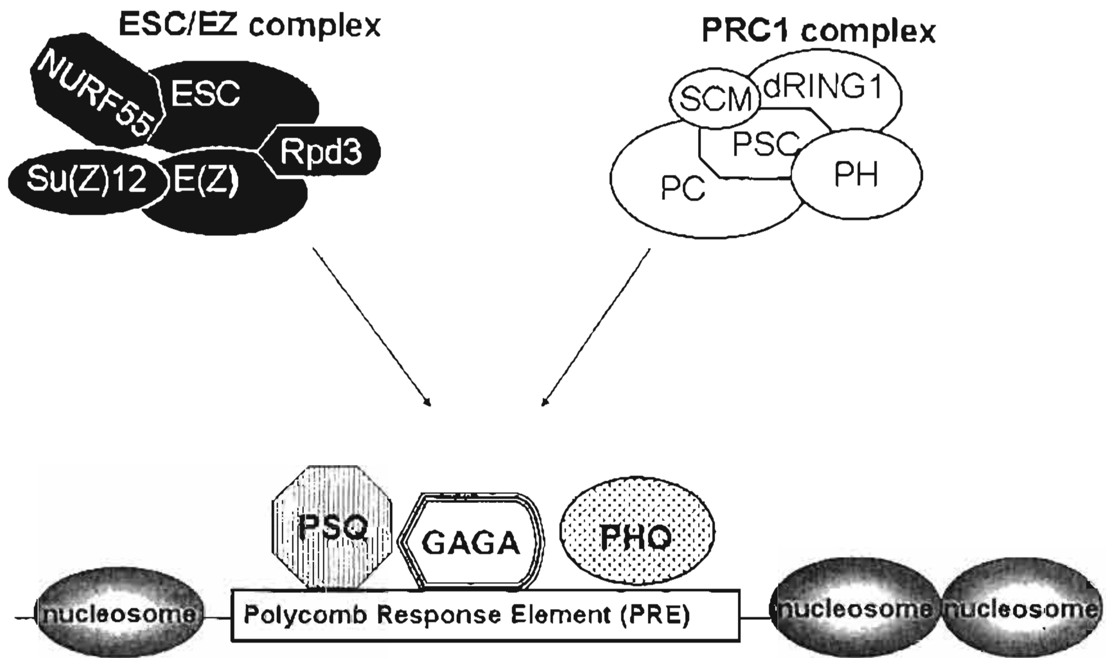


**Figure 1.2 Polycomb proteins associate in two complexes, PRC1 and ESC/E(Z).** Core components of PRC1 are Polycomb (PC), Polyhomeotic (PH) and Posterior sex combs (PSC). Core components of ESC/E(Z) are Extra sex comb (ESC) and enhancer of Zeste (E(Z). *cis*-regulatory DNA elements through which PcG proteins act are called Polycomb response elements (PRE). Polycomb proteins are recruited to PRE via DNA-binding proteins Pipsqueak (PSQ), GAGA factor and Pleiohomeotic (PHO).

ESC-E(Z) and PRC1 complexes are composed of different proteins at different targets and at different developmental time-points (Otte and Kwaks, 2003; Strutt and Paro, 1997). Another important notion is that PcG complex association with their target loci is dynamic throughout development (Ficz et al., 2005; Negre et al., 2006). There are two requirements for PcG proteins to silence a target. First, the target has to have a *cis*-regulatory DNA sequence known as a Polycomb Response Element (PRE). None of the PRC1 or ESZ-E(Z) proteins have sequence-specific DNA binding activity, but they are recruited to the PREs by DNA-binding proteins such as Zeste, GAGA factor and Pleiohomeotic (*PHO*) (Brown et al., 1998; Busturia et al., 2001; Horard et al., 2000; Mihaly et al., 1998). The second requirement is that the template has to be already transcriptionally silenced, as is the case for the homeotic genes that are initially repressed by the segmentation genes. In transgenes containing PREs, PcG silencing is established only in cells in which the enhancer has already been silenced before PcG function in the embryo (Poux et al., 1996; Poux et al., 2001).

There are a few possibly compatible models proposed as to how PcG proteins execute silencing: through chromatin packaging, inhibition of the transcription machinery and nuclear compartmentalization.

First, it has been suggested that PcG proteins form a chromatin structure that is topologically unfavorable for transcription. PcG complexes bound at PREs and at promoters along the chromosomes interact cooperatively with each other to form a series of loops throughout the repressed region (Barna et al., 2002; Pirrotta, 1995; Simon et al., 1995). These looped structures prevent interaction

between distantly located enhancers and promoters. More recently, it has been shown that a core PRC1 complex induces chromatin compaction by converting the "beads-on-a-string" organization of nucleosomes on DNA into a higher-order compacted structure (Francis et al., 2004).

The second mechanism suggests that PcG proteins do not create a chromatin environment inaccessible to the transcriptional machinery, but rather, they directly inhibit transcription. Crosslinking and immunoprecipitation studies have revealed that PcG proteins co-localize with the transcriptional machinery (Strutt et al., 1997). Moreover, artificially tethered PcG proteins repress transcription both in *Drosophila* embryos and in transiently introduced templates in tissue culture cells (Bunker and Kingston, 1994; Muller, 1995; Poux et al., 1996; Poux et al., 2001). It has been shown that for the *hsp26* gene, PcG proteins block transcription initiation by preventing the RNA polymerase from formin an initiation complex (Dellino et al., 2004).

The third mechanism of PcG-mediated silencing proposes that PcG proteins recruit their target genes to repressive subnuclear domains. In mammalian systems, genes are recruited to centromeric heterochromatin. In yeast, genes are recruited to telomeric heterochromatin (Francastel et al., 2001; Gasser et al., 1998). In human cell lines, the PcG proteins form unique discrete nuclear structures, named PcG bodies (Saurin et al., 1998). In *Drosophila*, the existence of PcG bodies is somewhat unclear. Some studies show that PC and PH localize into particular nuclear spots (Dietzel et al., 1999; Grimaud et al., 2006; Netter et al., 2001). Others argue against such discrete subnuclear

localization, although they do report some PSC-specific clusters which do not co-localize with other PcG proteins (Buchenau et al., 1998; Dietzel et al., 1999). In yeast, Andrulis et al. have shown that a transgene can be silenced after it is been anchored to the nuclear periphery (Andrulis et al., 1998). In conclusion, the three mechanisms mentioned above are not mutually exclusive, so it is possible that more than one mechanism is utilized at the same time.

### Silencing in heterochromatin: Position effect variegation

Position effect variegation (PEV) occurs when a chromosomal rearrangement translocates a euchromatic transcriptionally active gene close to heterochromatin. The relocated gene becomes transcriptionally silenced in some cells during development but not in others, thus giving a mosaic phenotype and hence the name PEV (Figure 1.3). The variegation phenomenon was first observed in *Drosophila* by Muller, and has since been seen in other animal species (Muller, 1930).

A classic example of the PEV phenomenon is the variegation silencing of the *white* gene (Figure 1.3). The *white* gene is located at the euchromatic tip of the X-chromosome (map position 1-1.5) and encodes a cell-autonomous protein required for making red pigment in the *Drosophila* eye. After a chromosome inversion ($w^{m4}$) the white gene is located approximately 25 kb distal to a broken piece of centromeric heterochromatin. This renders the *white* gene silenced in a subpopulation of cells, as seen by the lack of red pigmentation, but active in

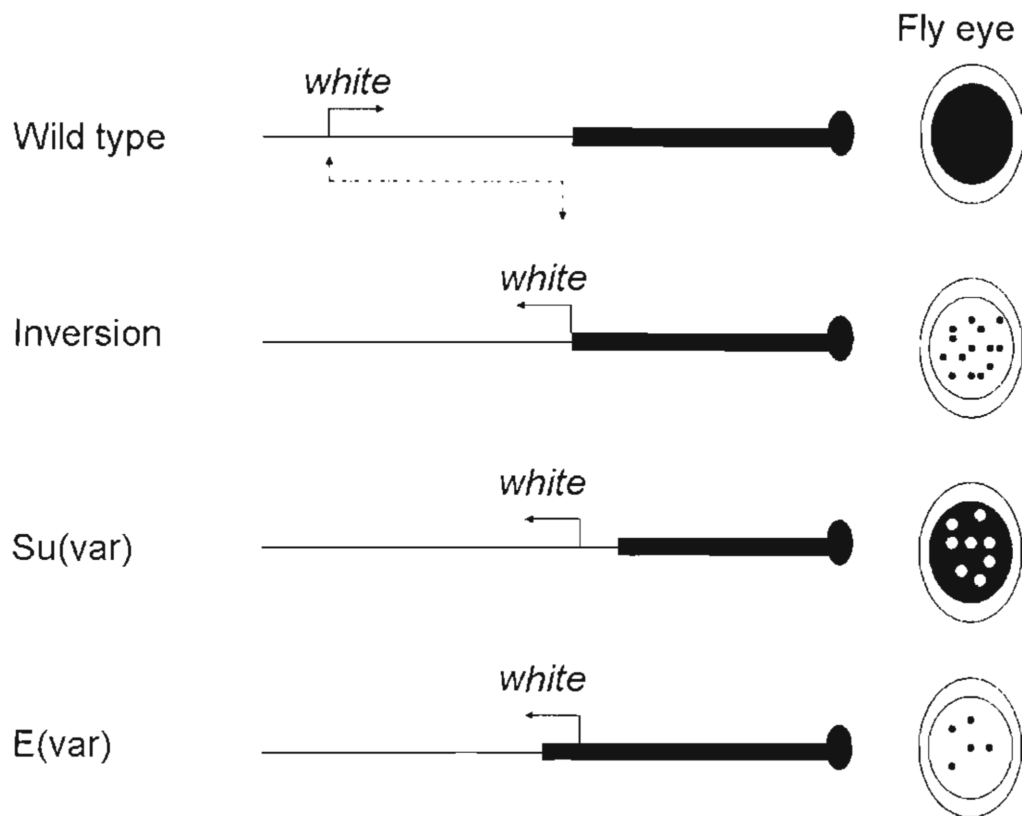


**Figure 1.3 Position effect variegation of the *white* gene.** In wild type, the *white* gene is located at the euchromatic tip of the X-chromosome, where it is actively transcribed, resulting in the red eye phenotype (shown in black). A chromosome inversion juxtaposes the white locus close to heterochromatin (thick black line), which renders it silenced in some cells but not in others, which gives a mosaic, "salt and pepper" eye phenotype. Mutations in genes that suppress PEV are called Suppressors of variegation (Su(var)) and mutations in genes that enhance PEV are called Enhancers of variegation (E(var)).

other cells giving a "salt and pepper" phenotype to the eye. There are mutations in gene that either enhance PEV, E(var)s, or suppress PEV, Su(var)s.

Among the models proposed to explain PEV are chromatin packaging and nuclear compartmentalization. Because chromosome segments containing the translocated gene change their banded morphology into more irregular or diffuse banding (Dimitri and Pisano, 1989), it has been suggested that the heterochromatin present at the translocation spreads over the translocated gene and renders it inactive. Locke et al. proposed that the transcriptional state of the gene depends on how far heterochromatin spreads along the chromosome, and that this is determined by the limiting amount of certain heterochromatin proteins (Locke et al., 1988). Supporting evidence comes from dosage effect studies in which when a PEV suppressor is present in one copy, it derepresses silencing, but when present in three copies, it enhances silencing (Wustmann et al., 1989) (Figure 1.3). Mutations in genes that affect PEV have turned out to be mutations in genes coding for chromatin-associated proteins or enzymes that modify chromatin.

Another line of evidence supporting the chromatin packaging model comes from studies reporting that PEV is sensitive to the amount of heterochromatin in the genome. For example, adding an extra Y-chromosome, which is mostly heterochromatic, leads to suppression of PEV. It has been suggested that this results from titrating out heterochromatic proteins (Dimitri and Pisano, 1989; Gowen and Gay, 1933; Zuckerkandl, 1974).

A second model for PEV silencing suggests that the variegating gene is

physically associated with a distinct silencing compartment of the nucleus that is unfavorable to transcription (Wakimoto and Hearn, 1990).

## Do euchromatic and heterochromatic silencing share a common molecular machinery?

The idea that PEV and PcG-mediated silencing share common molecular machinery has existed for a long time, but thus far no one has provided definitive evidence that this is the case (Grigliatti, 1991). Common to both PEV and PcG silencing is that both processes involve changes in chromatin structure, action at large distances, heritability of the silent state through many cell divisions and association with heterochromatin. Both PEV and PcG-mediated silencing involve changes in chromatin structure. So, if the PEV modifier genes encode nonhistone chromatin proteins or chromatin modifying factors, they might also influence the PcG silencing. In a PEV review (Grigliatti, 1991) was reported that Grigliatti's group examined the effect of six Su(var) and four E(var) mutations on the expression of several homeotic genes without naming the modifiers used and describing the phenotypes and interactions assayed. Grigliatti reports that one Su(var) mutation suppresses, and one E(var) mutation enhances the abdominal phenotype associated with the homeotic mutation Trithorax. The rest of the tested modifiers have no effect on the genes from the Antennapedia and Bithorax complexes. None of these studies have been published in detail since. Although this was not a comprehensive survey of the existing Su(var) mutations, Grigliatti concluded that some Su(var) loci might influence homeotic silencing but that

most are not involved in homeotic gene silencing. Later on, Sinclair et al. studied the effect of PcG genes on PEV and reported that Asx enhances PEV, E(Pc) suppresses PEV and nine other PcG genes had no effect on PEV (Sinclair et al., 1998a; Sinclair et al., 1998b). They concluded that there are fewer similarities between PcG proteins and PEV modifiers than initially thought.

However, there are more reasons to think that PEV and PcG silencing share similar molecular machinery. The PC protein shares a region of sequence similarity, the chromodomain, with SU(VAR)2-5 also known as heterochromatin protein 1 (HP1), a nonhistone chromosomal protein primarily associated with pericentric and telomeric heterochromatin (Paro and Hogness, 1991). It is via the chromodomain that PC and HP1 bind to methylated Lysine 9 on histone 3, a hallmark of inactive chromatin formation (Bannister et al., 2001; Lachner et al., 2001). Another domain, SET is shared by the PEV suppressor *Su(var)3-9*, the PcG gene *Enhancer of Zeste (E(z))* and the trx-G gene *trithorax* (Jones and Gelbart, 1993; Paro and Hogness, 1991; Tschiersch et al., 1994). The SET domain is evolutionarily conserved sequence motif present in chromosomal proteins with histone 3–specific methyl transferase activity implicated in heterochromatin establishment and maintenance.

Unpublished results from our lab and published results from others (Schwendemann and Lehmann, 2002) have shown that PSQ and the GAGA factor, two genes involved in homeotic gene silencing, bind to small (GA)n repeats within the PRE of the homeotic genes. They also bind to long (GA)n repeats within satellite DNAs, which make up most of the heterochromatin. Such

heterochromatic silencing is one of the hallmarks of PEV. Not only are both genes important for homeotic gene silencing but mutations in their loci enhance the PEV eye phenotype in $w^{m4h}$ flies.

The last line of evidence comes from an experiment in our lab demonstrating that PcG-mediated silencing is sensitive to the amount of heterochromatin in the genome. Shige Sakonju observed that flies carrying extra heterochromatin have an enhanced penetrance of $Pc^3$–induced homeotic transformations. As mentioned earlier, PEV is also sensitive to the amount of heterochromatin present in the genome.

Taken together, these data led us to speculate that PcG-mediated silencing of homeotic genes might share some aspect of silencing mechanisms with the heterochromatic PEV phenomenon.

## Thesis outline

My thesis research is focused on how Polycomb proteins implement silencing of homeotic genes in *Drosophila*. In Chapter 2, I describe the results of a screen designated to test for genetic interactions between a mutation in the eponymous *Pc* gene and a series of PEV modifiers. This screen revealed that most of the tested PEV modifiers enhanced $Pc^3$-induced homeotic transformations. One of the modifiers, *Su(var) 2-10,* enhanced the $Pc^3$ phenotype the most. In Chapter 3, I describe our genetic approach to asses whether *Su(var)2-10* behaves as a member of the PcG family. In Chapter 4, I describe experiments investigating the involvement of a protein modification pathway

called SUMOylation, in the PcG-mediated silencing of homeotic genes. In Chapter 5, I present data from our biochemical and *in vivo* approaches to functionally characterize the SU(VAR)2-10 protein. In Chapter 6, I describe SUMOylation of GAGA factor, a SU(VAR)2-10 interacting protein, and the biological significance of GAGA SUMOylation for its function in PEV.

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# CHAPTER 2

## SCREEN FOR GENETIC INTERACTION BETWEEN PEV MODIFIERS AND A *POLYCOMB* MUTATION

### Introduction

To test the hypothesis that PcG-mediated silencing shares molecular mechanisms with heterochromatic PEV silencing, we carried out a screen for genetic interactions between a series of modifiers of PEV and a $Pc^3$ mutation. If two genes are involved in the same biological pathway, then animals doubly heterozygous for mutations in both genes may display a stronger phenotype as compared to individually heterozygous animals. $Pc^3$ heterozygous mutants show homeotic phenotypes with low penetrance and expressivity. If PcG silencing works together with PEV, then flies doubly heterozygous for both $Pc^3$ and a certain PEV mutation will have enhanced penetrance and expressivity of homeotic transformations.

There are two types of second site mutations that modify PEV. Mutations in genes that suppress PEV and thus increase the red pigmented facets in the eye are called suppressors of variegation (Su(var)s). In contrast, E(var) mutations enhance the original mutation, which is seen as eyes with more white facets (Figure 1.3).

To date, there are up to 150 PEV modifiers identified. Most of them encode nonhistone chromatin proteins or chromatin modifying factors (Wallrath, 1998; Weiler and Wakimoto, 2002). Two of the best studied PEV modifiers are SU(VAR)3-9 and SU(VAR)2-5. SU(VAR)3-9 has a SET domain and methylates Lysine 9 on histone H3 (H3K9) (Rea et al., 2000; Schotta et al., 2002). This methylation mark is recognized by SU(VAR)2-5, also known as Heterochromatin protein 1 (HP1). HP1 is enriched in heterochromatin and telomeres, and it also localizes to about 200 sites within euchromatin. HP1 contains a conserved chromodomain through which it binds to the methylated H3K9 and consecutively recruits more heterochromatin proteins, which results in heterochromatin establishment (Ebert et al., 2006; Schotta et al., 2003). In general, SU(VAR) proteins create a repressive environment, such as at the centromeric heterochromatin, and E(VAR) proteins create active chromatin allowing gene expression.

## Materials and methods

### *Drosophila* stocks

All *Drosophila* stocks used in the genetic screen were obtained from the Bloomington stock center. The genotype of PEV modifiers used, molecular identity and references for them are listed in Table 2.1. *Su(var)2-10*$^{PEX2C}$ is a revertant allele kindly given to us by Gary Karpen (Hari et al., 2001). Flies were reared on a standard food medium at 25°C degrees under low crowded conditions. The genotypes of the flies that were used for the lacZ assay, are: [T8

MCP/PRE-lacZ ]/[T8 MCP/PRE-lacZ ], [T3 MCP/PRE-lacZ ]/[T3 MCP/PRE-lacZ ], and *Psq*$^{D91}$[T8 MCP/PRE-lacZ ]/*CyOGFP* . T3 is inserted in the second chromosome and T8 is on the third chromosome. All stocks were made in the lab by previous members.

Crosses

Female or male flies heterozygous for a PEV modifier were crossed to either male or female flies heterozygous for the *Pc*$^{3}$ mutation. As there were no significant differences observed, the data shown in this dissertation are derived from crosses between mothers that are mutant for a PEV modifier and fathers that are mutant for the *Pc* gene.

LacZ reporter assay and histochemical staining
forβ-galactosidase

PBX-MCP-Ubx promoter-*lacZ* reporter construct has been described previously (Busturia et al., 1997) (Figure 2.1). This reporter is built upon the *ry+ Ubxpp-lacZ* transformation vector described by Müller and Bienz (Muller and Bienz, 1991). It carries 3.1 kb of the *Ubx* promoter sequence upstream of the start site and it includes the *Ubx* transcribed sequence to the first seven amino acids of the ORF, where it is fused to the *lacZ* sequence. The PBX element is the 5.2 kb PBXO1 fragment, also described previously. Female third instar larvae were collected and divided in GFP and nonGFP batches and wing imaginal and haltere discs dissected. Staining for lacZ expression using the X-gal assay was

**Table 2.1 PEV modifiers used in the genetic screen**

| Suppressor of variegation | Molecular identity | References |
|---|---|---|
| $In(1)w^{m4h};Su(var)2\text{-}1^{1}/In(2L)t,In(2R)$ $Cy,Cy^{1}Roi^{1}pr^{1}cn$ | HDAC | Reuter et al., 1983<br>Sinclair et al., 1992 |
| $In(1)w^{m4h};Su(var)2\text{-}1^{1\text{-}13}$ *noc Sco/SM1* | HDAC | Wustmann et al., 1989 |
| $In(1)w^{m4h};Su(var)205^{5}/In(2L)Cy,In(2R)$ $Cy,Cy^{1}$ | HP1 | Grigliatti et al.,1991<br>Kellum, R., Alberts, B.M.1995<br>Eissenberg et al.,1992 |
| $cn^{1}\ P\{ry^{+t7.2}=PZ\}Su(var)2\text{-}10^{03697}/CyO;ry^{506}$ | dPIAS | Hari et al, 2001 |
| $In(1)w^{m4h};Su(var)2\text{-}10^{1}/In(2L)t,In(2R)$ $Cy,Cy^{1}Roi^{1}pr^{1}$ | dPIAS | Hari et al, 2001<br>Westphal and Reuter 2002 |
| $Su(var)2\text{-}10^{2}/CyO$ | dPIAS | Westphal and Reuter 2002 |
| $In(1)w^{m4h}$*,y, Df(2L) TE29Aa-11/CyO* | NA | Wustmann et al., 1989 |
| $Su(var)3\text{-}1^{3}/TM3\ Sb^{1}\ Ser^{1}$ | H3S10 kinase | Reuter et al., 1986 |
| $Su(var)3\text{-}4^{1}/TM3\ Sb^{1}\ Ser^{1}$ | NA | Reuter et al., 1986 |
| $Su(var)3\text{-}4^{2}/TM3\ Sb^{1}\ Ser^{1}$ | NA | Reuter et al., 1986 |
| $Su(var)3\text{-}9^{1}/TM3\ Sb^{1}\ Ser^{1}$ | H3K9 methylase | Schotta et al., 2002<br>Eber et al.,2004<br>Reuter et al., 1986 |
| $Su(var)3\text{-}9^{2}/TM3\ Sb^{1}\ Ser^{1}$ | H3K9 methylase | Schotta et al., 2002<br>Eber et al.,2004<br>Reuter et al., 1986 |
| $In(1)w^{m4h};Su(var)3\text{-}10^{2}/TM3\ Sb^{1}\ Ser^{1}$ | NA | Reuter et al., 1986 |
| $ry^{506}P\{ry^{+t7.2}=PZ\}mod^{07570}/TM3\ ry^{RK}Sb^{1}Ser^{1}$ | NA | Castrillon et al.,1993 |

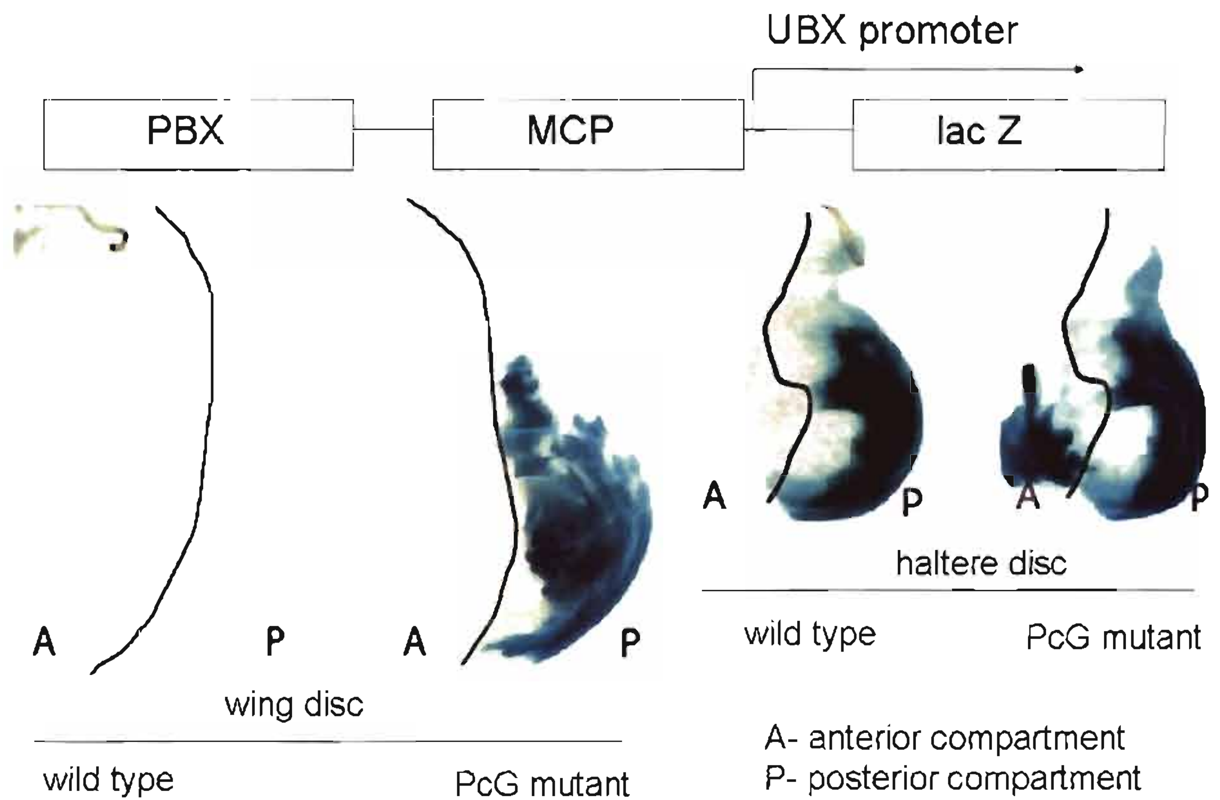


**Figure 2.1 Schematic of a LacZ reporter gene and derepression assay**. The reporter is a lacZ transgene driven from the Ubx promoter by the PBX enhancer and silenced by a PRE, called MCP (Busturia et al., 1997). The reporter is silenced in wing imaginal discs and in the anterior part of haltere discs in wild type larvae, but becomes derepressed in PcG mutant animals.

done as described by Busturia et al (1997). The discs were photographed using a Zeiss Axiophot microscope.

## Results

### Preliminary genetic screen

To test the hypothesis that PcG-mediated silencing and PEV share a common molecular machinery, we carried out a screen for genetic interactions between a PcG mutation called *Pc*$^3$ and a set of PEV suppressors that map on the second and third chromosomes, available from the Bloomington stock center. Female or male flies heterozygous for a PEV modifier were crossed to either male or female flies heterozygous for the *Pc*$^3$ mutation (Figure 2.2). Normally, *Pc*$^3$ heterozygous animals show homeotic transformations with low penetrance and expressivity. Thus, in the F1 progeny, we assayed for enhancement of *Pc*$^3$ phenotypes by PEV suppressors. The four examined homeotic transformations are depicted in Figure 2.3. These are: antenna-to-leg transformation, which results from derepression of the *Antp* gene in the antenna; wing-to-haltere transformation resulting from derepression of *Ubx* in the wing; T2-to-T1 leg transformation resulting from derepression of *esc* in the T2 legs, and abdominal segment A4-to-abdominal segment A5 transformation resulting from derepression of *Abd-B* in A4. The genetic interactions between each *Su(var)* and *Pc*$^3$ were assessed by measuring the penetrance and expressivity of the four

\

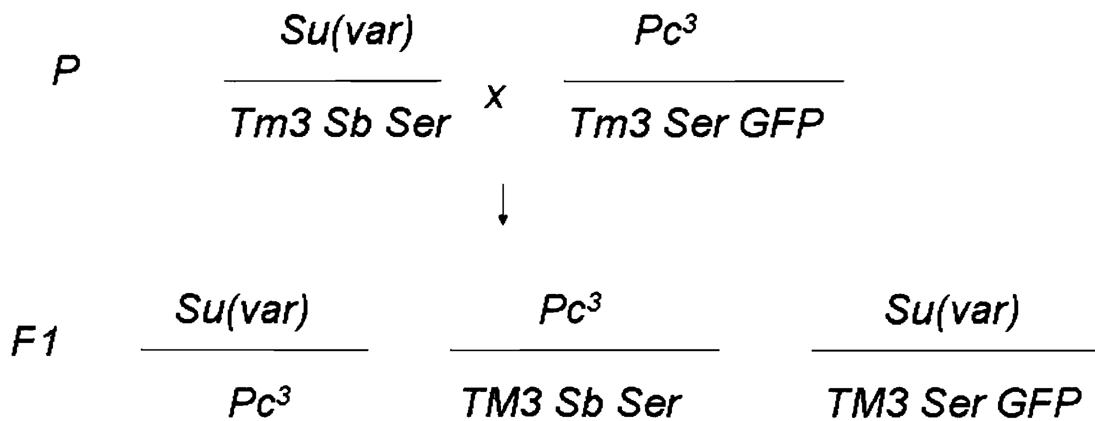


**Figure 2.2 Cross for the genetic interaction screen between *Pc*$^3$ and suppressors of PEV.** Shown is an example for a *Su(var)* from the third chromosome. Flies heterozygous for the *Pc*$^3$ mutation were crossed to flies heterozygous for a certain *Su(var)* mutation. Each mutation is balanced over a wild type chromosome carrying phenotypic markers which allow telling the different genotypes apart. In the F1 progeny, I assayed for enhancement of the *Pc*$^3$-induced homeotic transformations in the *Su(var)/ Pc*$^3$ double heterozygous mutant flies as compared to *Pc*$^3$/+ heterozygous flies. None of the *Su(var)/+* heterozygous flies showed homeotic transformations.

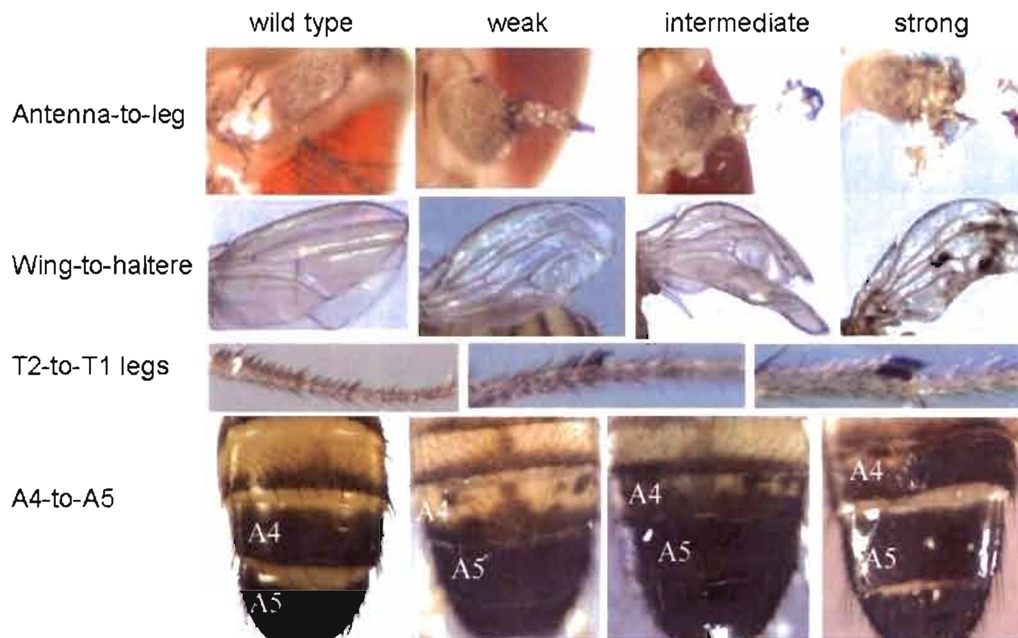


**Figure 2.3 Homeotic transformations assayed in the genetic screen and their expressivity classes.** The three expressivity classes are designated as weak, intermediate and strong. We used only two expressivity classes to asses the T2-to-T1 legs transformation, weak and strong. Each sex comb has ten teeth, but in the preliminary screen, instead of counting the number of teeth, we classified the T2-to-T1 transformations simply as weak (one to three extra teeth) or strong (about ten teeth).

homeotic transformations. Penetrance was scored as the number of animals of a given genotype showing each homeotic phenotype. Expressivity was classified as weak, intermediate or strong (Figure 2.3). The penetrance and expressivity for *Su(var)/* $Pc^3$ double heterozygotes were compared with $Pc^3$ heterozygotes. None of the *Su(var)/+* heterozygous animals showed homeotic transformations. The preliminary genetic screen revealed that the different *Su(var)s* enhanced both penetrance and expressivity of $Pc^3$-induced homeotic transformations to different extents (Tables 2.2, 2.3 and 2.4 ). Because, for a reason unknown to us, females and males show different extents of antenna-to-leg and wing-to-haltere transformations, the data for these tissues are presented separately. Three PEV suppressors from the third chromosome, *Su(var)3-4, Su(var)3-9* and *Su(var)3-10,* enhanced the $Pc^3$ phenotype with great penetrance and high expressivity. The second chromosome suppressors that showed the greatest interaction with $Pc^3$ were *Su(var)2-5* and *Su(var)2-10.* Some suppressors enhanced all transformations, while others enhanced only one or a few transformations.

Confirmation of the genetic screen data

Genetic background is very important for genetic interaction studies that involve chromatin proteins encoding genes, such as PEV modifiers. The presence of second site mutation in the genome could enhance the effect of a PEV modifier.

To confirm the results of this screen, I isogenised the genetic background of the most efficient modifiers that enhanced the $Pc^3$ phenotype. These were

**Table 2.2 Penetrance in percent (%) of antenna-to-leg transformation in *Pc³* heterozygous animals and *Pc³/Su(var)* heterozygous animals from the preliminary genetic screen.** For each genotype, n>100

| Su(var) used | females | | males | |
|---|---|---|---|---|
| | *Pc³/+* | *Pc³/Su(var)* | *Pc³/+* | *Pc³/Su(var)* |
| *DfTE29Aa-11* | 0 | 6 | 0 | 0 |
| *Su(var)2-1¹* | 4 | 28 | 0 | 0 |
| *Su(var)2-1¹⁻¹³* | 20 | 67 | 0 | 6 |
| *Su(var)2-5⁵* | 9 | 67 | 0 | 9 |
| modulo | 3 | 3 | 0 | 0 |
| *Su(var)2-10⁰³⁶⁹⁷* | 2 | 63 | 0 | 2 |
| *Su(var)2-10¹* | 0 | 12 | 0 | 0 |
| *Su(var)2-10²* | 26 | 28 | 2 | 0 |
| *Su(var)3-1³* | 6 | 7 | 1 | 0 |
| *Su(var)3-4¹* | 0 | 6 | 0 | 1 |
| *Su(var)3-4²* | 43 | 0 | 0 | 0 |
| *Su(var)3-9¹* | 32 | 0 | 0 | 0 |
| *Su(var)3-9²* | 47 | 5 | 0 | 0 |
| *Su(var)3-10²* | 63 | 11 | 2 | 4 |

**Table 2.3 Penetrance in percent (%) of wing-to-haltere transformation in *Pc³* heterozygous animals and *Pc³/Su(var)* heterozygous animals from the preliminary genetic screen.** For each genotype, n>100

| Su(var) used | females | | males | |
|---|---|---|---|---|
| | *Pc³/+* | *Pc³/Su(var)* | *Pc³/+* | *Pc³/Su(var)* |
| *DfTE29Aa-11* | 3 | 23 | 0 | 0 |
| *Su(var)2-1¹* | 25 | 100 | 17 | 84 |
| *Su(var)2-1¹⁻¹³* | 83 | 89 | 58 | 70 |
| *Su(var)2-5⁵* | 14 | 65 | 0 | 19 |
| modulo | 0 | 12 | 0 | 0 |
| *Su(var)2-10⁰³⁶⁹⁷* | 30 | 91 | 23 | 74 |
| *Su(var)2-10¹* | 36 | 64 | 14 | 18 |
| *Su(var)2-10²* | 44 | 68 | 27 | 38 |
| *Su(var)3-1³* | 4 | 30 | 0 | 9 |
| *Su(var)3-4¹* | 52 | 92 | 10 | 100 |
| *Su(var)3-4²* | 41 | 98 | 22 | 78 |
| *Su(var)3-9¹* | 0 | 26 | 0 | 27 |
| *Su(var)3-9²* | 0 | 84 | 0 | 92 |
| *Su(var)3-10²* | 73 | 100 | 49 | 100 |

**Table 2.4 Penetrance in percent (%) of T2-to-T1 transformation and A4-to-A5 in *Pc³* heterozygous animals and *Pc³/Su(var)* heterozygous animals from the preliminary genetic screen**. For each genotype, n>100

| Su(var) used | T2-to-T1 | | A4-to-A5 | |
|---|---|---|---|---|
| | *Pc³/+* | *Pc³/Su(var)* | *Pc³/+* | *Pc³/Su(var)* |
| *DfTE29Aa-11* | 0 | 5 | 0 | 0 |
| *Su(var)2-1¹* | 0 | 11 | 0 | 14 |
| *Su(var)2-1¹⁻¹³* | 5 | 13 | 6 | 16 |
| *Su(var)2-5⁵* | 6 | 70 | 0 | 18 |
| modulo | 0 | 44 | 0 | 36 |
| *Su(var)2-10⁰³⁶⁹⁷* | 15 | 38 | 12 | 88 |
| *Su(var)2-10¹* | 11 | 48 | 11 | 79 |
| *Su(var)2-10²* | 24 | 33 | 2 | 33 |
| *Su(var)3-1³* | 12 | 22 | 30 | 57 |
| *Su(var)3-4¹* | 0 | 45 | 0 | 75 |
| *Su(var)3-4²* | 0 | 4 | 12 | 6 |
| *Su(var)3-9¹* | 34 | 52 | 39 | 36 |
| *Su(var)3-9²* | 0 | 4 | 40 | 9 |
| *Su(var)3-10²* | 96 | 94 | 79 | 86 |

*Su(var)2-5, Su(var)3-4, Su(var)3-9, Su(var)3-10* and *Su(var)2-10* . All available alleles of these mutations, as well as *Pc³*, were crossed three times to a balancer stock that was wild type for the mutation of interest in order to eliminate any secondary modifiers and then the PEV modifiers were tested again for interaction with *Pc³*. The *w;Sco/CyO* balancer stock was used for modifiers on the second chromosome and *w;Sb/TM3Ser* for modifiers on the third chromosome. The results from this second genetic interaction study are shown in Table 2.5. We observed that the PEV modifiers, even after being isogenised, still enhanced the *Pc³* –induced homeotic transformations, as initially seen in the preliminary genetic screen. For unknown reasons, in some cases, the *Pc³* heterozygote flies had a higher penetrance of antenna-to-leg transformations as compared to the double heterozygotes. Such data were seen with *Su(var)3-4², Su(var)3-9¹* and *Su(var)3-10²* (Table 2.5). This discrepancy does not seem to be associated with the Sb- carrying balancer chromosome because *Su(var)3-4¹,* which is balanced over Sb did not show increased transformation . It should be mentioned that these results were seen many times in crosses for these particular modifiers. It is possible that the genetic interaction between *Pc³* and the aforementioned Su(var) mutations is such that instead of enhancement they cause repression of the *Pc³* phenotype.

<u>*Su(var)3-10* characterization</u>

For further studies, we chose initially to work with *Su(var)3-10,* as it was one of the PEV suppressors that showed the highest penetrance and strongest

**Table 2.5 Genetic interactions between isogenised PEV modifiers and *Pc*$^{3}$.**

| Genotype | Penetrance of homeotic transformations | | | | | |
|---|---|---|---|---|---|---|
| | females | | males | | | |
| | A-to-L | W-to-H | A-to-L | W-to-H | T2-to-T1 | A4-to-A5 |
| *Su(var)2-10*$^{03697}$/+; *Pc*$^{3}$/+ | 57%<br>+++<br>p=0.001 | 63%<br>+++<br>p=0.001 | 9%<br>+<br>p=0.001 | 3%<br>+<br>p=0.1 | 37%<br>+++<br>p=0.001 | 90%<br>+++<br>p=0.001 |
| *Pc*$^{3}$/+ | 17%<br>+ | 30%<br>+ | 1%<br>+ | 1%<br>+ | 0% | 17%<br>+ |
| *Su(var)2-10*$^{PEX2C}$/+; *Pc*$^{3}$/+ | 12%<br>+<br>p=0.6 | 5%<br>+ | 2%<br>+<br>p=1 | 1%<br>+ | 2%<br>+ | 12%<br>+<br>p=0.6 |
| *Pc*$^{3}$/+ | 14%<br>+ | 5%<br>+ | 1%<br>+ | 1%<br>+ | 2%<br>+ | 15%<br>+ |
| *Su(var)2-5*$^{5}$/+; *Pc*$^{3}$/+ | 36%<br>++<br>p= 0.8 | 74%<br>+++<br>p=0.01 | 2%<br>+<br>p=0.5 | 12%<br>+<br>p=0.1 | 7%<br>++<br>p=0.5 | 54%<br>++<br>p=0.02 |
| *Pc*$^{3}$/+ | 42%<br>+ | 21%<br>+ | 0% | 0% | 0% | 12%<br>+ |
| *Su(var)3-4*$^{1}$/ *Pc*$^{3}$ | 42%<br>++<br>p=0.1 | 55%<br>+++<br>p≤0.0001 | 4%<br>+<br>p=0.5 | 6%<br>+<br>p=0.1 | 39%<br>+<br>p≤0.0001 | 31%<br>++<br>p=0.04 |
| *Pc*$^{3}$/+ | 9%<br>+ | 4%<br>+ | 0% | 0% | 0% | 10%<br>+ |
| *Su(var)3-4*$^{2}$/*Pc*$^{3}$ | 3%<br>+<br>p≤0.1 | 46%<br>+++<br>p≤0.005 | 0% | 0% | 0% | 29%<br>+++<br>p=0.0001 |
| *Pc*$^{3}$/+ | 24%<br>+++ | 6%<br>+ | 0% | 0% | 0% | 3%<br>+ |
| *Su(var)3-9*$^{1}$/*Pc*$^{3}$ | 0.5%<br>++ | 79%<br>+++<br>p=0.0001 | 0% | 35%<br>+<br>p=0.0003 | 20%<br>+<br>p=0.01 | 25%<br>+<br>p=0.003 |
| *Pc*$^{3}$/+ | 31%<br>++ | 3%<br>+ | 0% | 4%<br>+ | 2%<br>+ | 2%<br>+ |

Table 2.5 continued

| Genotype | Penetrance of homeotic transformations | | | | | |
|---|---|---|---|---|---|---|
| | females | | males | | | |
| | A-to-L | W-to-H | A-to-L | W-to-H | T2-to-T1 | A4-toA5 |
| *Su(var)3-10*$^2$/*Pc*$^3$ | 14%<br>++<br>p=0.0001 | 98.5%<br>+++<br>p=0.0001 | 0% | 0% | 36.5%<br>+<br>p=0.008 | 39%<br>++<br>p=0.0016 |
| *Pc*$^3$/+ | 46%<br>+++ | 19%<br>+ | 0% | 0% | 4.5%<br>+ | 7%<br>+ |

1) A-to-L, antenna-to-leg transformation; W-to-H, wing-to-haltere transformation; T2-to-T1, transformation of second pair of legs into first; A4-to-A5, transformation of fifth abdominal segment, A5 into fourth abdominal segment
2) *Pc*$^3$ heterozygote control for each cross is given in a row below the data for the double heterozygotes.
3) PEV modifiers have been outcrossed three times to eliminate secondary modifiers from their genetic background
4) For each genotype more than 100 animals were screened
5) Expressivity classes are shown with plus, where + is a weak transformation, ++ moderate, +++strong
6) p-value is calculated between double heterozygotes and single *Pc*$^3$ heterozygotes, using two-tailed Fisher's exact test.

expressivity of homeotic transformations with *Pc*$^3$. The molecular identity of *Su(var)3-10* is not known as it has not been cloned, although Reuter's group has mapped it to position 89B9-13 on the right arm of chromosome 3 (Reuter, 1986) (Figure 2.4.). Another gene, dSAP18 has been mapped to the same region by Hanes' group (Zhu et al., 2001). They sequenced *Su(var)3-10* alleles but did not find any mutations in dSAP18, a result which does not rule out *Su(var)3-10.* To confirm where *Su(var)3-10* maps, I did a complementation test between *Su(var)3-10*$^2$ and three deficiencies from the same region: *Df(3R)sbd45*, *Df(3R)sbd104* and *Df(3R)sbd26* (Figure 2.4). The complementation test revealed that *Su(var)3-10*$^2$ is sub-lethal over the tested deficiencies (Table 2.6).

To further confirm that *Su(var)3-10* maps in the 89B9-13 region, I examined suppression of PEV. All of the deficiencies enhanced the *Pc*$^3$-induced homeotic transformations, which suggests that the homeotic gene silencing activity maps to the 89B9-13 region (Table 2.7). Also, all three deficiencies suppressed the PEV of the *w*$^{m4h}$ marker gene (Figure 2.5), which suggests that PEV modifying activity also maps to the same region. These experiments also demonstrated that *Su(var)3-10*$^2$ is a loss-of-function mutation. To further characterize *Su(var)3-10,* I tested for genetic interactions with another PcG gene besides *Pc*$^3$, namely *psq*$^{D91}$. To detect such an interaction with *psq*, I used a lacZ reporter assay instead of assaying adult homeotic phenotypes, for reasons explained below. I assayed the penetrance of lacZ reporter derepression in *Su(var)3-10*$^2$*/+; psq*$^{D91}$*/+* heterozygous larvae and compared it with that of *psq*$^{D91}$*/+* heterozygous larvae. The reporter is a lacZ transgene driven from the Ubx promoter by the PBX

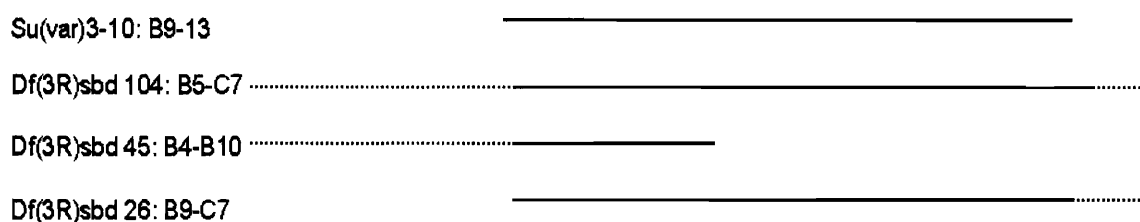


**Figure 2.4 Molecular map of the 89B9-13 region** The dark lines show the known breakpoints of the three deficiencies. The dashed lines represent the direction of the chromosomal deficiencies. The centromere-proximal region is to the left.

**Table 2.6 *Su(var)3-10*$^{2}$ is sublethal over the tested deficiencies**

| *Genotype* | Number of viable animals | | |
|---|---|---|---|
| | Df(3R)sbd104 | Df(3R)sbd45 | Df(3R)sbd26 |
| *Su(var)3-10*$^{2}$/Df* | 60 | 33 | 93 |
| *Df*/TM3 UBX* | 392 | 345 | 226 |

Shown here are the numbers of viable animals, doubly heterozygous either for *Su(var)3-10*$^{2}$ and a certain deficiency (Df*) or for the deficiency over a balancer chromosome.

**Table 2.7 Deficiencies for *Su(var)3-10*$^2$ enhance the *Pc*$^3$-induced homeotic transformations.**

| Genotype | Females | | Males | | | |
|---|---|---|---|---|---|---|
| | Antenna-to-leg | Wing-to-haltere | Antenna-to-leg | Wing-to-haltere | T2-to-T1 legs | A4-to-A5 |
| *Df(3R)Sbd104/Pc*$^3$ | 54% ++++ $p \leq 0.001$ | 60% +++ $p \leq 0.05$ | 11% ++ $p \leq 0.025$ | 15% +++ $p \leq 1$ | 6% ++ $p \leq 1$ | 15% ++ $p \leq 0.05$ |
| *TM2 ry UBX/Pc*$^3$ | 10% + | 30% + | 0% | 10% + | 2% + | 0% |
| *Df(3R)Sbd45/Pc*$^3$ | 17% +++ $p \leq 1$ | 62% +++ $p \leq 0.025$ | 4% ++ $p \leq 0.01$ | 27% +++ $p \leq 1$ | 0% | 7% + |
| *TM6 UBX/Pc*$^3$ | 9% + | 27% + | 0% | 19% + | 0% | 7% |
| *Df(3R)Sbd26/Pc*$^3$ | 6% + $p \leq 1$ | 94% ++++ $p \leq 0.005$ | 0% | 68% +++ $p \leq 0.01$ | 4% + $p \leq 1$ | NA |
| *TM6 UBX/Pc*$^3$ | 2% + | 42% + | 0% | 11% + | 0% | NA |

Compared is the penetrance of homeotic transformations in double heterozygous *Pc*$^3$/*Df(3R)* animals (upper row) versus percentage of homeotic transformations in *Pc*$^3$/ + heterozygotes (lower row).

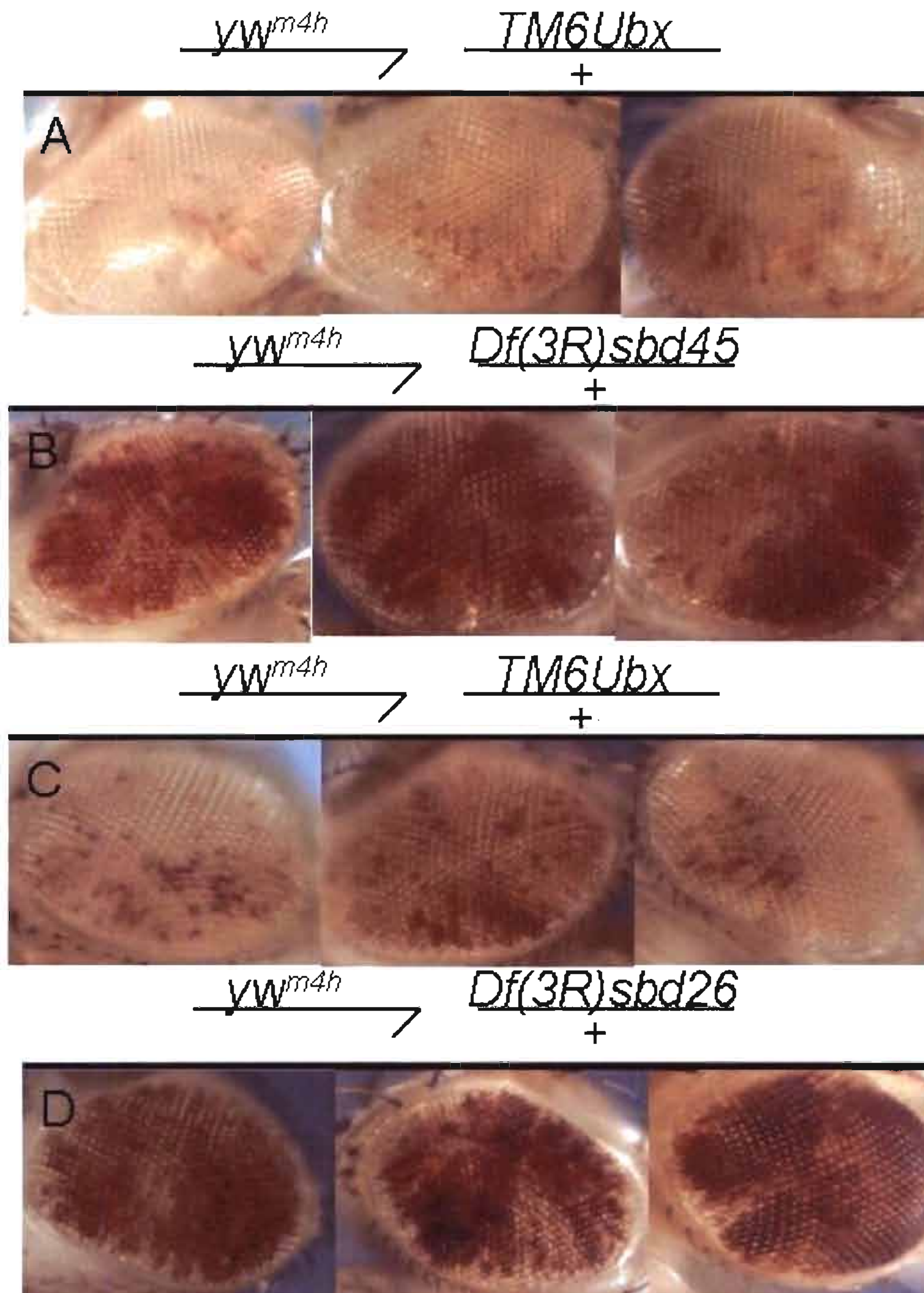


**Figure 2.5 The PEV suppression activity of Su(var)3-10 maps to 89B9-13 region.** Panels A and C show the PEV eye phenotype of the control animals. Panels B and D, show animals with suppressed PEV in animals heterozygous for Df(3R)sbd45 and Df(3R)sbd26.

enhancer and silenced by a single PRE, called MCP (Busturia et al., 1997) (Fig.2.1). The reporter is silenced in wing imaginal discs and in the anterior part of haltere discs in wild type larvae, but becomes derepressed in PcG mutant animals. Because the transgene silencing depends on a single PRE, this reporter assay is a very sensitive indication of a compromised PcG silencing as opposed to scoring of adult homeotic phenotypes, where silencing depends on multiple PREs. First, flies heterozygous for *Su(var)3-10*$^{2}$ were crossed to flies heterozygous for the *psq*$^{D91}$*[T8]* transgene. The LacZ expression assay revealed that the wing and haltere discs from double heterozygous *Su(var)3-10*$^{2}$*/+; psq*$^{D91}$*[T8]/+* flies had strong X-gal staining as compared to *psq*$^{D91}$*[T8]* heterozygotes (Table 2.8.). Thus, *Su(var)3-10*$^{2}$ enhances *psq*$^{D91}$– induced derepression of a PRE-dependent reporter gene. Second, I asked whether *Su(var)3-10*$^{2}$ on its own would be able to derepress two reporter genes inserted in different chromosome locations. Again, *Su(var)3-10*$^{2}$ heterozygous flies were crossed to flies homozygous for the T3 or T8 lacZ reporters and penetrance of lacZ derepression was scored. *Su(var)3-10*$^{2}$ was able to derepress both reporters both in wing and haltere discs. This result further confirmed that *Su(var)3-10* behaves as a PcG member.

## Discussion

The results described in this chapter present genetic evidence that the euchromatic PcG- mediated silencing shares molecular machinery with the heterochromatic PEV.

**Table 2.8 *Su(var)3-10$^2$* derepresses PRE- dependent reporters**

| Genotype | wing discs | haltere discs |
|---|---|---|
| *Su(var)3-10$^2$/+;Psq$^{D91}$/+* | 86% | 42% |
| | p=0.025 | p=0.001 |
| *Psq$^{D91}$/+* | 54% | 15% |
| *Su(var)3-10$^2$/+;T3 /+* | 64% | 20% |
| | p=0.01 | p=0.001 |
| *T3 /+* | 38% | 0% |
| *Su(var)3-10$^2$/ T8* | 25% | 8% |
| | p=0.01 | p=0.025 |
| *T8/+* | 12% | 4% |

1) for each genotype more than 200 wing discs were counted
2) p-value is calculated using two-tailed Fisher's exact test

I have shown that a series of PEV modifiers enhance *Pc*$^{3}$-induced homeotic transformations. Some *Su(var)s* enhanced the *Pc*$^{3}$ phenotype more than others. Different modifiers enhanced different homeotic transformations to different extents. For example, *Su(var)2-5*$^{5}$ induced strong wing-to-haltere and T2-to-T1 transformations, while *Su(var)3-9*$^{1,2}$ induced strong wing-to-haltere, T2-to-T1, and A4-to-A5 transformations. These results are not surprising as it had been shown that the composition of PcG complexes and the silencing mechanisms utilized by them vary in different tissues (Ficz et al., 2005). Thus, silencing of the *Ubx* gene in wings might require interaction of *Pc* with both *Su(var)2-5* and *Su(var)3-9,* while silencing of Abd-B in the A4 segment requires only *Pc* and *Su(var)3-9.* Also, when assayed for interaction with *Ph*$^{d}$ and *Scm*$^{D1}$, *Su(var)2-10*$^{03697}$ enhanced only the T3-to-T1 leg transformation.

The preliminary genetic screen was carried out with stocks that had not been outcrossed and thus might have accumulated secondary modifiers affecting the genetic interactions (Lloyd et al., 2003). This is seen by the high numbers of homeotic transformations in *Pc*$^{3}$ heterozygous animals. When all stocks were outcrossed three times to a balancer stock, and the genetic crosses repeated, the penetrance of transformations usually decreased. The tendency of the modifiers to enhance PcG-induced transformations, however, remained the same. This confirmed that the genetic interactions observed in the preliminary screen were indeed due to an interaction between the PEV modifiers and *Pc*$^{3}$. Interestingly, animals heterozygous for *Pc*$^{3}$ and *Su(var)3-4*$^{1}$ ,*Su(var)3-4*$^{2}$, *Su(var)3-9*$^{1}$, or *Su(var)3-10*$^{2}$, had lower penetrance of antenna-to-leg

transformation compared to $Pc^3$/+ heterozygotes alone (Table 2.2 and Table 2.5). I have observed this tendency both in the preliminary genetic screen and after outcrossing the *Su(var)* stocks, which suggests that this discrepancy is not due to the genetic background. Similar observations were made with *Su(var)3-10*$^2$, where many genetic interactions have been assayed, using different balancer chromosomes and backgrounds, and $Pc^3$ heterozygotes always had higher antenna-to-leg transformation. Thus far, we have no explanation why this is the case.

The molecular identity and function of only few *Su(var)s* tested here are known (Table 2.1). SU(VAR)2-1 is a HDAC, SU(VAR)3-1 is a histone kinase, SU(VAR)3-9 is a histone methyltransferase and SU(VAR)2-5 is a heterochromatin binding protein (HP1). SU(VAR)3-9 creates a methylation mark on Histone H3 which is recognized and bound by SU(VAR)2-5, which results in recruiting of more heterochromatin proteins and establishing of a silent heterochromatin environment. Our results suggest that proteins involved in heterochromatin establishment would also be involved in mediating the PcG silencing.

In the beginning our attention was focused on *Su(var)3-10*$^2$, one of the modifiers that enhanced $Pc^3$ phenotype to the greatest extent. *Su(var)3-10* has not been cloned, but its cytological location is known and thus we were able to test a few deficiencies that have been shown to map to the same region. Complementation tests revealed that *Su(var)3-10*$^2$ is sub-lethal over the tested deficiencies. Genetic interaction tests between the deficiencies and $Pc^3$ showed

enhanced *Pc*$^{3}$-homeotic transformations, which confirmed that the homeotic gene silencing activity also maps within the same region. Tests for PEV modifications of the *w*$^{m4h}$ marker gene, revealed that the PEV suppressing activity of *Su(var)3-10*$^{2}$ also maps within the same region. We decided not to pursue *Su(var)3-10*$^{2}$ because it does not have other alleles available. *Su(var)3-10*$^{2}$ is an EMS caused mutation, which cannot be used to obtain revertant alleles in order to control for genetic background.

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# CHAPTER 3

## GENETIC CHARACTERIZATION OF SU(VAR)2-10

### Introduction

*Su(var)2-10* is one of the suppressors that greatly enhanced the $Pc^{3}$-induced homeotic transformations in the preliminary genetic screen. In this introduction I will present some background information on genetic and molecular studies done on *Su(var)2-10* and, in Chapter 5, I will introduce what is known about the protein structure and potential mode of operation of SU(VAR)2-10.

*Su(var)2-10* mutations have been reported to have diverse phenotypes, which suggests multiple roles in the cell. In addition to being identified as suppressor of PEV, *Su(var)2-10* was identified as a negative regulator of *stat92E* from the Drosophila JAK-STAT pathway (Betz et al., 2001). SU(VAR) 2-10 physically interacts with STAT92E, and this has implications in regulating blood cell and eye development. One extra copy of *Su(var)2-10* results in small and rough eyes, while most of the flies with two extra copies have no eyes. This phenotype is rescued by overexpression of *stat92E*. Crossing flies heterozygous for the $stat92E^{06346}$ loss-of-function allele with flies carrying one extra copy of *Su(var)2-10* results in developing of additional antennae instead of eyes. In terms of blood development, $Su(var)2\text{-}10^{03697}$ loss-of-function enhances melanotic

tumorigenesis caused by a mutation in *hopscotch (HOP)*, the single Drosophila tyrosine kinase of the JAK family. In summary, it is the correct *Su(var)2-10/stat92E* ratio that is crucial for blood cell and eye development.

More information about *Su(var)2-10* comes from studies carried out in Gary Karpen's lab (Hari et al., 2001). They identified *Su(var)2-10* in a screen for PEV modifiers with effects on chromosome inheritance. It was reported that *Su(var)2-10* mutations caused defects in the proper inheritance of a J21A minichromosome and of endogenous chromosomes. Furthermore, they demonstrated that *Su(var)2-10* is required for proper chromosome structure, as mutants have a disrupted banding pattern of their polytene chromosomes, and for chromosome condensation, as injecting embryos with anti-SU(VAR)2-10 antibodies yields massive condensation effects, which results in prophase/prometaphase arrest. Another interesting observation made was that *Su(var)2-10* mutant nuclei have disrupted telomere-telomere and telomere-lamina associations and thus *Su(var)2-10* has a role in the organization of chromosomes in interphase nuclei. In addition, Hari et al. showed that SU(VAR)2-10 proteins colocalize with the nuclear lamin in the nucleoplasm and are not concentrated along condensed mitotic chromosomes. In summary, *Su(var)2-10* regulates chromosome structure and function by establishing/maintaining chromosome organization in interphase nuclei.

In this chapter, using genetic approaches I further assayed the contribution of *Su(var)2-10* to PcG-mediated silencing and I established that *Su(var)2-10* mutations on their own compromise homeotic gene silencing.

## Materials and methods

### *Drosophila* stocks

The *Su(var)2-10* and $Pc^3$ stocks used in this chapter are described in Chapter 2. For the lacZ assay, I used flies that were either homozygous for the lacZ reporter or heterozygous for a certain PcG gene recombined with the T8 or T3 reporter. T3 is inserted in the second chromosome and T8 is on the third chromosome. The genotypes used were: [T8 MCP/PRE-lacZ ]/ [T8 MCP/PRE-lacZ ], [T3 MCP/PRE-lacZ ]/ [T3 MCP/PRE-lacZ ], $Pc^3$ [T8 MCP/PRE-lacZ]/*TM3SerGFP*, $Psc^{h27}$ [T3 MCP/PRE-lacZ]/*CyOrGFP*, $Trl^{R85}$ [T8 MCP/PRE-lacZ]/*TM3SerGFP* and $Psq^{D91}$ [T3 MCP/PRE-lacZ]/*CyOrGFP*. All of the lacZ reporter flies were made in the lab by previous members.

### Wing imaginal discs staining

For antibody staining, the imaginal discs were dissected in PBS and fixed in 4% paraformaldehyde for 1-2 hours. After washing with PBS and blocking with PBST for one hour, discs were incubated overnight at 4°C with the FP.3.38 monoclonal anti-UBX antibody kindly provided by Rob White (White and Wilcox, 1984)(1:10 dilution). The discs were washed in PBS and incubated with 1:500 anti-mouse Cy3-conjugated secondary antibody (Jackson ImmunoResearch) and analyzed and photographed using Axioskop microscope. The procedure for X-gal staining to assay lacZ expression is described in Chapter 2.

## Results

### Mutations in *Su(var)2-10* compromise PcG- mediated silencing of homeotic genes.

To eliminate the possibility that the observed genetic interactions with $Pc^3$ are allele specific, I tested three mutant alleles and one revertant allele of Su(var)2-10. They were assayed for genetic interaction not only with $Pc^3$, but also with another Polycomb allele, $Pc^4$, and with two other PcG genes- Polyhomeotic, $Ph^D$, and Sex combs on the middle leg, $Scm^{D1}$. The data for the penetrance of homeotic transformations are presented in Table 3.1 and their expressivity in Figure 3.1. As a control, I used the $Su(var)^{PEX2C}$ allele, which is a precise excision of the P-element in the $Su(var)2\text{-}10^{03697}$ allele (Hari et al., 2001). Both $Su(var)2\text{-}10^{03697}$ and $Su(var)^{PEX2C}$ have identical genetic backgrounds and thus any genetic interactions observed are due to the presence of the P-element in the mutant allele.

All mutant alleles enhanced the $Pc^3$-induced homeotic transformations to different extents. The weak homeotic transformations observed in the $Su(var)^{PEX2C}/+;Pc^3/+$ double heterozygous animals are due to the presence of $Pc^3$. $Su(var)2\text{-}10^{03697}$ enhanced the $Ph^D$- induced T3 –to-T2 leg transformation, and $Scm^{D1}$ – induced T2-to-T1 leg transformation ( Figure 3.1 and Table 3.1) but no phenotypes were seen for the other three tissues.

**Table 3.1 Genetic interactions between *Su(var)2-10* alleles and different PcG-genes.**

| Genotype | Penetrance of homeotic transformations | | | | | |
|---|---|---|---|---|---|---|
| | females | | males | | | |
| | A-to-L | W-to-H | A-to-L | W-to-H | T2-to-T1 | A4-to-A5 |
| *Su(var)2-10$^{03697}$/+; Pc$^{3}$/+* | 57% * +++ | 63%* +++ | 9% * + | 3%** + | 37% * +++ | 90%* +++ |
| *Pc$^{3}$/+* | 17% + | 30% + | 1% + | 1% + | 0% | 17% + |
| *Su(var)2-10$^{PEX2C}$/+; Pc$^{3}$/+* | 12% ** + | 5% + | 2%** + | 1% + | 2% ++ | 12% ** + |
| *Pc$^{3}$/+* | 14% + | 5% + | 1% + | 1% + | 2% ++ | 15% + |
| *Su(var)2-10$^{03697}$/+; Pc$^{4}$/+* | 10% * + | 92% * +++ | 0% | 42% * +++ | 97% * +++ | 72% * +++ |
| *Pc$^{4}$/+* | 0% | 24% + | 0% | 0% | 13% + | 20% + |
| *Su(var)2-10$^{1}$/+; Pc$^{3}$/+* | 25% * ++ | 77% * +++ | 2% ** + | 4% ** + | 72.5%* +++ | 100%* +++ |
| *Pc$^{3}$/+* | 0% | 20% + | 0% | 3%* | 8.5% + | 24% + |
| *Su(var)2-10$^{2}$/+; Pc$^{3}$/+* | 18% ** +++ | 73%* +++ | 0% | 0% | 4%** + | 48% * +++ |
| *Pc$^{3}$/+* | 15% + | 6% + | 0% | 0% | 0% | 3% + |
| *Su(var)2-10$^{2}$/+; Pc$^{4}$/+* | 0% | 79% * +++ | 0% | 0% | 64% * +++ | 95% * +++ |

Table 3.1 continued

| Genotype | Penetrance of homeotic transformations | | | | | |
|---|---|---|---|---|---|---|
| | females | | males | | | |
| | A-to-L | W-to-H | A-to-L | W-to-H | T2-to-T1 | A4-to-A5 |
| *$Pc^4$/+* | 0% | 16% + | 0% | 0% | 8% + | 14% + |
| *$ph^d$;Su(var)2-$10^{03697}$/+* | 0% | 0% | 0% | 0% | 4.2 teeth /T3 leg | 37% * +++ |
| *$ph^d$ ; +/+* | 0% | 0% | 0% | 0% | 1.9 teeth/T3 leg | 0% |
| *Su(var)2-$10^{03697}$/+; $Scm^{D1}$/+* | 0% | 0% | 0% | 0% | 68%* ++ | 0% |
| *$Scm^{D1}$/+* | 0% | 0% | 0% | 0% | 28% + | 0% |

1) Compared is the penetrance of homeotic transformations in double heterozygous *$Pc^3$/Su(var)* animals (upper row) versus percentage of homeotic transformations in *$Pc^3$/+* or *$Ph^d$/+* or *$Scm^{D1}$/+* heterozygotes (lower row).
2) The penetrance of homeotic transformation is given in percents. Expressivity is shown with pluses: + weak, ++ moderate, +++ strong.
3) N=200-400
4) p-value is calculated using two-tailed Fisher's exact test and labeled with asterisks; * penetrance significantly different from the PcG control ($p < 0.5$); ** penetrance not significantly different from internal control ($p > 0.5$); p-values are calculated for percentage of homeotic transformation of double heterozygous animals (*$Pc^3$/Su(var)*) versus *$Pc^3$* heterozygous (or *$Ph^d$* or *$Scm^{D1}$* heterozygotes). For the first two rows, p-value is for the mutant *Su(var)2-$10^{03697}$* and the revertant *Su(var)$^{PEX2C}$*.
5) In the double heterozygotes for *$Pc^4$*, *$Ph^d$* and *$Scm^{D1}$*, extra sex combs on the third pair of legs (T3) were counted instead on the second pair (T2) as *$Pc^4$/+*, *$Ph^d$/+* and *$Scm^{D1}$/+* heterozygous already have extra sex combs on the T2 legs.

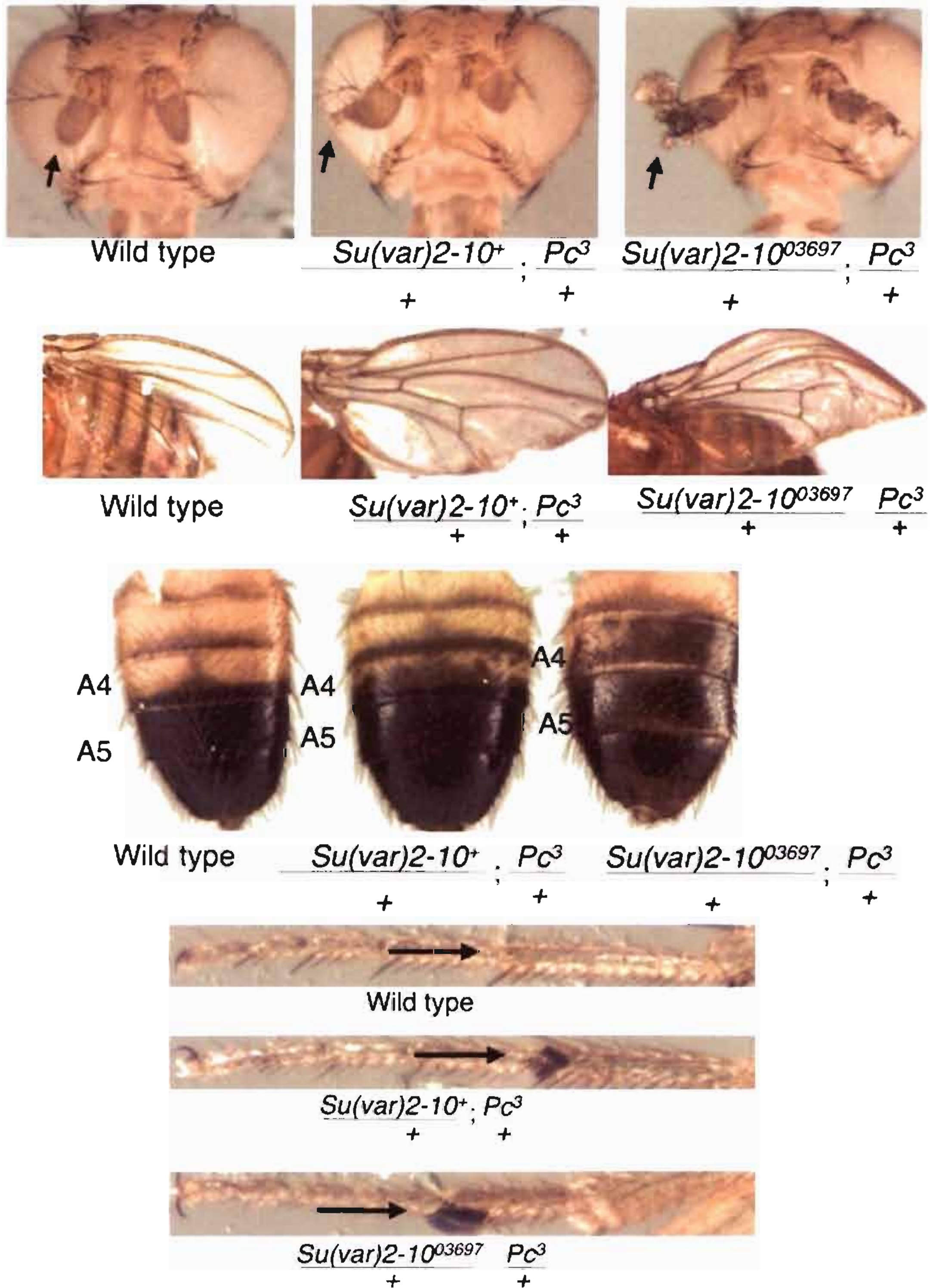


**Figure 3.1 *Su(var)2-10$^{03697}$* strongly enhances *Pc$^{3}$*-induced homeotic transformations**. The weak homeotic transformations observed in the *Su(var)$^{PEX2C}$/+;Pc$^{3}$/+* double heterozygous animals are due to the presence of *Pc$^{3}$*.

Su*(var)2-10*$^{03697}$ derepresses endogenous homeotic gene

To obtain molecular evidence that *Su(var)2-10* mutations indeed derepress homeotic genes, I examined the expression of an endogenous homeotic *Ubx* gene in wing imaginal discs where *Ubx* is normally silenced. Derepression of *Ubx* in wing discs, leads to transformation of wings into halteres. Immunohistochemistry revealed that *Su(var)2-10*$^{03697}$ heterozygous flies lack Ubx-derepression similarly to the absence of adult homeotic phenotype (Figure 3.2). In *Pc*$^{3}$ heterozygous flies *Ubx* becomes derepressed within a very small cluster of cells. In Su*(var)2-10*$^{03697}$/+; *Pc*$^{3}$/+ double heterozygotes, *Ubx* derepression becomes greatly enhanced. Thus, at molecular level, Su*(var)2-10*$^{03697}$ interacts with *Pc*$^{3}$ to derepress at least one of the homeotic genes, *Ubx*.

Su*(var)2-10* mutant alleles enhance PcG- gene induced derepression of a lacZ reporter

When looking at adult phenotypes, *Su(var)2-10* alleles were able to enhance homeotic transformations induced only by *Pc* alleles, *Ph*$^{d}$ and *Scm*$^{D1}$ (Table 3.1). To gain evidence that *Su(var)2-10* interacts with more PcG genes, I took advantage of the more sensitive MCP/PRE-lacZ reporter system. For reasons explained earlier, the reporter assay can detect a genetic interaction that cannot be detected when looking at adult homeotic phenotypes. I tested whether *Su(var)2-10* mutations would enhance PcG-induced reporter derepression by

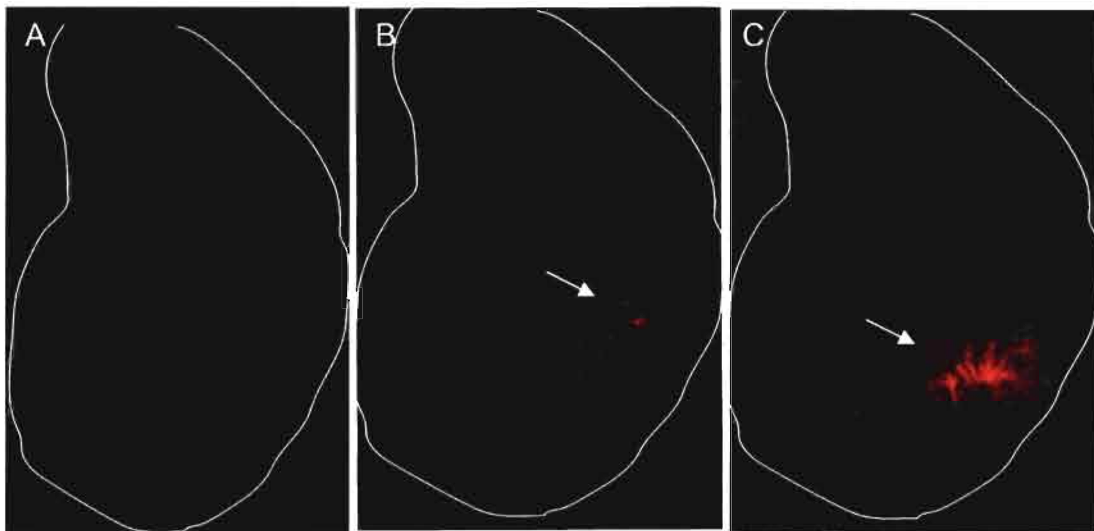


**Figure 3.2 *Su(var)2-10$^{03697}$* strongly enhances *Pc$^{3}$* induced *Ubx*-derepression**. Shown is anti-UBX staining of third instar larval imaginal wing discs. A) *Su(var)2-10$^{03697}$/+;* none of the 20 examined discs had Ubx-derepression; B) *Pc$^{3}$/+;* 14 out of 26 examined discs showed weak Ubx-derepression; C, *Su(var)2-10$^{03697}$/+; Pc$^{3}$/+* ; 29 out of 32 examined discs had very strong Ubx-derepression. The expressivity shown in these photos is representative of the derepression patterns observed among the discs. Arrows point to cells expressing *Ubx.*

using lacZ reporter genes recombined to $Psq^{D91}$, $Psc^{h27}$ and $Trl^{R85}$ (Figure 3.3). To establish whether the observed *Su(var)2-10* genetic interaction with $Pc^3$ is mediated through PRE elements, a lacZ assay was done also with $Pc^3$ [T8 MCP/PRE-lacZ ] reporter. The increase in lacZ expression showed that indeed, such interaction exists, which further confirmed that *Su(var)2-10* contributes to PcG-mediated silencing and it acts through PREs.

Su*(var)2-10* mutant alleles derepress PcG-mediated silencing on their own.

Next, I assayed whether *Su(var)2-10* mutations would derepress a PRE-dependent reporter gene in imaginal wing and haltere discs on its own. Expressivity of the observed lacZ reporter derepression is shown in Figure 3.3A and the penetrance data are shown in Figure 3.3B. *Su(var)2-10*$^{03697}$ derepressed both the [T8 MCP/PRE-lacZ] and [T3 MCP/PRE-lacZ] reporter genes. The fact that a *Su(var)2-10* mutation derepresses a PRE- dependent reporter gene on its own, suggests that *Su(var)2-10* behaves as a PcG member.

## Discussion

*Su(var)2-10* alleles enhanced both $Pc^3$- and $Pc^4$- induced transformations very strongly, and they also interacted with $Ph^d$ and $Scm^{D1}$. The revertant allele, *Su(var)2-10*$^{PEX2C}$ did not enhance any PcG-induced phenotypes. All genetic interaction data presented earlier suggested that *Su(var)2-10* contributes to the PcG-mediated silencing of homeotic genes.

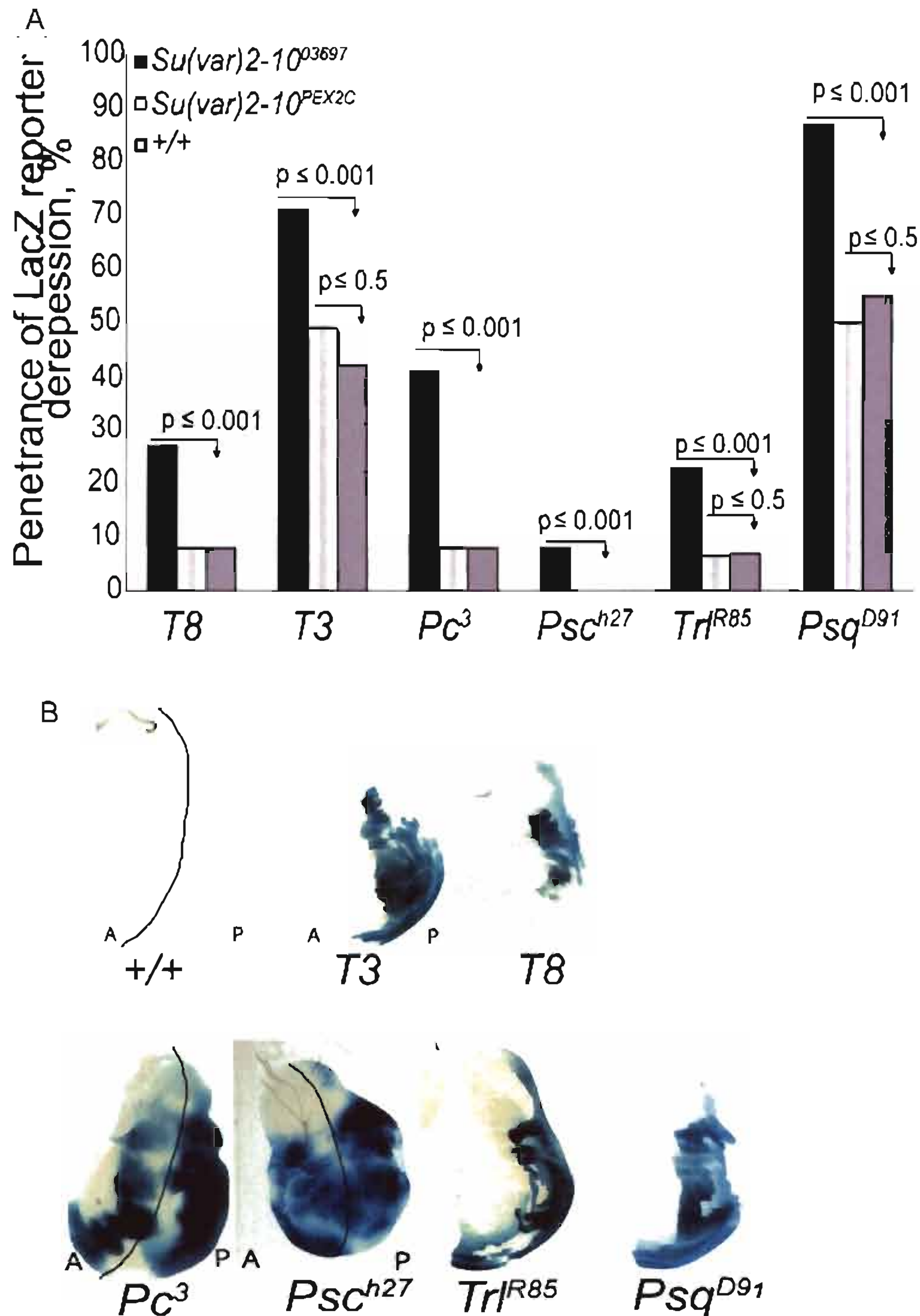


**Figure 3.3** ***Su(var)2-10*** **derepresses lacZ reporters on its own and it enhances PcG-induced reporter derepression.** A) Penetrance of lacZ expression for *Su(var)2-10* mutant (black bars) and revertant allele (grey bars). Due the leakiness of the transgene promoter, penetrance of lacZ expression for each promoter is also shown in dark grey bars. C) Representative photos of lacZ staining of imaginal wing discs from the examined genotypes. A-anterior part, P-posterior part.

In this chapter, I have also shown that *Su(var)2-10* is a novel PcG member. Mutations in this gene enhance the $Pc^3$-induced derepression of an endogenous homeotic gene, *Ubx*. Thus, the adult homeotic phenotypes seen when *Su(var)2-10* was tested for the genetic interaction with $Pc^3$ are indeed due to the contribution of *Su(var)2-10* to homeotic gene silencing.

I have demonstrated that *Su(var)2-10* acts on homeotic gene silencing via a PRE-dependent mechanism, which is suggested from the fact that *Su(var)2-10* derepresses PRE-dependent reporter genes on its own (Figure 3.3A, B).

In conclusion, I have shown that *Su(var)2-10* contributes to homeotic gene silencing and is a novel PcG member. In Chapter 5, using biochemical and *in vivo* approaches I will try to functionally characterize *Su(var)2-10.*

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# CHAPTER 4

# SUMOYLATION IN PCG SILENCING AND IN PEV

## Introduction

SU(VAR)2-10 shares a high degree of sequence identity with the mammalian family of Protein Inhibitor of Activated STATs (PIAS) proteins (Betz et al., 2001; Hari et al., 2001). In mammalian systems, PIAS proteins have been reported to function as SUMO E3 ligases in a protein modification pathway called the SUMO pathway (Figure 4.1).

SUMOylation is a process whereby a Small Ubiquitin-Like Modifier protein (SUMO) is attached covalently to a short consensus sequence, ΨKXE, where Ψ is a large hydrophobic amino acid, generally isoleucine, leucine or valine; K is the lysine residue that is modified; X is any residue, and E is a glutamic acid. SUMO proteins share only ~ 18% sequence identity with Ubiquitin, but they both have the very characteristic ubiquitin-fold tertiary structure ((Bayer et al., 1998). SUMOs are 11 kDa proteins, which run as 20 kDa on SDS-PAGE gels. Although both SUMOylation and Ubiquitination have very similar mechanistic characteristics, their physiological consequences are quite different. While in most cases, Ubiquitination targets its substrates for degradation, SUMOylation, results in altering protein function by changing its conformation, recruitment of

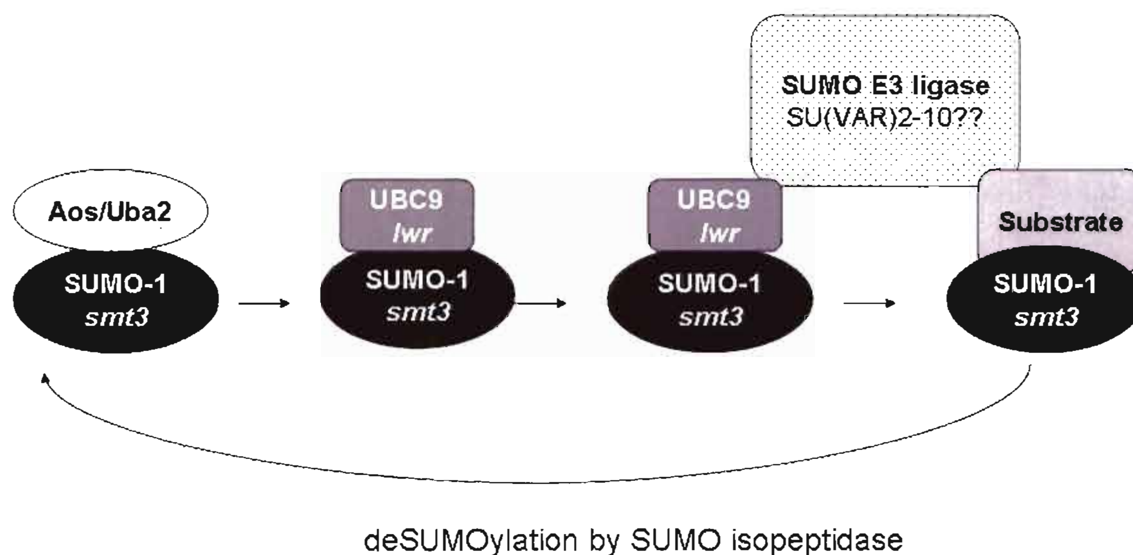


**Figure 4.1 SUMO pathway schematic**. The SUMOylation pathway starts with activation of the SUMO peptide from a SUMO activating E1 called Aos/Uba2. SUMO-1 is then transferred to a SUMO conjugating E2, called UBC9, which covalently conjugates SUMO-1 on a substrate with the help of a SUMO E3 ligase. With italics are shown the name of the *Drosophila* genes; *smt3* encodes SUMO-1; *lwr* encodes E2. SUMOylation is reversible as SUMO isopeptidases remove SUMO from the substrates.

binding partners, subcelullar localization or antagonizing other post-translational modifications. An interesting feature of SUMOylation is that at any given time only a small fraction (less than 1%) of the substrate is modified.

Like Ubiquitination, the SUMOylation pathway starts with a SUMO-activating enzyme (E1, called Uba2/Aos1), which catalyses an ATP-dependent activation of the SUMO C terminus and then transfers the activated SUMO to a SUMO-conjugating enzyme (E2, called UBC9) (Figure 4.1). From E2, SUMO is transferred to the ΨKXE consensus sequence of the substrate with the assistance of one of the many SUMO E3-ligases. Both E2 and E3 contribute to the substrate specificity. SUMOylation is a reversible process, and removal of SUMO is carried out by SUMO isopeptidases that specifically cleave at the C terminus of SUMO. The same proteases (as carboxy-terminal hydrolases) cleave a short peptide blocking the C terminus in SUMO precursors to generate mature SUMO.

Similarly to Ubiquitin pathway, the SUMO pathway also has E3 ligases, which function by bridging the substrate and SUMO E2 together. Since SUMOylation can occur *in vitro* without the presence of SUMO E3 ligases, this raises the possibility that *in vivo*, SUMO E3 function might be to confer substrate specificity.

Mammalian PIAS proteins have been reported to function as SUMO E3 in the SUMOylation of proteins such as p53 (Kahyo et al., 2001; Schmidt and Muller, 2002), Jun (Schmidt et al., 2002), Lef-1 (Sachdev et al., 2001) and the

nuclear androgen receptor (Kotaja et al., 2000). More information about the mechanisms utilized by mammalian PIAS proteins will be given in Chapter 5.

Additional evidence to suggest that SUMOylation might be utilized by PcG-mediated silencing came from a report that the mammalian Polycomb protein PC2 is SUMO modified and it is a SUMO E3 ligase (Kagey et al., 2003). PC2 enhances the SUMO modification of the co-transcriptional repressors CtBP and CtBP2.

The SUMO modification pathway is well conserved in *Drosophila*. The genes encoding components of the mammalian SUMOylation pathway have been also identified in Drosophila and these are *DmAos/Uba2*, *smt3* and *lwr* (Bhaskar et al., 2000). Mutations in all these genes are homozygous lethal. Female escapees, trans-heterozygous for *smt3* mutant alleles, produce a few late-stage egg-chambers, and these rare eggs have defects in their dorsal appendages (Schnorr et al., 2001). Homozygous mutations in *lwr* are late embryo or first instar lethal and the mutant animals lose the anterior half of their body structures (Epps and Tanda, 1998).

Thus far, we showed that *Su(var) 2-10* contributes to PcG silencing and mutations in *Su(var) 2-10* compromise homeotic gene silencing. If SU(VAR)2-10 is a SUMO E3 ligase like the mammalian PIAS proteins, then mutations in components of the SUMO pathway should compromise homeotic gene silencing as well. In this Chapter, using genetic approaches I will study whether the SUMO pathway contributes to PcG silencing and PEV (as *Su(var)2-10* was primarily isolated as suppressor of PEV). I will also test whether mutations in components

of the SUMO pathway interact genetically with *Su(var)2-10* mutations to derepress expression of endogenous homeotic genes.

## Materials and methods

### *Drosophila* stocks and genetic techniques

*Drosophila* stocks were obtained from the Bloomington stock center. $Pc^{3}$ and $w^{m4h}$ stocks used here are described in previous chapters. Follows a list of used genotypes and references for information: $Psmt3^{04493}cn^{1}/CyO;ry^{506}$ (Schnorr et al., 2001), $y^{1}w^{67c23}$;$P\{w^{+mC}=lacW\}smt3^{k06307}/CyO$ (Milchanowski et al., 2004), $P\{ry^{+t7.2}=PZ\}\ lwr^{05486}cn^{1}/CyO;ry^{506}$ (Apionishev et al., 2001) and $P\{ry^{+t7.2}=PZ\}\ lwr^{02858}cn^{1}/CyO;ry^{506}$ (Epps and Tanda, 1998). *l(2)04493* maps to a region about 10bp upstream of the first exon of *smt3*. *l(2)05486* is inserted in the 5' regulatory region of *lwr* while *l(2)02858* is inserted in the 5' UTR of *lwr.* Shige Sakonju generated the precise excisions of the $smt3^{04493}$ and $lwr^{05486}$ P-elements, by genetically introducing a source of transposase to fly lines bearing the P-element and selecting for loss of the $rosy^{+}$ eye color marker. I confirmed the P-element excisions using primers starting outside both ends of the $smt3^{04493}$ or $lwr^{05486}$ P-element insertions to check for presence or lack of P-element. I also used standard primers to amplify either the 5' or 3' end of the PZ P-elements and the sequence outside the P-element insertion. For list of the primers used see Table 4.1. The DNA sequence adjacent to the P-element inserted in *l(2)05486* has been reported in Accession number G00739 and the two corresponding ESTs have Accession numbers AA247001 and AA392936. The DNA sequence

adjacent to the P-element inserted in *l(2)04493* has been reported in Accession number AQ025627. Double mutants of $smt3^{04493}Su(var)2\text{-}10^{03697}$ and $lwr^{05486}Su(var)2\text{-}10^{03697}$ were generated by Shige Sakonju, using genetic recombination approaches.

LacZ reporter assay

See Chapter 2 for description of the [T3 MCP/PRE-lacZ] and [T8 MCP/PRE-lacZ] reporters used.

Antibody staining of whole embryos

Eggs were collected 11h to 16h AEL and prepared for staining using standard protocols. Monoclonal mouse antibodies against Scr,Abd-B and Antp were purchased from Developmental Studies Hybridoma Bank at the University of Iowa. The FP.3.38 monoclonal anti-UBX antibody was kindly provided by Rob White and used at 1:20 dilution (White and Wilcox, 1984). The other dilutions were as follows: anti-AbdB was used at 1:10, anti-Antp 8C11 was used at 1:10 and anti-Scr from ascites at dilution 1:1000. The embryos were incubated with 1:500 anti-mouse Cy3-conjugated secondary antibody (Jackson ImmunoResearch) and analyzed and photographed using Axioskop microscope.

PEV assay

Males heterozygous for the mutant or revertant allele of $smt3^{04493}$ and $lwr^{05486}$ were crossed to females carrying the PEV marker, $w^{m4h}$. Because $w^{m4h}$ is on the X chromosome, in F1 we compared the PEV of $w^{m4h}$ in males

**Table 4.1 List of primers used to confirm excisions of the P-element in *lwr* or *smt3* stocks**

| Primer Name | Sequence |
|---|---|
| lwr-FW | CAAGCGTTCATTCCTGA |
| lwr-RV | TAAATCCAGCTGTACGGCTACTGACGACC |
| smt3-FW | GTAGCTGTAGCAGAAGCAAAAGGAAG |
| smt3-RV | GTTATTTACGCACACAGACGC |
| Plac1 | CACCCAAGGCTCTGCTCCCACAAT |
| Pry2 | CTTGCCGACGGGACCACCTTATGTTATT |

The pair lwr-FW/lwr-RV or smt3-FW/smt3-RV were used to amplify the sequence outside the P-element. Lack of P-element gives a short product. Combinations of lwr-FW/Plac1 and smt3-FW/Plac1 were used to amplify the sequence at the site of P-element insertion through the 5' end of the element. Combinations of lwr-RV/Pry2 and smt3-RV/Pry2 were used to amplify the sequence at the site of P-element insertion through the 3' end of the element.

heterozygous for $w^{m4h}$;$smt3^{04493}$/+ (or $w^{m4h}$;$lwr^{05486}$/+ ) versus $w^{m4h}$ ;+/+ males.

## Results

### Test for genetic interaction between mutations from the SUMOylation pathway and PcG mutations

To assay whether SUMOylation is involved in PcG-mediated silencing, two SUMOylation pathway mutant alleles, *lwr* and *smt3* were tested for genetic interactions with $Pc^3$ and $Ph^d$. As controls, we used isogenic revertant alleles of $smt3^{04493}$ and $lwr^{05486}$ (see Material and Methods). The *lwr* mutant allele did not enhance the $Pc^3$- and $Ph^d$- induced homeotic transformations (data for $smt3^{k06307}$, $lwr^{02858}$ and $Ph^d$ not shown) (Table 4.2). We observed that *smt3* significantly enhanced the penetrance of $Pc^3$-induced phenotype (Table 4.3). Nevertheless, the expressivity of the homeotic transformations was very weak. These results suggest that at least one of the SUMOylation pathway mutations, namely *smt3* interacted genetically with $Pc^3$ albeit not strongly.

### LacZ expression assay of SUMO pathway mutations

To further examine whether the SUMOylation pathway contributes to PcG-mediated silencing, mutations in *lwr* and *smt3* were assayed for derepression of [T3 MCP/PRE-lacZ] and [T8 MCP/PRE-lacZ] reporters. None of the mutant alleles was able to derepress either of the lacZ reporters significantly more than the corresponding internal controls. Data are shown only for [T8MCP/PRE-lacZ]

**Table 4.2 Test for genetic interactions between *lwr* and *Pc*$^3$**

| Genotype | Females | | Males | | | |
|---|---|---|---|---|---|---|
| | A-to-L | W-to-H | A-to-L | W-to-H | T2-to-T1 | A4-to-A5 |
| *lwr*$^{05486}$/+; *Pc*$^3$/+ | 33%<br>++<br>p=0.6 | 50%<br>++<br>p=1 | 1%<br>+<br>p= 1 | 6%<br>+<br>p=0.05 | 9%<br>++<br>p=0.02 | 18%<br>++<br>p=0.4 |
| *Pc*$^3$/+ | 35%<br>++ | 49%<br>++ | 0% | 14%<br>+ | 28%<br>++ | 12%<br>++ |
| *lwr*$^{rev\#24}$/+; *Pc*$^3$/+ | 50%<br>++<br>p=0.7 | 40%<br>++<br>p=0.5 | 0% | 0%<br><br>p=0.1 | 6%<br>+<br>p=0.3 | 33%<br>+<br>p=0.3 |
| *Pc*$^3$/+ | 55%<br>++ | 50%<br>++ | 0% | 3% | 11% | 22% |

Penetrance of homeotic transformations is given in percents. Expressivity of the observed homeotic transformations is given with pluses, where + weak, ++ intermediate, +++strong. P-values are calculated for the *lwr*/+;*Pc*$^3$ double heterozygotes and *Pc*$^3$/+ single heterozygotes.

**Table 4.3 Test for genetic interactions between *smt3* and *Pc*$^3$**

| Genotype | Females | | Males | | | |
|---|---|---|---|---|---|---|
| | A-to-L | W-to-H | A-to-L | W-to-H | T2-to-T1 | A4-to-A5 |
| *smt3*$^{04493}$/+; *Pc*$^3$/+ | 42% ++ p=0.1 | 55% +++ p≤ 0.0001 | 4% + p=0.5 | 6% + p=0.5 | 39% + p≤0.0001 | 31% ++ p=0.04 |
| *Pc*$^3$/+ | 40% ++ | 10% ++ | 3% + | 5% + | 6% + | 4% ++ |
| *smt3*$^{rev\#38}$/+; *Pc*$^3$/+ | 9% + | 4% + | 0% | 0% | 0% | 10% + p=0.6 |
| *Pc*$^3$/+ | 9% + | 4% + | 0% | 0% | 0% | 9% + |

Penetrance of homeotic transformations is given in percents. Expressivity of the observed homeotic transformations is given with pluses, where + weak, ++ intermediate, +++strong. P-values are calculated for the *smt3/+;Pc*$^3$ double heterozygotes and *Pc*$^3$/+ single heterozygotes.

and *smt3*$^{04493}$, *lwr*$^{05486}$ and their revertant alleles (Table 4.4). Also *smt3*$^{04493}$ and *lwr*$^{05486}$ did not enhance lacZ expression induced either by *Pc*$^{3}$ [T3] or *psq*$^{D91}$[T3] (data not shown). Lack of lacZ reporter derepression suggests that the SUMO pathway does not contribute to PcG silencing or that it cannot be detected using the sensitive lacZ assay. It is also possible that the genetic interactions we are trying to detect are very weak, and we can see phenotype only when the SUMOylation pathway mutations are combined with *Pc*$^{3}$, as is it was shown earlier with *smt3* in Table 4.3.

Double recombinants of *Su(var)2-10* mutation with SUMO pathway mutations

To further sensitize the system in order to detect any contribution of the SUMOylation pathway to the PcG silencing, we made double mutants of *smt3*$^{04493}$*Su(var)2-10*$^{03697}$ and *lwr*$^{05486}$*Su(var)2-10*$^{03697}$ and assayed them for enhancement of either *Pc*$^{3}$- or *Ph*$^{d}$- induced homeotic phenotypes. I have already shown that *Su(var)2-10*$^{03697}$ significantly enhances both *Pc*$^{3}$- and *Ph*$^{d}$- induced homeotic transformations. If *Su(var)2-10*$^{03697}$ was acting on homeotic gene silencing via the SUMOylation pathway, one would expect that double recombinants of *smt3*$^{04493}$*Su(var)2-10*$^{03697}$ or *lwr*$^{05486}$*Su(var)2-10*$^{03697}$ would enhance *Pc*$^{3}$- or *Ph*$^{d}$- induced phenotypes to a greater extent than does *Su(var)2-10*$^{03697}$ alone. The double recombinants did not enhance the *Pc*$^{3}$ phenotype more than *Su(var)2-10*$^{03697}$ does (Table 4.5). *Ph*$^{d}$ heterozygotes show T3-to-T1 transformation with average of 4.2 teeth per leg (maximum number of

**Table 4.4 LacZ expression assay for *lwr* and *smt3* mutant and revertant alleles**

| Genotype | % discs with T8 reporter lacZ derepression (n) | |
|---|---|---|
| | wing discs | haltere discs |
| *$lwr^{05486}$/CyO* | 25 (81) | 7 (86) |
| *CyO/+* | 18 (94) | 3 (94) |
| *$lwr^{rev\#24}$/CyO* | 17 (115) | 2 (114) |
| *CyO/+* | 18 (84) | 1 (84) |
| *$smt3^{04493}$/CyO* | 22 (41) | 0 (40) |
| *CyO/+* | 19 (53) | 2 (45) |
| *$smt3^{rev\#38}$/CyO* | 16 (57) | 0 (67) |
| *CyO/+* | 16 (38) | 0 (38) |

$p \geq 0.5$ is for all crosses in the table; p-value is calculated for each allele and its internal control *(CyO/+)*;. (n), number of counted imaginal discs

**Table 4.5 Test for genetic interactions between *Pc*$^{3}$ and *lwr*$^{05486}$ *or smt3*$^{04493}$ double recombinants with *Su(var)210*$^{03697}$*/CyOGFP*.**

| Genotype | Females | | Males | | | |
|---|---|---|---|---|---|---|
| | A-to-L | W-to-H | A-to-L | W-to-H | T2-to-T1 | A4-to-A5 |
| *Su(var)2-10*$^{03697}$*/+; Pc*$^{3}$*/+* | 57% +++ p=0.001 | 63% +++ p=0.001 | 9% + p=0.001 | 3% + p=0.1 | 37% +++ p=0.001 | 90% +++ p=0.001 |
| *Pc*$^{3}$*/+* | 17% + | 30% + | 1% + | 1% + | 0% | 17% + |
| *lwr*$^{05486}$ *Su(var)2-10*$^{03697}$*/+; Pc*$^{3}$*/+* | 54% + p=0.03 | 86% +++ p=0.0001 | 0% | 3% + p=0.3 | 40% +++ p=0.001 | 84% +++ p=0.001 |
| *Pc*$^{3}$*/+* | 31% + | 20% + | 0% | 0% | 3% + | 9% + |
| *smt3*$^{04493}$ *Su(var)2-10*$^{03697}$*/+; Pc*$^{3}$*/+* | 21% + p=0.1 | 53% +++ p=0.001 | 0% | 4% + p=1 | 31% +++ p=0.0001 | 75% +++ p=0.0001 |
| *Pc*$^{3}$*/+* | 13% + | 15% + | 0% | 3% + | 8% + | 7% + |

A) Shown is the penetrance in percent of four different transformations in *Pc*$^{3}$ heterozygotes, *Pc*$^{3}$*;Su(var)210*$^{03697}$double heterozygotes and in animals carrying all three mutant alleles: *Pc*$^{3}$, *smt3*$^{04493}$ and *Su(var)210*$^{03697}$.
B) For each phenotype n>100;
C) expressivity shown in pluses; + weak, ++ intermediate, +++ strong

teeth per leg is ten) (Table 4.6). Animals heterozygous for *ph*$^{D}$;*Su(var)2-10*$^{03697}$ had 4.2 teeth/T3 leg. Animals heterozygous for *ph*$^{D}$; *smt3*$^{04493}$*Su(var)2-10*$^{03697}$/+ or *ph*$^{D}$; *lwr*$^{05486}$*Su(var)2-10*$^{03697}$/+ had correspondingly 4.2 and 4 teeth/T3 leg. We also compared the A4-to-A5 transformation in double and in triple mutants. It should be noted that because all animals had 100% penetrance of A4-to-A5 transformation, we decided to count only these animals that have strong transformation (Table 4.6). 37% of the animals heterozygous for *ph*$^{D}$;*Su(var)2-10*$^{03697}$ showed strong A4-to-A5. 67% of the *ph*$^{D}$; *smt3*$^{04493}$*Su(var)2-10*$^{03697}$/+ heterozygotes had strong A4-to-A5. The penetrance for *ph*$^{D}$; *lwr*$^{05486}$*Su(var)2-10*$^{03697}$/+ was 59%. For both crosses, the difference between the triple mutants and the double mutants was statistically significant. This experiment suggests, that mutations in *lwr* and *smt3* contribute to the *Su(var)2-10*$^{03697}$ enhancement of *ph*$^{D}$ –induced A4-to-A5 transformation.

To determine whether interaction between SUMOylation pathway mutations and *Su(var)2-10*$^{03697}$ can be seen earlier in developmental time, we examined embryos homozygous for *smt3*$^{04493}$*Su(var)2-10*$^{03697}$ or *lwr*$^{05486}$*Su(var)2-10*$^{03697}$ for derepression of endogenous homeotic genes. Antibody staining for Ubx, Scr, Abd-B and Antp did not reveal any derepression of these homeotic genes outside their specified segments (Figure 4.2). One limitation with this experiment is that there is a maternal contribution, which might explain the lack of phenotype. Thus we can conclude that when the zygotic contribution is eliminated, mutations in *lwr* or *smt3* do not interact with *Su(var)2-10* mutation to derepress endogenous homeotic genes.

**Table 4.6 Test for genetic interactions between *$ph^d$* and *$lwr^{05486}$ Su(var)2-$10^{03697}$/CyOGFP*,or *$smt3^{04493}$Su(var)2-$10^{03697}$* double recombinants**

| Genotype | Number of teeth/ T3 leg (n) | %,Penetrance of strong A4-to-A5 (n) |
|---|---|---|
| *$ph^d$;Su(var)2-$10^{03697}$/+* | 4.2 (397) | 37% (241) |
| *$ph^d$;$lwr^{05486}$Su(var)2-$10^{03697}$/+* | 4.2 (272) | 67% (215) p=0.0003 |
| *$ph^d$;$smt3^{04493}$Su(var)2-$10^{03697}$/+* | 4 (272) | 59% (202) p=0.007 |

A) Shown is the number of teeth in each extra sex comb per T3 leg; Maximum number of teeth per leg is five; B) Shown is the penetrance of only the strong A4-to-A5 transformation. As all animals have A4-to-A5 transformation, the only difference comes if one accounts for the expressivity. Thus we have counted only the animals with strong A4-to-A5 transformation.

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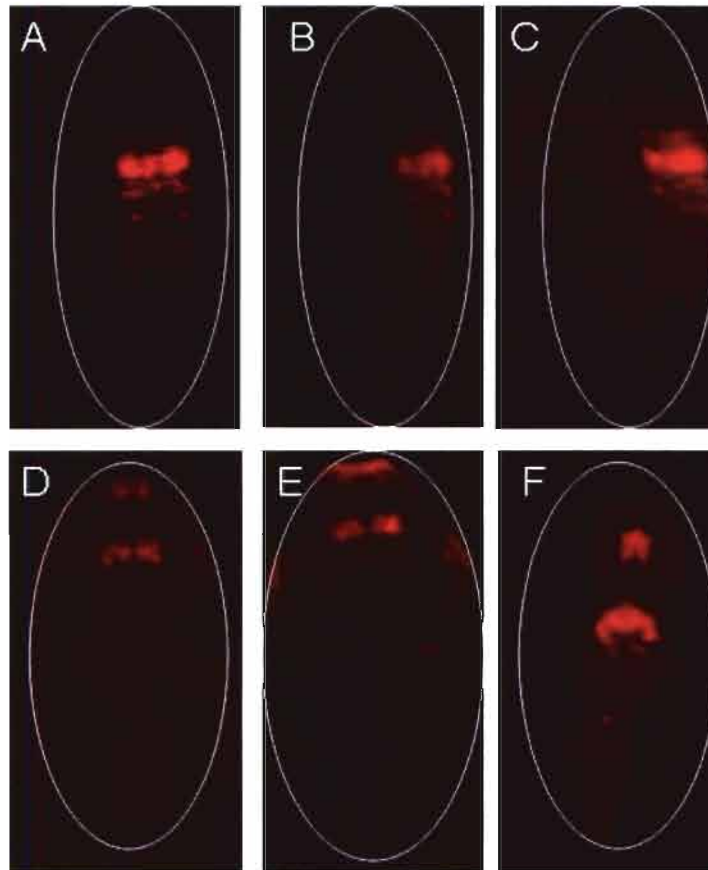


**Figure 4.2 Antibody staining against UBX and SCR in *smt3$^{04493}$Su(var)2-10$^{03697}$* and *lwr$^{05486}$Su(var)2-10$^{03697}$* homozygous embryos.** A, B, C show staining against UBX (lateral view) and D,E,F show staining against SCR (top view). A and D are wild type, B and E are *smt3$^{04493}$Su(var)2-10$^{03697}$* homozygous embryos and C and F are *lwr$^{05486}$Su(var)2-10$^{03697}$* homozygous embryos.

Do SUMO pathway mutations affect PEV?

*Su(var)2-10* was originally isolated as a suppressor of PEV. If *Su(var)2-10* is a SUMO E3 ligase, we would expect that SUMO pathway mutations would also affect PEV. Thus, we assayed whether $smt3^{04493}$ and $lwr^{05486}$ act like *Su(var)2-10* as suppressors of PEV. We observed that males heterozygous for $lwr^{05486}$ or $smt3^{04493}$ had redder eyes compared to their internal control (CyO/+) or the revertant alleles ($lwr^{rev\#24}$ or $smt3^{rev\#38}$) (Figure 4.3). This result shows that both mutations suppressed the PEV phenotype and suggests that the SUMOylation pathway contributes to PEV.

## Discussion

Using genetic approaches we could not find a definitive evidence for a strong SUMO pathway contribution to PcG-mediated silencing of homeotic genes. We observed that only one SUMO pathway mutation, namely $smt3^{04493}$ enhanced weakly the $Pc^3$-induced homeotic transformations. Double recombinants for either *lwr* or *smt3* with *Su(var)2-10* did not enhance $Pc^3$ phenotype more than *Su(var)2-10* does alone. Nevertheless, the double recombinants enhanced the $ph^D$ –induced A4-to-A5 phenotype to a greater extent than does *Su(var)2-10* alone. Also, we were not able to detect derepression of homeotic genes in embryos homozygous for $lwr^{05486}Su(var)2\text{-}10^{03697}$ and $smt3^{04493}Su(var)2\text{-}10^{03697}$, a result that might be due to the presence of maternal contribution.

Although we do not have definitive genetic data for a SUMO pathway

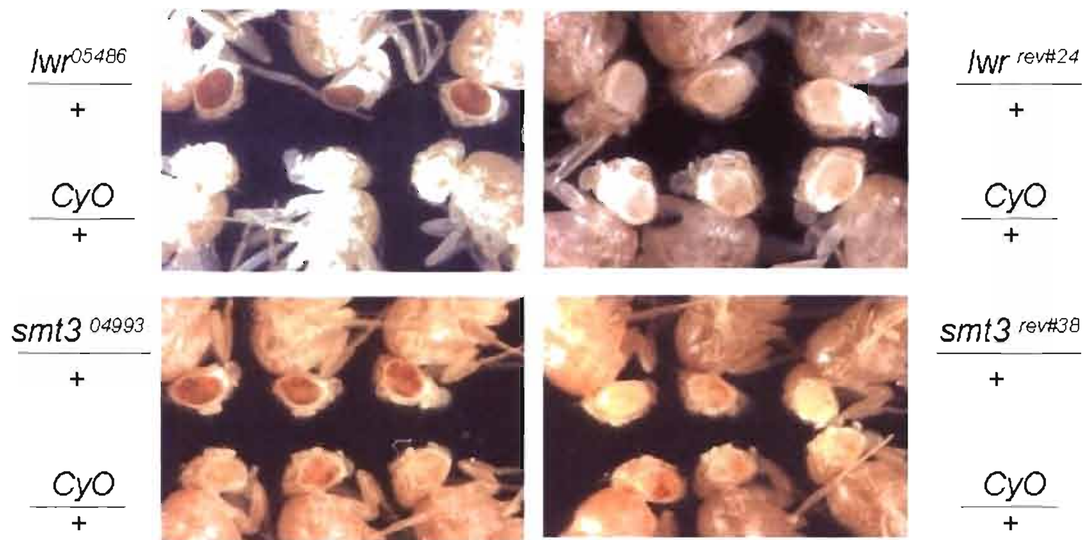


**Figure 4.3 Effect of *lwr* and *smt3* mutations on PEV**. Shown are photos of PEV eye phenotype in mutant or revertant alleles of *smt3* and *lwr* and their internal CyO/+ controls.

contribution to PcG silencing we cannot eliminate such a possibility. This is mostly due to the observations that the $Pc^3$ phenotype is enhanced by $smt3^{04493}$ and that the $ph^D$ -induced A4-to-A5 is greater in triple mutants for $ph^D;lwr^{05486}$ $Su(var)2\text{-}10^{03697}$ and $ph^D;smt3^{04493}Su(var)2\text{-}10^{03697}$. This result suggests that Su(var)2-10 might interact with SUMOylation pathway mutations to enhance $ph^D$ -induced transformation.

Regarding PEV, we observed that mutations in *lwr* and *stm3* suppressed PEV, which led us to conclude that the SUMOylation pathway has implications for PEV.

In summary, our genetic interaction studies suggest that the SUMOylation pathway may be involved in PcG mediated silencing and PEV.

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# CHAPTER 5

## BIOCHEMICAL AND *IN VIVO* APPROACHES TO FUNCTIONALLY CHARACTERIZE SU(VAR)2-10

### Introduction

SU(VAR)2-10 is a PIAS protein, and PIAS proteins have been shown to function as SUMO ligases in mammalian systems. Thus we tested the notion that SU(VAR)2-10 is such a ligase by using biochemical approaches in parallel with the genetic ones described in the previous chapter. PIAS proteins were originally identified as negative regulators of cytokine signaling that inhibits STAT (Signal Transducers and Activators of Transcription) transcription factors. STATs are involved in variety of events in embryology, hematopoiesis, immune responses and growth control. As it is more relevant to my dissertation, I will give background information regarding the SUMOylation aspect of their function.

#### SUMO E3 ligases

Thus far, three types of SUMO ligases have been described. One includes Zn-binding RING finger proteins (such as the PIAS proteins) (Hochstrasser, 2001) . Another is the vertebrate nuclear pore protein RanBP2 (Pichler et al., 2002); and third is the PcG protein PC2 (Kagey et al., 2003). For a

protein to be classified as a SUMO ligase, it must (a) bind UBC9, (b) bind a substrate directly or indirectly and (c) augment the transfer of SUMO from the conjugating enzyme to the substrate *in vitro* (Hershko and Ciechanover, 1998).

The exact molecular mechanism of PIAS function in the SUMOylation pathway is still unclear. Ubiquitin ligases belong to two major classes: HECT-domain ligases and RING-domain ligases (Weissman, 2001). HECT E3s catalyze ubiquitination by forming thiol-ester intermediates between ubiquitin and a conserved cysteine residue in the HECT domain. RING ligases simply function as adaptor molecules that tether E2 to the substrate. The RING ligases fall into two groups: monomeric and multimeric. The monomeric ligases have binding sites for both E2 and the substrate, while in the multimeric ligases the RING finger binds E2, and additional protein binds the substrate. SUMO ligases resemble the ubiquitin RING ligases in that they function as docking sites positioning UBC9-SUMO to facilitate transfer of SUMO to substrate. SUMO E3s may also be involved in activation of UBC9.

Structure of PIAS proteins

The eukaryotic family of PIAS proteins is evolutionarily conserved from yeast to humans. In mouse and man, there are at least five PIAS genes and/or splice variants: *PIAS1, PIAS3, PIASy, PIASxα* and *PIASxβ,* all of which localize to nuclear dots/bodies (Kotaja et al., 2002a; Sachdev et al., 2001). PIAS1 and PIAS3 inhibit the DNA binding activity of STAT1 and STAT3 respectively (Chung et al., 1997; Liu et al., 1998). PIASy represses STAT1, LEF1, Smad4 and AR

without inhibiting their DNA binding activity (Chung et al., 1997; Gross et al., 2001; Liu et al., 2001; Long et al., 2003; Sachdev et al., 2001). PIASx$\alpha$ interacts with AR (Wu et al., 1997) and PIASx$\beta$ interacts with the homeodomain protein Msx2 (Moilanen et al., 1999) and with STAT4 (Arora et al., 2003). Among the many interacting partners of PIAS proteins are c-Jun (various PIASes), p53 (PIAS1) ,Sp3 (PIAS1), HMGI-C (PIAS3), Cfi-1 (PIAS3), IRF-1 (PIAS3), and TFII-I (PIASx$\beta$) (Nakagawa and Yokosawa, 2002; Rodel et al., 2000; Sapetschnig et al., 2002; Schmidt and Muller, 2002; Zentner et al., 2001). Although in most of the cases, PIAS proteins exert repression on the transcription factors, they can also augment gene expression by associating with other transcriptional co-activators (Kotaja et al., 2000; Yamamoto et al., 2003).

Except for a variable 100-450 residue C-terminal region, the PIAS proteins are highly homologous, showing nearly 50-60% identity at the amino acid level. The ~400 residue N-terminal region consists of a SAP domain (SAF, Acinus, PIAS) and an SP-RING domain (Siz/PIAS-Really Interesting Gene). A common feature of SAP-containing proteins is their ability to bind chromatin. The SAP domain in SAF-A (Scaffold Attachment Factor) recognizes distinct AT-rich DNA sequences known as scaffold or matrix attachment regions (MARs/SARs) (Kipp et al., 2000). MARs are associated with the proteinaceous meshwork of the nuclear matrix, which is involved in maintaining a higher-order chromatin structures. The SAP domains of PIAS1 and PIASy bind *in vitro* to synthetic MAR/SAR sequences, but not to mutant ones, and PIASy has been reported to associate with the nuclear matrix *in vivo* (Sachdev et al., 2001; Tan et al., 2002).

SAP domains mediate binding of PIAS proteins to their protein targets: PIASy binds to LEF1 (Sachdev et al., 2001) and STAT-1 (Liu B, 2001, PNAS) via the SAP domain, and PIASxα binds to glucocorticoid and androgen receptors (Kotaja et al., 2002b). Deletion of the SAP domain in PIASy abrogates its ability to relocate LEF1 to nuclear bodies and thus, it is possible that the SAP module is necessary for targeting of PIASes to nuclear bodies (Sachdev et al., 2001). SU(VAR)2-10 interacts physically with the STAT ortholog stat92E via its RING domain to regulate blood cell and eye development (Betz et al., 2001). In conclusion, SAP domains may mediate interaction with different target proteins, DNA and chromatin or mediate the subcelullar localization of PIAS proteins.

The SP-RING domain resembles the RING domains found in many ubiquitin RING ligases as it has zinc-binding cysteine/histidine residues. Unlike the canonical RING fingers, the SP-RING lacks two of the cysteine residues, critical for binding to zinc and thus it is unclear whether SP-RING domain can acquire the so called “cross-brace” arrangement of the RING domains (Schmidt and Muller, 2003). Like ubiquitin RING domains, the SP-RING domain binds directly to the conjugating enzyme, UBC9, and is therefore required for the ligase activity of PIAS proteins.

In addition to SAP and SP-RING domains, PIAS proteins also contain a short motif of hydrophobic amino acids followed by acidic amino acids, called SUMO interaction motif (SIM). As the name suggest, SIM has been reported to bind to SUMO (Minty et al., 2000) and to be involved in localization and

transcriptional effects of PIAS proteins (Kotaja et al., 2002a; Sachdev et al., 2001).

PIAS proteins as SUMO ligases

PIAS proteins have been implicated in SUMOylation of several targets. Some substrates are unique for a certain PIAS protein, while others can be SUMOylated in the presence of different PIASes. Coexpression of PIASy and LEF1 results in SUMOylation of LEF1 and other proteins and translocation of LEF1 to nuclear bodies, such as the PML bodies (Sachdev et al., 2001).

SU(VAR)2-10

*Su(var)2-10* is alternatively spliced to produce at least twelve different transcripts, six of which have been confirmed by Northern analysis by Gary Karpen's group (Hari et al., 2001). Conceptual translation of *Su(var)2-10* transcripts yields at least nine polypeptides, which are identical across a 514-amino-acid domain, but differ in their COOH termini. SU(VAR)2-10 bears a high percent of sequence identity to vertebrate PIAS proteins : 40% within the SAP domain and 84% within the SP-RING domain and it also contains a SIM sequence (Figure 5.1).

In this chapter, I assayed whether SU(VAR)2-10 satisfies the three requirements for a protein to be a SUMO ligase: (a) binding to UBC9, (b) binding

| | |
|---|---|
| **dPIAS** | VQMLRVVELQKILSFLNISFAGRKTDLDSPILSFL |
| **PIAS1** | VMSRLRVVSELQVLLCYAGRNKHGRKHLLTKALHLL |
| **PIAS3** | VMSRVSELQVLLCFAGRNKSGRKHELLAKALHLL |
| **PIASx** | VSSFRVSELQKILCFAGRNKHGRKHDLDSPILLMKALHLL |
| **PIASy** | VMSFRVSELQMLLGFVGRSKSGLKHELVTRAILQLV |

| | |
|---|---|
| **dPIAS** | CPLGKMKMLLPCRASTCSHLQCFDASLYLQMNERKPTWMCPYC |
| **PIAS1** | CPLGKMRLTIPCRALTCSHLQCFDATLYLQMNERKPTWMCPYC |
| **PIAS3** | CPLGKMRLTVPCRALTCAHLQCFDAALYLQMNERKPTWMCPYC |
| **PIASx** | CPLGKMRLTIPCRAVTCTHLQCFDAALYLQMNERKPTWMCPYC |
| **PIASy** | CPLGKMRLSVPCRAETCAHLQCFDAVLYLQMNERKPTWMCPYC |

**Figure 5.1 SU(VAR2-10 shows a high degree of sequence identity to the PIAS family of proteins.** Shown is an alignment between SU(VAR)2-10 and human PIAS proteins within their SAP (upper panel) and RING (lower panel) domains. Conserved sequences are marked in grey.

to a substrate and (c) augmenting the transfer of SUMO from the conjugating enzyme to the substrate *in vitro.* I found that SU(VAR)2-10 binds to UBC9 and to GAGA, but does not enhance SUMOylation of GAGA.

## Materials and methods

### Plasmids and their construction

A 1529 fragment from Su(var)2-10 (CG8068-PE), was amplified from cDNA library (Brown and Kafatos, 1988) using primers that encompass the region common for all 12 isoforms. The forward primer introduces an NcoI site and the reverse primer introduces a Hind III site and a stop codon. The sequences of the primers used are as follows: FW: GTCGACTCCATGGACCTGCAGAGCCGCATCCTCTCGTTC and RV: GTCGACAAGCTTTTACATGTCGTCGTCTGAATCGCTTAACGTTAG. The NcoI-Su(var)2-10 fragment was then ligated into the NcoI and Hind III sites of Novagen pET-GST 42a (+) vector (kindly provided by Carl Thummel's lab members). The pET- Su(var)2-10-ΔRING mutant and the pET- Su(var)2-10-W362A/G mutants were constructed by site-directed mutagenesis of the pET-Su(var)2-10. The primers used to construct the pET- Su(var)2-10-ΔRING were Δ-FW: GCCATCTATGACCAGGTTGTCCCTTCAGCA TAGTGGTGGCTAT and Δ-RW: ATAGCCACCACTATG CTGAAGGACAACC TGGTCATAGATGGC. The primers used to introduce a point mutation W362A and W362G were as follows: to amplify the "left" PCR products I used a point mutation introducing A-FW: TGAATGAGCGTAAGCCCACGGCGAACTGCC CTGTATGCGACAAG and RV:

GCCTAGGTAT TAATCAATTAGTGG. To amplify the "right" PCR product I used A-RV: CTTGTCGCATACAGGGCAGTTCGCCGT GGGCTTACGCTCATTCAT and RV: GTCGACAAGCTTTTACATGTCGTCGTC TGAATCGCTTAACGTTAG. To create a W362G mutation, I used: G-FW: ATGAATGAGCGTAAGCCCACG GGGAACTGCCCTGTATGCGACAAG and G-RV: CTTGTCGCATACAGGGCA GTTCCCCGTGGGCTTACGCTCATTCAT. The other pair of primers is the same as the ones used for introducing W362A. After amplifying the left and right products carrying the mutation, they were annealed and the bigger product was cloned into the NcoI – Hind III site of the pET42a(+) vector just as the wild type Su(var)2-10 construct.

For *in vitro* translation of the Su(var)2-10 constructs, the above described constructs were digested from pET plasmid with KpnI and Hind III and subsequently ligated into KpnI-Hind III digested Bluescript pSK+ plasmid.

To construct pET-UBC9, the Uc9 fragment from pKS-Ubc9 construct made by Hua Xin in our lab, was amplified with primers introducing restriction site and sub-cloned into pET42a(+).

For the purpose of expressing GAGA in vitro, the GAF ORF encoding 519 amino acid polypeptide was amplified from the GAF cDNA cloned into pCK vector kindly provided by T. Kornberg (Soeller et al., 1993) and cloned into SacI-Hind III sites of pET-42a(+). To construct pET-GAGA$^{mSUMO}$, mSUMO fragment cloned into pSK+ from Hua Xin was amplified using SacI and Hind III restriction sites introducing primers and cloned into pET42a(+). The primers used for these constructs were: Sac-FW: AGGCCTGAGCTCATGTCGCTGTGCCCAA

TGAATTCG, Sac-RV: GTCGACAAGCTTATGTCGCTGCCAATGAATTCG, Hind-FW: GTCGACAAGCTTATGTCGCTGCCAATGAATTCG and Hind-RV: GTCGAC ACAAGCTTCTACTGCGGCTGCGGC.

*In vivo* studies

For *in vivo* studies, Su(var)2-10, Su(var)2-10ΔRING, Su(var)2-10ΔSAP and Su(var)2-10W362A constructs were digested from pET-Su(var)2-10 templates blunt-ligated into pSK+ and subsequently cloned into NotI sites of pP{GS[hsp70,EGFP, $ry^{+}$]} vector system, kindly provided by Gunter Reuter (Schotta and Reuter, 2000) also see http://www2.biologie.uni-halle.de/genet/drosophila/pGS/modules.html. Germline transformation was carried out according to Rubin and Spradling (Rubin and Spradling, 1982). Transgenic flies were generated using *ycnry* as host and pUChsΔ2-3 as helper plasmid. Emerging flies were mated to the host *ycnry* flies and the progeny screened for $ry^{+}$ transformants. Chromosome integration was determined genetically and clones for wild type pGS-Su(var)2-10 were isolated on second and fourth chromosomes, designated as $h2\text{-}10^{5}$ and $h2\text{-}10^{6}$. Stocks were maintained either as homozygous or balanced over CyO chromosome by mating to *[iab26,ry+]/CyO* stock. To induce transgene expression, flies were maintained at 25°C and heat shocked every day for 1h at 37°C until adult flies emerge. Expression of transgenes was confirmed by detecting GFP on GFP scope and by Western blotting using polyclonal antibodies against GFP (MBL, kindly provided

by Thummel's laboratory) diluted 1:200 and visualized with ECL detection system.

PEV analysis and pigment extraction

To assay PEV, transgenic flies were first mated to *yw$^{m4h}$;Sco/Cyo* to balance them over *Sco* chromosome and then males were mated to *yw$^{m4h}$Su(var)2-10$^{2}$ /CyO*. Parents were kept for 3 days in a vial and then removed. Eggs were heat shocked for 1h at 37°C until adult flies emerge. Eye color of male offspring was assayed either immediately or they were let to age for few days. Pigment quantification was done according to Rabinow et al. (Rabinow et al., 1991). Several samples of 40 to 50 heads per genotype were collected, homogenized in 1 ml of methanol, acidified with 0.1% HCl (Ephrussi and Herold, 1944) and centrifuged. The absorbance of the supernatant was measured at 480nm.

GST protein expression, purification and
GST pull-down assays

GST fusion proteins were produced in Escherichia coli BL21 (Stratagene, kindly provided by Mat Hockin) and purified with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer instructions. Protein purification of SU(VAR)2-10 constructs was done as previously described by Kotaja et al. (2002b) and protein purification of GAGA constructs and UBC9 was done as described in Vikis and Guan (2004). The $^{35}$S-labelled full-length and

truncated or mutant Su(var)2-10, Su(var)2-10-ΔRING, Su(var)2-10-W362A, Su(var)2-10-W362G, UBC9, GAGA and $GAGA^{mSUMO}$ were *in vitro* translated from pBluescript SK+ (Stratagene) using rabbit reticulolysate (Promega). Protein-protein affinity chromatography with purified GST fusion proteins bound to glutathione-Sepharose and 10µl $^{35}S$-methionine-labeled *in vitro* translated protein was carried out as described by Vikis and Guan (2004). After electrophoresis, the gels were fixed in methanol (45%) - acetic acid (10%), dried and visualized by fluorography.

*in vitro* SUMOylation assays

GST fusion proteins were produced as described above. Purified proteins were eluted in buffer containing 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 10% glycerol, and 20 mM glutathione. *In vitro* translated and $^{35}S$-methionine-labeled proteins were obtained as described above. The SUMOlation assay using the SUMO kit from LAE-Biotech was carried out following the manufacturer instructions. 1-1.5 µl of the *in vitro* translated product was incubated with 1µg SUMO-1, 150 ng E1, and 20 ng E2 at 30°C for 1hr in the presence of 1 mM dithiothreitol, 4mM $MgCl_2$ and 2 mM ATP. The amount of GST-SU(VAR)2-10 used in the reactions was 100 ng or 150 ng or 200 ng. The reaction was terminated be adding 2X SDS sample buffer. The samples were heated at 95°C for 5 min, resolved by SDS-PAGE, and visualized by fluorography. The SUMOlation assay using all recombinant proteins was carried out as follows: GST-SU(VAR)2-10, GST-SU(VAR)2-10-ΔRING, GST-SU(VAR)2-10 W362A,

GST-SU(VAR)2-10-WS62G, GST-GAGA, and GST-mGAGA were produced and purified as described above. Aos/Uba2, UBC9 and SUMO-1 recombinant proteins were kindly provided by Adam Blaszczak from Barbara Graves' lab. 10 µl GST-GAGA or GST- $GAGA^{mSUMO}$ were incubated with 3 µl UBC9, 3 µl SUMO, 2 µl E1 at 30°C for 90 min in the presence of 1 mM dithiothreitol, 4mM $MgCl_2$ and 2 mM ATP. After the first 45 min more E1, SUMO and DTT was added to the reactions and they were let go for 45 min more. The amount of GST-SU(VAR)2-10 proteins used was below, equal or above the molar ratio of GST-GAGA. The samples were resolved by SDS-PAGE, and visualized by immunoblotting using anti-GST (kindly provided by Adam Blaszczak) or anti-GAGA antibodies.

## Results

### Assay for protein interaction between SU(VAR)2-10 and GAGA Factor

According to unpublished results from John Lis' lab cited by Hari et al. SU(VAR)2-10 binds to GAGA factor in a yeast two-hybrid screen(Hari et al., 2001). Using SUMO™plot prediction program available from the Abgen website, we found that GAGA factor has seven potential SUMO acceptor sites and thus GAGA presents a potential target for SUMOylation. GAGA is important for us, because it is involved in homeotic gene silencing, both as a repressor and activator. GAGA binds to short $GA_n$ repeats within the PRE and to longer $GA_n$ repeats within heterochromatin. As an activator of homeotic gene silencing, GAGA was reported to have nucleosome remodeling activity. In Chapter 3, I

showed that *Su(var)2-10* alleles interact genetically with a mutant allele of the GAGA factor encoding gene *trl* (Figure 3.2) to derepress a PRE-dependent lacZ reporter gene. The ability of SU(VAR)2-10 to interact with GAGA was confirmed by a GST pull down assay. $^{35}$S-methionine labeled *in vitro* translated GAGA protein was incubated with GST control protein or GST-SU(VAR)2-10 bound to glutathione-Sepharose. As shown in Figure 5.2, GAGA bound efficiently SU(VAR)2-10 but failed to bind GST alone.

Assay for protein interaction between
SU(VAR)2-10 and SUMO E2

GST pull-down experiments were used to assay whether SU(VAR)2-10 interacts with UBC9 *in vitro*. The GST assays were carried out under two different conditions: a) recombinant GST-SU(VAR)2-10 proteins were immobilized to glutathione-Sepharose and incubated with $^{35}$S-labeled UBC9, or b) recombinant GST-UBC9 pre-bound to glutathione-Sepharose was incubated with $^{35}$S-labeled SU(VAR)2-10. In addition to the full- length GST-SU(VAR)2-10 protein, I used three mutant proteins: GST-SU(VAR)2-10-ΔRING, GST-SU(VAR)2-10 W362A, GST-SU(VAR)2-10-W362G (Figure 5.3). Deletion of the RING domain in mammalian PIAS proteins has been shown to abrogate their binding to UBC9. Mutating the conserved Tryptophan codon at position 362 within the RING domain, to either Alanine or Glycine codon does not interfere with binding to UBC9 but compromises the E3 ligase activity (probably because

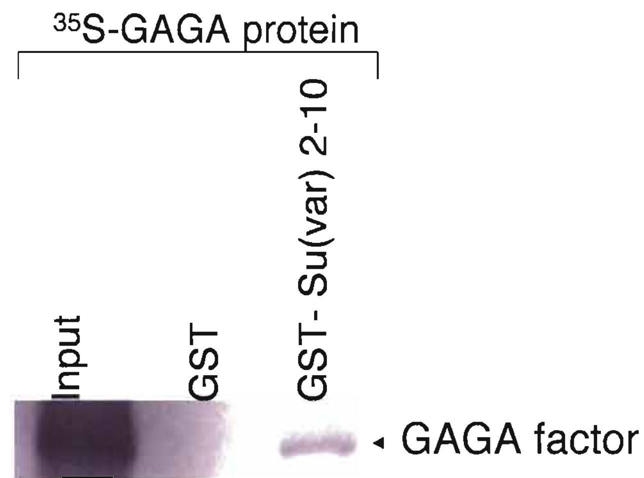


**Figure 5.2 SU(VAR)2-10 interacts with GAGA *in vitro*.** First lane shows the input of $^{35}$S-methionine labeled *in vitro* translated GAGA into each reaction. Middle lane shows lack of GAGA binding to GST only protein. Third lane shows that GAGA protein binds to GST-SU(VAR)2-10 fusion protein.

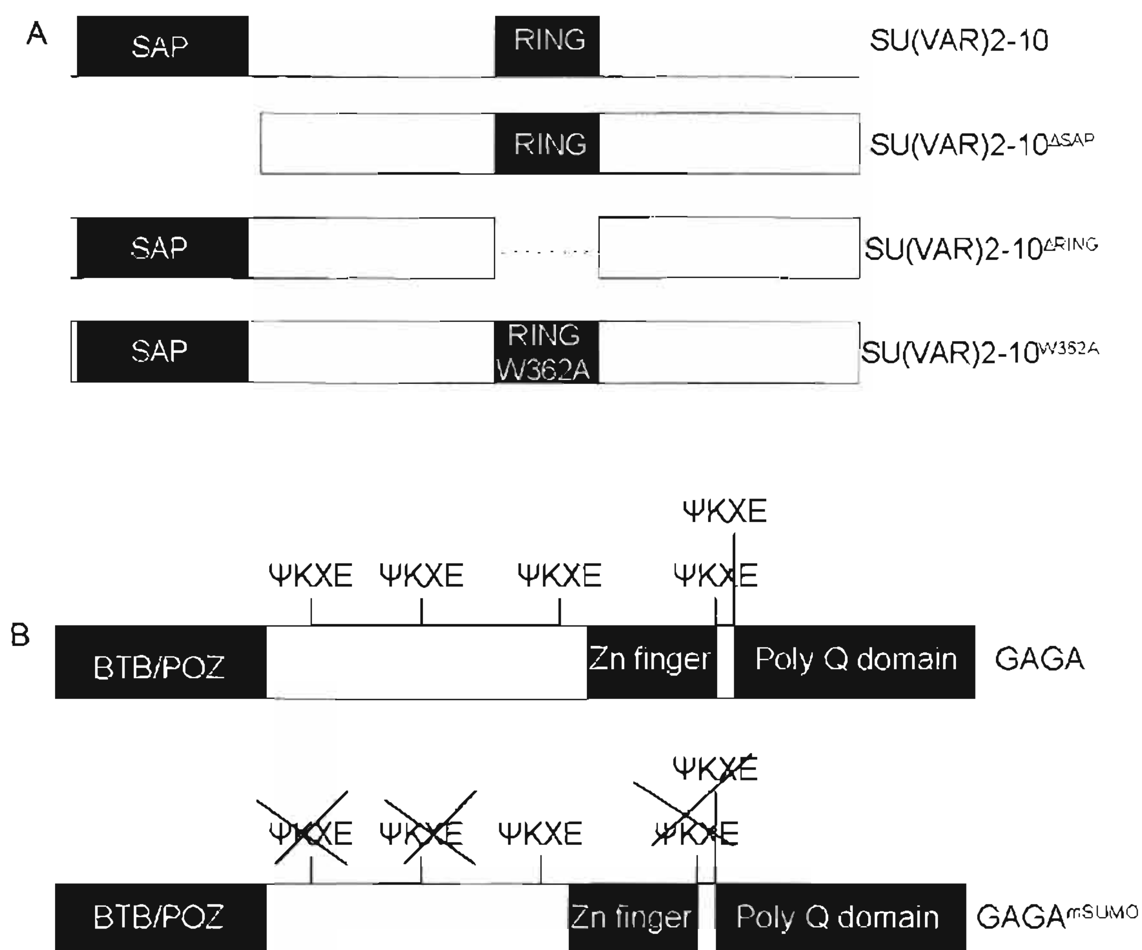


**Figure 5.3 Schematic diagrams of SU(VAR)2-10 (A)and GAGA (B) constructs used in these studies.** A) SU(VAR)2-10 contains a putative chromatin-binding SAP domain, a C2HC3 RING domain. The SU(VAR)2-10-ΔSAP construct lacks the SAP domain and the SU(VAR)2-10-ΔRING lacks the RING domain. SU(VAR)2-10W362A constructs has the tryptophan at position 362 substituted with alanine. B) GAGA contains a BTB/POZ domain, Zn finger and a polyQ domain. The GAGA$^{mSUMO}$ construct has the SUMO acceptor (ΨKXE) sites deleted.

the Tryptophan plays an essential role in the RING domain structure). Several different protocols were utilized that either provide low-stringency or high-stringency conditions. Under low-stringency conditions (no pre-clearing of $^{35}S$-labeled lysates and no BSA in the reaction mix, 50mM Tris-HCl, 150mM NaCl ), I was able to detect non-specific binding of UBC9 to both the GST control protein and SU(VAR)2-10 (Figure 5.4A). This interaction was eliminated under higher-stringency conditions (BSA added to reaction mix, 50mM Tris-HCl,150mM NaCl or 4mM Tris-HCl used) (Figure 5.4B). These results showed that SU(VAR)2-10 is not capable of direct physical interaction with UBC9.

Does SU(VAR)2-10 enhance SUMOylation of GAGA Factor?

To determine whether SU(VAR)2-10 has SUMO E3 ligase activity, we used an *in vitro* reconstituted SUMOylation system with purified proteins and GAGA factor as a substrate for SUMOylation. Two different SUMOylation assays were used. The first one was based on the LAE Biotech *in vitro* SUMOylation kit (providing human E1, E2 and SUMO-1 proteins), recombinant GST-SU(VAR)2-10 and $^{35}S$- methionine labeled *in vitro* translated GAGA. As shown on Figure 5.5A, GAGA SUMOylation is dependent upon the presence of E2 and it does not require SU(VAR)2-10. SUMO modification of substrates without E3 ligase has been observed in many other *in vitro* SUMOylation systems (Bhaskar et al., 2002; Desterro et al., 1999; Johnson, 2004; Kotaja et al., 2002a; Reindle et al., 2006; Sachdev et al., 2001). Adding a ligase usually results in more robust

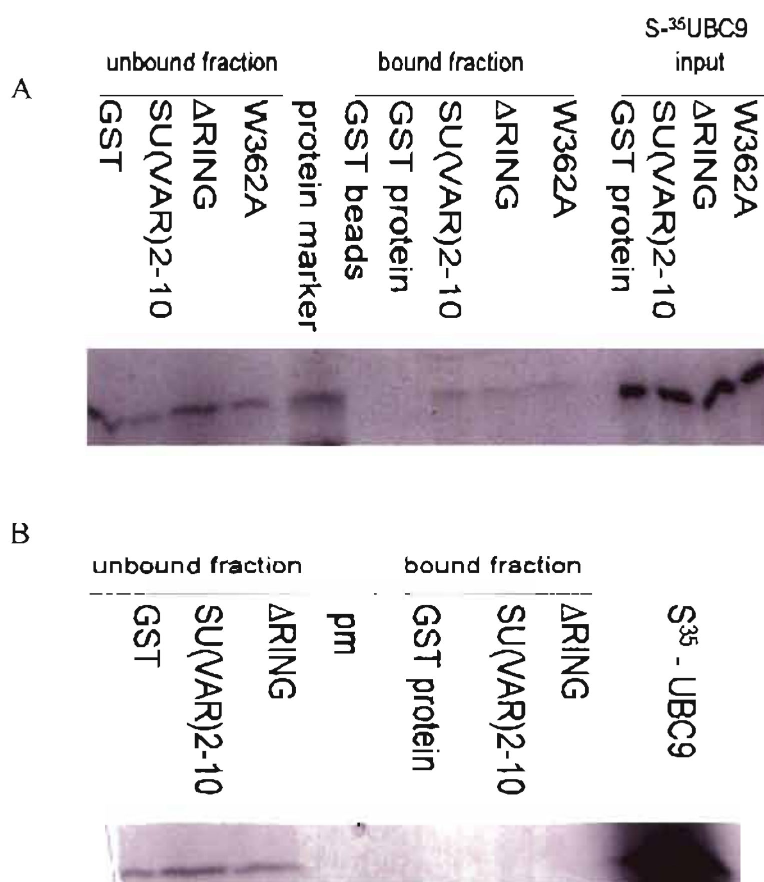


**Figure 5.4. GST pull-down assay for protein interaction study between SU(VAR)2-10 and UBC9.** For both panels SU(VAR)2-10 proteins are bacterially expressed GST-fusion proteins and UBC9 is $^{35}$S-labeled and *in vitro* translated. A) GST assay under low stringency conditions. UBC9 binds to all SU(VAR)2-10 variants. B) under higher-stringency conditions UBC9 does not bind to either GST nor SU(VAR)2-10 proteins.

SUMOylation, as seen by the enhanced intensity of the slowly migrating modified forms. That the observed high molecular GAGA bands are SUMO modified GAGA species, was confirmed by Hua Xin (see Chapter 6) in our lab who designed a mutant form of GAGA that has five out of seven SUMO acceptor sites eliminated (Figure 5.3B). Incubation of $GAGA^{mSUMO}$ with the SUMOylation kit did not result in the appearance of the high-molecular species seen with the wild type GAGA (see Figure 5.5B). Adding GST-SU(VAR)2-10 to the SUMO conjugation assay containing $^{35}$S-GAGA, did not result in enhancing of GAGA modified species. In addition, adding amounts of SU(VAR)2-10 above the molar concentration of GAGA resulted in inhibiting the SUMO modification. In this assay, GAGA was *in vitro* translated in Rabbit Reticulocyte Lysate (RRL) and directly added to the conjugation mixture. This raises a possibility that some enzymatic activity from the RRL might interfere with the SUMOylation assay. Another disadvantage of this assay is that the GAGA protein might not be in a large amount to detect a SUMO modification (the RRL system is not advantageous for obtaining high protein concentration). It has been shown for many substrates, that only very small fraction is being modified (Kotaja et al., 2002a). To avoid bringing contaminating activity from the RRL system and to obtain high concentrations of GAGA, I prepared GST-His tagged fusions of both proteins GAGA and $GAGA^{mSUMO}$ and carried out a different SUMOylation assay. Instead of the LAE kit, I used yeast E1, human E2 and SUMO-1 proteins, kindly provided by Adam Blaszczak (Macauley et al., 2006). As seen in Figure 5.5B, GAGA is SUMOylated in the abscence of SU(VAR)2-10. Adding increasing

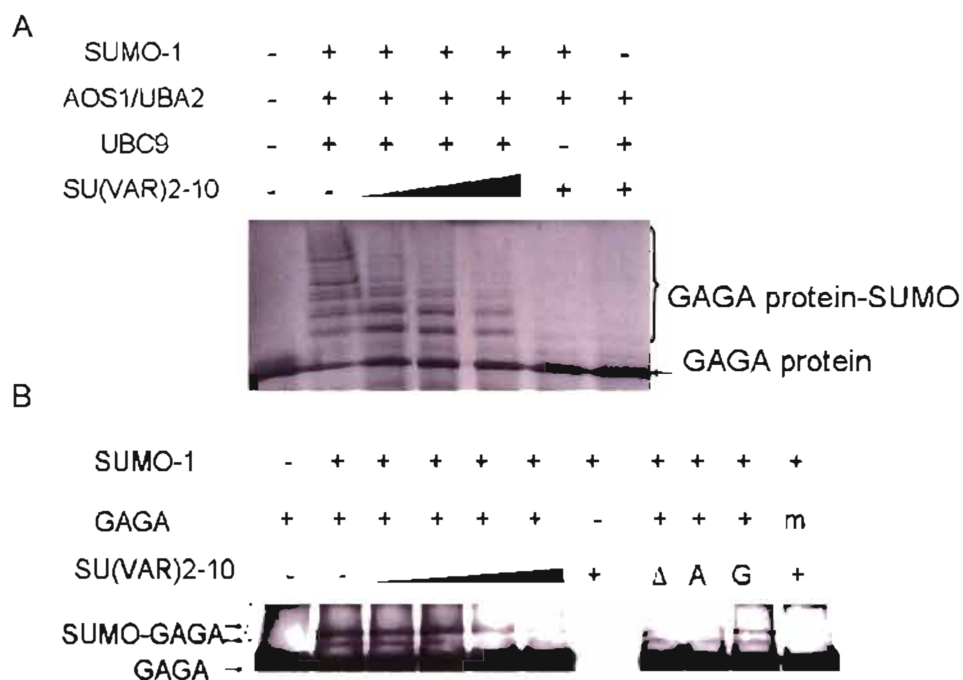


**Figure 5.5 SU(VAR)2-10 does not enhance SUMOylation of GAGA.**
A) SUMOylation of GAGA using LAE kit, recombinant SU(VAR)2-10 and $^{35}$S-methionine labeled *in vitro* translated in Rabbit Reticulocyte Lysate GAGA (1h at 37°C). GAGA SUMOylation is dependent upon presence of UBC9 (compare lanes 2 and 6). Adding increasing concentrations of SU(VAR)2-10 seems to inhibit the modification process. Equimolar ratio of SU(VAR)2-10 to GAGA is as follows: 1:2 (lane 3),1:1(lane 4), 2:1 (lane 5).
B) SUMOylation assay with recombinant GST-GAGA proteins. Wild type GAGA is SUMOylated in the absence of SU(VAR)2-10 (lane 2). Mutant GAGA (m) lacks the high-molecular weight species (the very last lane). Δ,SU(VAR)2-10-ΔRING mutant; A, SU(VAR)2-10-W362A; G, SU(VAR)2-10-W362G.

concentrations of SU(VAR)2-10 up to an equimolar ratio with GAGA, resulted in inhibition of the modification. The same result was observed when adding equimolar amounts of the mutant forms SU(VAR)2-10-ΔRING and SU(VAR)2-10-W362A. SU(VAR)2-10-ΔRING lacks the RING domain required for binding to UBC9, while SU(VAR)2-10-W362A should bind to UBC9 but should not have ligase activity (Kotaja et al., 2000; Kotaja et al., 2002a). SU(VAR)2-10 did not enhance GAGA modification in any other conditions tried, such as providing UBC9 at suboptimal concentrations or incubating the reaction at lower temperature (30°C) or for shorter time (30 min instead of 1.5h or 2h). Thus, none of the SUMOylation assays revealed that SU(VAR)2-10 enhances SUMOylation of GAGA.

*In vivo* assay of *Su(var)2-10*

To further study the mode of action of *Su(var)2-10* we undertook an *in vivo* approach to resolve our lack of definitive genetic and biochemical data. I attempted to create transgenic flies that express GFP fusions of wild type Su(var)2-10, or mutant Su(var)2-10ΔRING, Su(var)2-10W362A and Su(var)2-10ΔSAP. Su(var)2-10ΔRING and Su(var)2-10W362A have mutations that abrogate the SUMO E3 ligase activity. Su(var)2-10ΔSAP has the SAP domain deleted in order to determine whether this domain is important for functioning of SU(VAR)2-10. These constructs were cloned into the pP[GS] vector system that has GFP and $ry^{+}$ cassettes driven by the hsp75 promoter. pGS-Su(var)2-10 was yielded two separate transgenic lines- one on the second chromosome (h2-$10^{5}$)

and one on the fourth chromosome (h2-$10^6$). For the other three mutant constructs more than 1000 eggs were injected, and about ten to twenty *$ry^+$* flies were obtained but none of them gave any progeny after being mated to the host *y cn ry* stock. Both *h2-$10^5$* and *h2-$10^6$* transgenic flies showed GFP expression when observed under GFP microscope but none of them were positive for GFP protein when assayed by Western blotting with anti-GFP antibody. Nonetheless, both *h2-$10^5$* and *h2-$10^6$* were assayed for rescuing the red eye phenotype caused by suppression of PEV from the *Su(var)2-$10^2$* mutation. To do so, we used *Su(var)2-$10^2$* in the *$w^{m4}$* background. *In(l)$w^{m4}$* is a rearrangement where the *white* locus is juxtaposed close to the heterochromatin as a result from an inversion and thus becomes silenced in some cells but not in all, which gives the salt and pepper eye phenotype. To assay the PEV, the eye pigment was extracted and quantified at 480nm as described in Materials and Methods. As shown in Table 5.1, *h2-$10^5$* and *h2-$10^6$* were not able to rescue *$yw^{m4h}$;Su(var)2-$10^2$* red eye phenotype, as flies heterozygous for the mutant allele and the transgene (*$yw^{m4h}$; 2-$10^2$/h2-$10^5$*) have the same O.D.480 as the mutant flies (*$yw^{m4h}$; 2-$10^2$/Sco*), 0.776 and 0.787 correspondingly. The *h2-$10^6$* flies used were kept as homozygous stock, as they carry the transgene on the fourth chromosome which is mostly heterochromatic and thus unfavorable for gene expression. Nevertheless we decided to assay this transgene as well and we observed that *$yw^{m4h}$; 2-$10^2$/h2-$10^6$* flies had O.D.480 of 1.077 which is similar to the O.D.480 of 1.089 of *$yw^{m4h}$; 2-$10^2$* control flies.

**Table 5.1 Lack of phenotypic rescue of *Su(var)2-10$^2$* mutation by *h2-10$^5$* and *h2-10$^6$* transgenics**

| Genotype of males assayed | n | O.D. 480 | Ratio |
|---|---|---|---|
| ***yw$^{m4h}$; 2-10$^2$/CyO x h2-10$^5$/Sco*** | | | |
| *yw$^{m4h}$; 2-10$^2$/h2-10$^5$* | 6 | 0.776 ± 0.088 | 2.14 |
| *yw$^{m4h}$; h2-10$^5$/CyO* | 4 | 0.476 ± 0.115 | 1.3 |
| *yw$^{m4h}$; 2-10$^2$/Sco* | 5 | 0.787 ± 0.112 | 2.17 |
| *yw$^{m4h}$; Sco/Cyo* | 3 | 0.362 ± 0.04 | |
| ***yw$^{m4h}$; 2-10$^2$/CyO* x *h2-10$^6$/h2-10$^6$*** | | | |
| *yw$^{m4h}$; 2-10$^2$/h2-10$^6$* | 7 | 1.077 ± 0.066 | 2.6 |
| *yw$^{m4h}$; h2-10$^6$/CyO* | 6 | 0.410 ± 0.065 | |
| ***yw$^{m4h}$; 2-10$^2$/CyO* x *ycnry*** | | | |
| *yw$^{m4h}$; 2-10$^2$/cn; +/ry* | 6 | 0.768 ± 0.076 | 1.47 |
| *yw$^{m4h}$; CyO/cn; +/ry* | 4 | 0.520 ± 0.015 | |
| ***yw$^{m4h}$; 2-10$^2$/CyO*** | | | |
| *yw$^{m4h}$; 2-10$^2$/CyO* ( heat shocked) | 5 | 1.089 ± 0.08 | 1.8 |
| *yw$^{m4h}$; 2-10$^2$/CyO* (nonheat shocked) | 3 | 0.600 ± 0.05 | |

1) Pigment assays were performed as described in Material and Methods.
2) The O.D. 480 values are means ± standard error on *n* number of assays (50 flies each) for each genotype.
3) In bold is shown the parental lines used to carry out the crosses. The Su(var) chromosome is paternally originated in all genotypes. PEV assay was performed only on the male progeny.
4) The first two cross are for *h2-10$^5$* and *h2-10$^6$* transgenes. The third cross is a control for how the host stock *ycnry* used for the embryo injection might affect the variegation assay. The fourth set is to detect how much does raising the temperature affects the control *yw$^{m4h}$;2-10$^2$* stock, as it is know the PEV is sensitive to the temperature.

## Discussion

The definition for a SUMO E3 ligase is that E3 binds to the E2 conjugating enzyme, it binds to a substrate and it enhances the conjugation of SUMO peptide to the substrate. The experiments described above showed that SU(VAR)2-10 binds to GAGA factor, which suggests the latter to be a potential target. I was not able to detect interaction between SU(VAR)2-10 and the E2 conjugase in the GST pull-down assay. This suggests that either SU(VAR)2-10 is not an E3 ligase or it is possible that SU(VAR)2-10 is a part of a multiprotein E3 complex similarly to some Ubiquitin E3 complexes, and it is the interacting partner of SU(VAR)2-10 that interacts with UBC9, while SU(VAR)2-10 binds only the substrate.

Using *in vitro* SUMOylation systems we did not detect any ligase activity of SU(VAR)2-10 towards GAGA for which there might be a few possible explanations. Either SU(VAR)2-10 does not have an E3 activity, or GAGA is not the right substrate for SU(VAR)2-10. As it was mentioned earlier it is possible that SU(VAR)2-10 is a part of a bigger multiprotein E3 ligase complex which other components are not present in the *in vitro* systems we have used. One might raise the question as to whether the *Drosophila* SU(VAR)2-10 is able to cooperate with the human UBC9 that we have used. The RING domain of SU(VAR)2-10, with which interaction with UBC9 is mediated, has 84 % sequence identity with the RING domains of the human PIAS proteins. In addition, it has been reported that SUMO modification of many substrates in mammalian systems can be stimulated by several different PIAS proteins, both *in vitro* and in cell cultures. For example, SUMOylation of p53 is enhanced by three different

PIAS proteins: PIAS1, PIAS3 and PIASy (Kahyo et al., 2001; Schmidt and Muller, 2002). In yeast, the two Siz/PIAS SUMO ligases, Siz1 and Siz2, enhance SUMOylation of several proteins, such as PCNA, Cdc3, Cdc11 and Shs/Sep7, (although each ligase has preferences toward a unique substrate *in vivo*) (Reindle et al., 2006). More importantly, Lehembre et al. have shown that *Drosophila* SUMO-1 can be processed and conjugated to its substrates in human cell lines and that human PML protein can be modified in *Drosophila* cells (Lehembre et al., 2000). Thus, the SUMO modification pathway is evolutionarily conserved and SU(VAR)2-10 should be able to work with the human proteins used in our *in vitro* assays.

It is interesting to speculate why adding increasing amounts of SU(VAR)2-10 leads to lessening the SUMOylation of GAGA. SU(VAR)2-10 has several SUMO acceptor sites and thus might be competing with GAGA for the SUMO peptide. This can be argued against, simply because SUMO-1 was added in excess at two time points-first in the beginning of the incubation and 45 min later. Another possible explanations is that SU(VAR)2-10 binds to GAGA in a way that makes its acceptor sites inaccessible. Although, the GST pull-down assays did not detect any interaction between SU(VAR)2-10 and UBC9, one might argue that SU(VAR)2-10 might compete with GAGA for binding to UBC9, which was added in suboptimal concentrations.

Our attempt to characterize *in vivo* the mode of action of SU(VAR)2-10 was not successful, because the wild type transgene failed to be expressed. No

transgenic flies carrying mutant versions of *Su(var)2-10* were obtained due to sterility of the transgenic clones.

To resolve some of the questions arising from the biochemical studies, one should identify the interacting partners of SU(VAR)2-10. Thus far, it has been reported that SU(VAR)2-10 physically interacts with STAT92E as a part of the JAK/STAT pathway involved in *Drosophila* eye formation and determination (Betz et al., 2001). John Lis's lab also reported that SU(VAR)2-10 binds to GAGA in a yeast-two hybrid screen, something that I have confirmed with in a GST pull-down experiment. Identifying proteins that bind SU(VAR)2-10 would reveal either potential substrates for ligase activity or that SU(VAR)2-10 is a part of a bigger SUMO E3 ligase complex.

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# CHAPTER 6

# SUMO MODIFICATION OF GAGA FACTOR AND ITS BIOLOGICAL SIGNIFICANCE

## Introduction

In our effort to study SU(VAR)2-10 we identified GAGA Factor (GAGA) as an interacting partner in a GST pull-down. Hypothesizing that SU(VAR)2-10 might be a SUMO E3 ligase, and GAGA its target for SUMOylation, we decided to study in detail whether GAGA is SUMOylated and the biological significance of this modification.

### GAGA is a protein with many functions

GAGA was initially identified as an *in vitro* transcriptional activator of the *Ultrabithorax(Ubx)* and *engrailed(en)* genes, and was later shown to bind GA-rich sequences found in the promoter regions of numerous *Drosophila* housekeeping and developmentally regulated genes (Adkins et al., 2006; Biggin et al., 1988; Gilmour et al., 1989; Granok et al., 1995; Soeller et al., 1993; Tsukiyama et al., 1994). Moreover, GAGA has also been reported to associate with specific heterochromatin regions throughout the cell cycle (Raff et al., 1994). It is

estimated that there are as many as 250 potential *in vivo* targets of GAGA in *Drosophila* (Adkins et al., 2006; Benyajati et al., 1997; Tsukiyama et al., 1994; van Steensel et al., 2003). GAGA binding sites have also been found in Polycomb response elements (PRE) and in Trithorax response elements (TRE) in the *Drosophila* homeotic genes (Farkas et al., 1994; Horard et al., 2000). Thus GAGA binds to multiple $GA_n$ repeats within the euchromatin and heterochromatin and has numerous roles, both as an activator and a repressor. Consistent with these observations, mutations in the GAGA-encoding gene, *Trithorax-like (Trl),* affect the expression of several developmental loci (Bhat et al., 1996; Farkas et al., 1994), and chromosome segregation and division (Bhat et al., 1996) and also behave as dominant enhancers of PEV (Farkas et al., 1994).

GAGA as an activator

Tsukiyama et al. have reported that GAGA is involved in nucleosome remodeling by cooperating with NURF in disrupting histone octamers deposited over GAGA sites on the proximal region of the *hsp70* promoter region (Tsukiyama et al., 1994; Tsukiyama and Wu, 1995) which coincides with DNAase I hypersensitive sites (Cartwright and Elgin, 1986; Lis and Wu, 1993; Shopland et al., 1995; Wu, 1980). GAGA facilitates RNA polymerase II activity by counteracting the effects of transcriptional repressors, such as linker histone H1, or by remodeling chromatin through a partnership with remodeling complexes, such as NURF or FACT (Espinas et al., 2000; Kerrigan et al., 1991; Okada and Hirose, 1998; Orphanides et al., 1998), and potentially via recruitment of HDAC

(Espinas et al., 2000; Salvaing et al., 2003). Thus, despite its interaction with the transcriptional machinery and other coactivators, it seems that GAGA functions more like an antirepressor rather than as a classical transcriptional activator (Adkins et al., 2006; Croston et al., 1991; Kerrigan et al., 1991).

GAGA as a repressor

GAGA has been found to colocalize with several PcG proteins at PREs (Cavalli and Paro, 1998; Strutt et al., 1997), to interact with PcG proteins at PREs (Horard et al., 2000), and to be required for silencing (Busturia et al., 2001; Hodgson et al., 2001; Horard et al., 2000; Mishra et al., 2003; Mishra et al., 2001). Mahmoudi et al. presented the biochemical evidence that GAGA efficiently binds to a chromatinized PRE and facilitates recruiting of the PcG repressor protein PHO (Mahmoudi et al., 2003). Although it does not stably associate and copurify with PRC1 components, GAGA has been shown to recruit PRC1 to nucleosomal templates and thus to enhance the activity of PRC1 complex to repress remodeling of a nucleosomal template (Mulholland et al., 2003).

Protein structure of GAGA

GAGA protein has a single zinc finger with an adjacent basic region (Benyajati et al., 1997; Soeller et al., 1993), which binds with high-affinity to the GAGA target sequences (Pedone et al., 1996). GAGA has a conserved protein-protein interaction motif, called BTB/POZ domain, at its N-terminus. It has been

proposed that via this domain GAGA interacts with other proteins (Bardwell and Treisman, 1994; Zollman et al., 1994) and with itself (Espinas et al., 1999; Katsani et al., 1999; Read et al., 2000). At the C-terminus there is a glutamine-rich domain (Q domain) which is the least understood. It has been reported to be involved in promoter distortion, single-strand binding, and multimerization (Wilkins and Lis, 1999), to act as a transactivation domain (Vaquero et al., 2000) or to have a role in binding site choice *in vivo* (Greenberg and Schedl, 2001).

GAGA isoforms

GAGA has two major isoforms, GAGA-519 and GAGA-581, which differ only in the length and sequence of their Q domain. Purified recombinant GAGA-519 and GAGA-581 proteins form homomeric complexes, which bind specifically to a single GAGA sequence *in vitro* (Benyajati et al., 1997). The two isoforms function similarly in transient transactivation assays in tissue culture cells and in chromatin remodeling experiments *in vitro.* Although they have overlapping activities, they are not identical; and thus they are not interchangeable. Only GAGA-519 accumulates during the first 6h of embryogenesis, as later in development both isoforms are present in nearly equal amounts. In nuclei of larval salivary glands they completely colocalize to specific euchromatic regions (Benyajati et al., 1997). According to Greenberg and Schedl, both isoforms localize to mitotic chromosomes, while only GAGA-519 localizes to centromeric heterochromatin in preblastoderm nuclei (Benyajati et al., 1997; Greenberg and

Schedl, 2001). They also report that the isoforms differ with regard to rescuing different *Trl* phenotypes. In our studies, we work with the GAGA-519 isoform.

In this chapter, we assayed that GAGA is SUMOylated and that it interacts with UBC9. I also show that SUMOylation of GAGA has biological significance for GAGA function in PEV. Hua Xin carried out the biochemical experiments, while I did the *in vivo* ones.

## Materials and methods

### Fly stocks

$Trl^{R85}$ is an imprecise excision of the 13C P-element inserted into the first intron of the gene, and because it removes an extensive region of the transcription unit is considered to be a null (Farkas et al., 1994).

### Plasmids and protein studies

For *in vitro* synthesizing of GAGA, the GAGA ORF encoding a 519 amino acid polypeptide was amplified from the GAGA cDNA cloned in pCK vector (Soeller et al., 1993) (kindly provided by T. Kornberg) with primers introducing Bgl II and then blunt ligated into EcoRV restriction site of pBluescript-SK vector (Stratagene). The primers were GAGA-FW:
ACGTAGATCTCGATGTCGCTGCCAATGAATTCG and GAGA-RV2:
ACGTAGATCTGTCGACTGGCTACTGCGGCTGCGGC.

The UBC9 ORF was amplified from the Brown cDNA library (Brown and Kafatos, 1988) and cloned into the XhoI site of pKS. $GAGA^{mSUMO}$ was designed

by using three pairs of primers introducing specific mutations in three SUMO acceptor sites and introducing BglII and EcoRI sites. GAGA constructs were cut with BglII and EcoRI and ligated into BamHI site of pSK+. All three proteins were synthesized in vitro in TNT coupled transcription/translation reticulocyte lysate (Promega) from the T7 promoter according to the recommended Promega protocol.

For yeast two hybrid assay, the wild type GAGA coding sequence in pBluescript $SK^{+}$ was cut out by Bgl II and was ligated into Bam HI digested pAS2 vector (yeast two hybrid vector containing Gal4 DNA-binding domain sequence driven by ADH promoter). The GAGA sequence was fused in frame to the downstream of Gal4 DNA-binding domain. pAS1 and pACT2 constructs were expressed in YI90 cells, and pGEX-5X-2 constructs were expressed in BL21(DE3) (Invitrogen) according to the manufacturer protocols.

Four GAGA coding region fragments including four amino acid mutations for potential SUMO acceptor sites were produced by PCR by using four pairs of primers and *Pfu* DNA polymerase. The first pair of primers is GAGA-FW described above and GAGA-1RVm: GTCTCTACCCTTTGTCCTTCGCTCCTGATGATCGGTG (where the two underlined nucleotides are substitutions from T to C, so that K268R and K273R can be introduced in the PCR product). This pair of primers generated the GAGA-1 fragment. The second pair of primers is GAGA-1FWm: CACCGATCATCAGGAGCGAAGGACAAAGGGTAGAGAC and GAGA-2RVm: CTTTTCTTCTCCTTCCTCACGCCGGGTTTGGC. This pair of primers generated

a GAGA partial coding region including K268R, K273R and K373R. It is called the GAGA-2 fragment. The third pair of primers is GAGA-2FWm: GCCAAACCCGGCGTGAGGAAGGAGAAGAAAAG and GAGA-3RVm: CCCTCATGTCTGATGTATGCCTGTCCACCACCC. This pair of primers generated a GAGA partial coding region including K373R and K474R. It is called the GAGA-3 fragment. The fourth pair of primers is GAGA-3FWm: GGGTGGTGGACAGGCATACATCAGACATGAGGG and GAGA-RV2 (same as the one used for cloning wild type GAGA). This pair of primers generated a GAGA partial coding region including K474R, it is called the GAGA-4 fragment. Then GAGA-1 and GAGA-2 DNA fragments were put together with PCR by using the primer pair of GAGA-FW and GAGA-2RVm to generate 5' piece of GAGA coding sequence including K268R, K273R and K373R. It is called GAGA-5'. GAGA-3 and GAGA-4 DNA fragments were put together via PCR by using the primer pair of GAGA-2FWm and GAGA-RV2 to generate 3' piece of GAGA coding sequence including K474R. It is called GAGA-3'. GAGA-5' and GAGA-3' DNA fragments were put together via PCR by using the primer pair of GAGA-FW and GAGA-RV2 to get full length GAGA coding sequence with four potential SUMO acceptor sites being mutated (K268R, K273R, K373R and K474R). The mutated GAF DNA fragment was digested by Bgl II restriction enzyme (Both GAGA-F and GAGA-R2 primers introduce BglII sites) and ligated into Bam HI cut pBluescript $SK^+$. The correct mutant GAF clones were picked by diagnostic restriction digestion and sequencing verification.

*Drosophila lwr* (UBC9) gene was amplified from an embryo cDNA library with primers lwr-FW: ACGTCTCGAGGAATTCTAATGTCCGGCATTGCTATTACACG that introduces two restriction sites for XhoI and EcoRI enzymes and lwr-RV: ACGTCTCGAGTTACTACTCAGTGGCCGCCATGGC, introducing an XhoI site. The PCR product was digested by XhoI and ligated into XhoI pGEX-5X-2 vector DNA. The *lwr* (UBC9) gene was fused in frame downstream of GST protein coding sequence in pGEX-5X-2. The correct clones were picked by diagnostic restriction digestion and sequencing verification. The PCR product of the lwr gene was also digested by EcoRI and XhoI and ligated into pACT2 vector DNA double digested by EcoRI and XhoI. The pACT2 vector contains Gal4 activation domain driven by the ADH promotor. The *lwr* gene was fused in frame downstream of Gal4 activation domain. The correct clones were picked by diagnostic restriction digestion and sequencing verification.

SUMOylation assay

SUMOylation assays were done with the SUMOylation kit from LAE Biotech and carried out according to their recommended protocol. 4μl *in vitro* translated $^{35}$S-GAGA or $^{35}$S-GAGA$^{mSUMO}$ were incubated for 30 min or 1h at 30°C.

Electrophoretic mobility shift assay

GAGA and GAGA$^{mSUMO}$ proteins were *in vitro* synthesized using TNT coupled transcription/translation RRL (Promega). The oligonucleotide probes used and the EMSA procedure were described previously (Busturia et al., 2001).

*In vivo* studies

For *in vivo* studies, GAGA and GAGA$^{mSUMO}$ were digested from pET-GAGA and pET-GAGA$^{mSUMO}$ templates, blunt-ligated into pSK+ and subsequently cloned into NotI sites of pP{GS[hsp70,EGFP, ry$^{+}$]} vector system, kindly provided by Gunter Reuter (Schotta and Reuter, 2000) also see http://www2.biologie.uni-halle.de/genet/drosophila/pGS/modules.html. Germline transformation was carried out according to Rubin and Spradling (Rubin and Spradling, 1982). Transgenic flies were generated using *y cn ry* as host and pUChsΔ2-3 as helper plasmid. Emerging flies were mated to the host *y cn ry* flies and the progeny screened for *ry*$^{+}$ transformants. Chromosome integration was determined genetically. I obtained one clone for wild type pGS-GAGA on the third chromosome (hG3), one mutant transgene (hmG1) was inserted into the third chromosome, and one (hmG7) on the second. Stocks were maintained either as homozygotes or balanced over second or third chromosome balancers. To induce transgene expression, flies were maintained at 25°C and heat shocked every day for one hour at 37°C until adult flies emerged. Expression of transgenes was confirmed by visually detecting GFP on GFP scope and by Western blotting using polyclonal antibodies against GFP (MBL, kindly provided

by Carl Thummel's laboratory) diluted 1:200 and visualized with ECL detection system.

Confocal microscopy

Salivary glands from third instar larvae were dissected in PSB and mounted in Vectashield mounting media to prevent rapid loss of fluorescence (Vector Laboratories Inc.). Confocal laser scanning microscopy was performed with an inverted Leica TCS NT microscope and image processing was done as described elsewhere (Buchenau et al., 1998; Dietzel et al., 1999).

PEV analysis and pigment extraction

To assay PEV, male transgenic flies were mated to females carrying $yw^{m4h}$*;Sco/Cyo* or $y^{wm4h}$*; Trl*$^{R85}$ */MKRS,Sb.* Parents were kept for three days in a vial and then removed and eggs were heat shocked for 1h at 37°C until adult flies emerge. Eye color of male offspring was assayed either immediately or they were let to age for few days. Pigment quantification was done according to Rabinow et al. (1991). Several samples of forty heads per genotype were collected, homogenized in one ml of methanol, acidified with 0.1% HCl (Ephrussi and Herold, 1944) and centrifuged. The absorbance of the supernatant was measured at 480nm.

## Results

### Wild type GAGA protein but not GAGA$^{mSUMO}$ is SUMOylated *in vitro*

We examined the sequence of GAGA-519 for the consensus motif common at SUMO targets, ψKXE (Rodriguez et al., 2001; Sampson et al., 2001), where ψ represents a large hydrophobic residue, using the online tool SUMOplot provided by Abgent available at http://www.abgent.com/doc/sumoplot. Although SUMOylation is not always confined to such sites, the data presented below show that the SUMOplot analysis provided a useful match in this case. The program identified five high probability motifs and two low probability motifs. The candidate sequences with the best match to the consensus were IKHE surrounding K474, IKSE surrounding K268, VKKE surrounding K373, AKHD surrounding K196, and AKHP surrounding K325 labeled with red in Figure 6.1A and 6.1B. The two low probability motifs were AKPG surrounding K369 and IKHE surrounding K474 (Figure 6.1). To mutate some of these SUMO acceptor sites, the Lysine residue in four of these consensus sites was substituted with Arginine, namely K268R, K273R, K373R and K474R and the protein was named GAGA$^{mSUMO}$. We mutated three motifs that have the highest score of probability to accept GAGA. The fifth mutated motif has a low score, but it was mutated because it is conveniently located to a high probability motif.

We first tested whether GAGA protein as a potential substrate for SUMO modification using an *in vitro* SUMOylation assay kit from LAE Biotech. In the experiment presented on Figure 6.2 we incubated a $^{35}$S-labeled form of GAGA or

A

1 MSLPMNSLYSLTWGDYGTSLVSAIQLLRCHGDLVDCTLAAGGRSFPAHKI
51 VLCAASPFLLDLLKNTPCKHPVVMLAGVNANDLEALLEFVYRGEVSVDHA
101 QLPSLLQAAQCLNIQGLAPQTVTKDDYTTHSIQLQHMIPQHHDQDQLIAT
151 IATAPQQTVHAQVVEDIHHQGQILQATTQTNAAGQQQTIVTTDA**AKHD**QA
201 VIQAFLPARKRKPRVKKMSPTAPKISKVEGMDTIMGTPTSHGSGSVQQV
251 LGENGAEGQLLSSTPI**IKSE**G**QKVE**TIVTMDPNNMIPVTSANAATGEITP
301 AQGATGSSGGNTSGVLSTPKAKR**AKHP**PGTEKPRSRSQSEQPATCPICY
351 VIRQSRNLRRHLELRHF**AKPGVKKE**KKSKSGNDTTLDSSMEMNTTAEGDN
401TVGSDGAGGAGSAGGQSSGTTPTRVISNAPQAAGAPAILAQGVLPQQQQQ
451 QQLQQQHQQHLTATLAGGGQAY**IKHE**GGGGGGTGQQQQQQAAQQQGM
504 QNV IHIVGDQVFI PQQQQPQPQ

B

| No. | Position | Group | Score | Lysine mutated in our assay to Arginine |
|---|---|---|---|---|
| 1 | K474 | GGQAY **IKHE** GGGGG | 0.94 | K474R |
| 2 | K268 | SSTPI **IKSE** GQKVE | 0.94 | K268R |
| 3 | K373 | FAKPG **VKKE** KKSKS | 0.93 | K373R |
| 4 | K196 | VTTDA **AKHD** QAVIQ | 0.79 | not changed |
| 5 | K325 | PKAKR **AKHP** PGTEK | 0.69 | not changed |
| 6 | K369 | ELRHF **AKPG** VKKEK | 0.62 | not changed |
| 7 | K273 | IKSEG **QKVE** TIVTM | 0.50 | K273R |

**Figure 6.1 Protein domains and SUMOplot prediction of GAGA.** A) Shown in bold are the SUMO acceptor consensus sites. B) Listed are the SUMO acceptor sites and their corresponding score of probability as estimated by SUMOplot™ and also are listed the four lysines that are substituted with arginine in the GAGA$^{mSUMO}$.

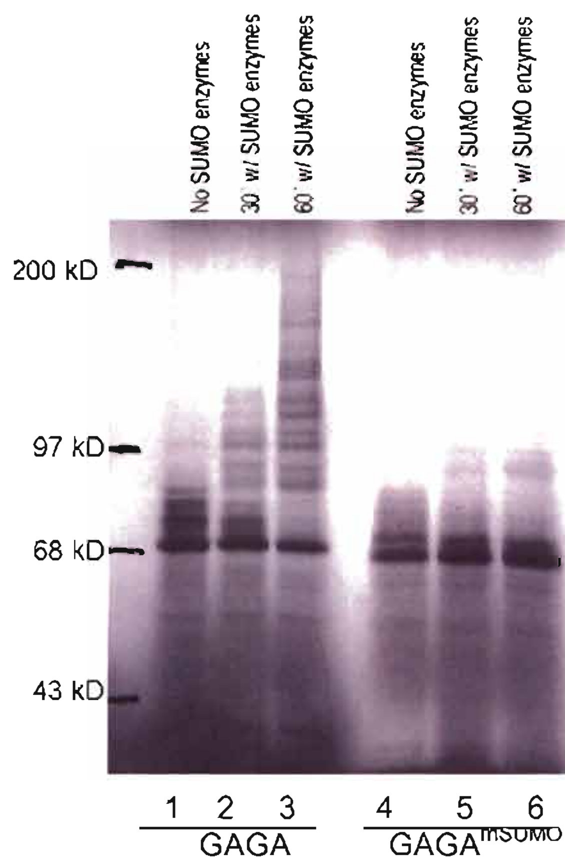


**Figure 6.2 GAGA but not GAGA$^{mSUMO}$ is SUMOylated.** When GAGA is subjected to an *in vitro* SUMOylation assay for either 30 min or 1h, high molecular weight bands appear indicative of modification (lanes 2 and 3). These high Mw bands are lacking in the control experiments where no SUMO modification enzymes are added (lanes 1 and 4) or when GAGA$^{mSUMO}$ is used (lanes 5 and 6).

GAGA$^{mSUMO}$ with the LAE Biotech SUMOylation kit. After 30 min or 60 min of incubation, we observed the appearance of high-molecular weight species with the wild type protein but not with the mutant one, suggesting that these are SUMOylated forms of GAGA. As not all of the SUMO acceptor sites are mutated, one could see some residual modification of GAGA$^{mSUMO}$.

GAGA binds to UBC9 in a yeast-two hybrid assay
and in a GST pull-down

As SUMO modified substrates have to bind the SUMO E2 conjugating enzyme in order for SUMO to be conjugated, we assayed whether GAGA binds to UBC9, by performing a yeast two hybrid assay and a GST pull-down. Using UBC9 as a bait and GAGA as an interacting protein we detected a strong interaction between the two proteins (Figure 6.3A). The interaction was confirmed when $^{35}$S-GAGA was pulled down only by recombinant GST-UBC9 pre-coupled to GST Sepharose (Figure 6.3B) and not by the GST control.

SUMOylation of GAGA does not change its binding activity
and GAGA$^{mSUMO}$ has the same DNA binding activity
as wild type GAGA

SUMOylation could negatively regulate transcription factor activity through altering its interactions with DNA, either stimulating or repressing binding to DNA (Goodson et al., 2001; Hong et al., 2001). To determine whether SUMOylation of

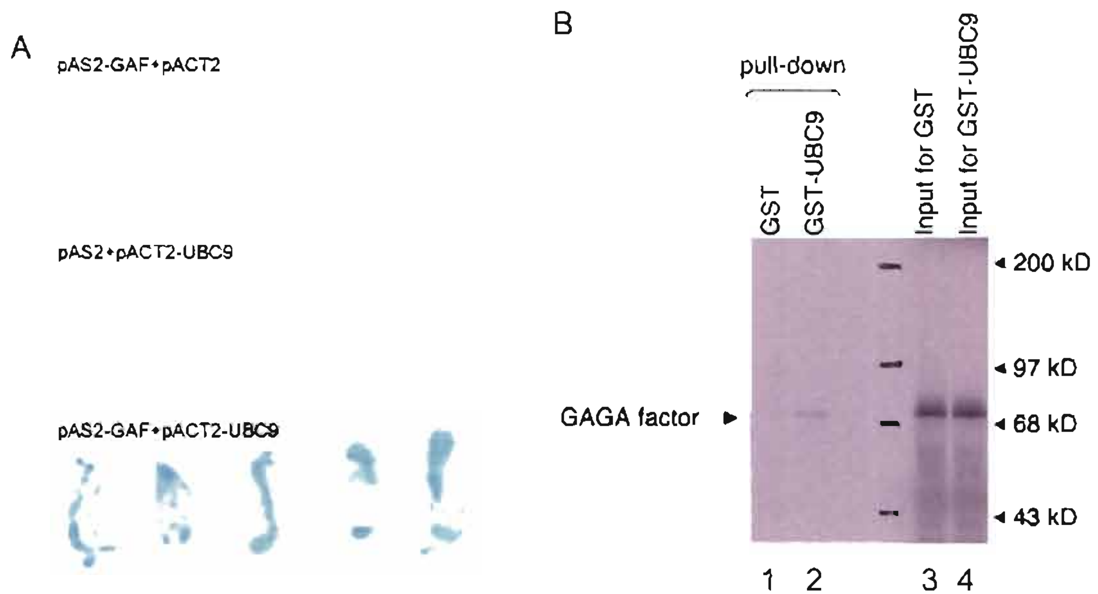


**Figure 6.3 Protein interaction studies between GAGA and UBC9.** A) Yeast two-hybrid assay between GAGA and UBC9. Top panel shows the control for GAGA not binding to the pACT2, and middle is a control for UBC9 not binding to the pAS2. Bottom shows that GAGA and UBC9 interact strongly. B) GST pull-down assay: GAGA does not bind to control GST protein (lane 1), GAGA binds to UBC9 (lane 2), input for GST (lane 3) and input for GST-UBC9 (lane 4). GAGA is $^{35}$S-labeled and *in vitro* translated.

GAGA changes its binding to DNA, oligonucleotides representing GAGA binding sites were tested in electrophoretic mobility shift assays (EMSA) using in vitro synthesized GAGA proteins, either SUMOylated or not. As shown on Figure 6.4A, there is no change in the DNA binding specificity when GAGA is SUMOylated using two different oligonucleotides probes – $(GAGAG)_8$ or MCP 548/558 (Busturia et al., 2001) which differ in the number of GAGA binding sequences. MCP 548/558 has two potential GAGA binding sites, GAGAG and GAGA at residues 548 and 558 (Busturia et al., 2001). Mutating the SUMO acceptor sites in $GAGA^{mSUMO}$ protein does not change binding to DNA either, as shown on Figure 6.4B. Wild type and mutant GAGA proteins give the same profile of shifted bands for both oligonucleotide probes used. The shifted bands are competed by excess unlabelled competitors with the same sequence as the labeled oligos. The presence of three or four shifted bands might correspond to protein-DNA complexes formed by different length GAGA polypeptides synthesized in the *in vitro* translation system.

Assaying the biological significance of GAGA SUMOylation i*n vivo*

To assay the biological significance of GAGA SUMOylation *in vivo*, I constructed transgenic lines expressing an EGFP-tagged version of either wild type GAGA or $GAGA^{mSUMO}$ using the pP{GS[$ry^+$, hsp70EGFP]} vector modular system (Schotta and Reuter, 2000). I obtained one transgenic line for GAGA, (hG3, inserted on the third chromosome), and two lines for the mutant

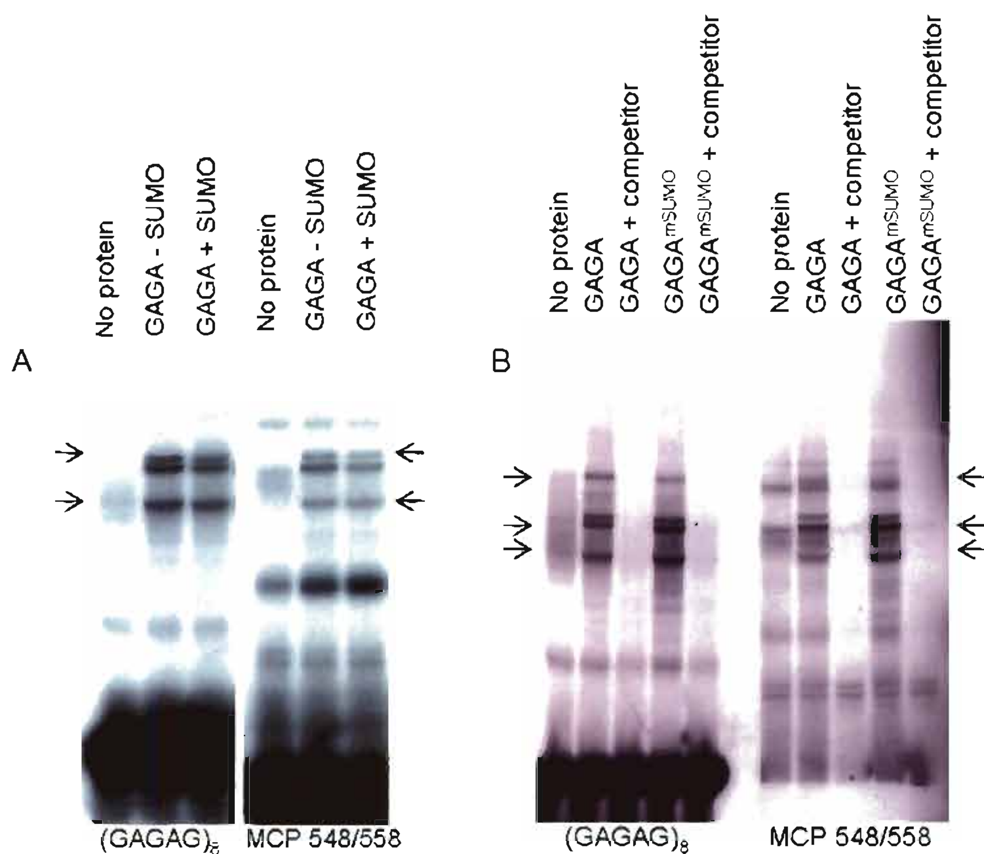


**Figure 6.4 Binding of *in vitro* synthesized GAGA to $GA_n$ oligonucleotides.** A)DNA binding assay using SUMOylated or non-SUMOylated GAGA protein and two different oligonucleotides probes: $(GAGAG)_8$ oligonucleotide and MCP 548/558 which differ in the number of GAGA binding sites. When $(GAGAG)_8$ is used three shifted bands are present both in the SUMOylated and un-SUMOylated GAGA proteins (shown with arrows, as the upper arrow points to a doublet of bands). These shifted bands are missing in the control reaction where no protein has been added. When MCP probe is used, both the modified and un-modified GAGA proteins give the same three shifted bands.
B) DNA binding assay of GAGA and $GAGA^{mSUMO}$. Mutating the SUMO acceptor sites in GAGA does not change its binding specificity to DNA. Four shifted bands (arrows) are present in lanes 3 and 4, but are competed by excess cold competitor that has the same sequence as the labeled oligo (middle arrows points to a doublet).

GAGA$^{mSUMO}$ (hmG1 on the third and hmG7 on the second chromosome). Western analysis of crude nuclear extracts from homozygous transgenic lines revealed that all three proteins are expressed in approximately equal amount (Figure 6.5A).

SUMO modification has been shown to regulate subcelullar localization of many proteins including RanGAP, NEMO, PML, CBP, Sp100 (Huang et al., 2003; Matunis et al., 1996; Stade et al., 2002), or their subnuclear localization (Johnson, 2004; Sachdev et al., 2001; Schmidt and Muller, 2003). GAGA protein has been reported to have very specific chromatin localization. In mitotic chromosomes, it binds to more than 100 euchromatic sites but it is excluded from the heterochromatin (O'Brien et al., 1995; Platero et al., 1998). To assess whether SUMOylation of GAGA is essential for its subcellullar or chromatin localization, we examined the GAGA protein localization in the transgenic lines expressing wild type (*hG3*) or mutant (*hmG1,hmG7*) GAGA proteins (Figure 6.5B). Salivary glands were dissected from third instar larvae and the unfixed cells were analyzed by confocal imaging. We detected no difference between the localization of wild type and mutant forms: they were both nuclear (Figure 6.5B, upper panel), and more specifically they were localized within euchromatin but excluded from the heterochromatic chromocenter (Figure 6.5B).

Mutations in the GAGA encoding gene, *Trl*, are dominant enhancers of the PEV (Farkas et al., 1994). The PEV marker that we work with, $w^{m4h}$, results when the active euchromatic *white* gene is juxtaposed to a heterochromatic

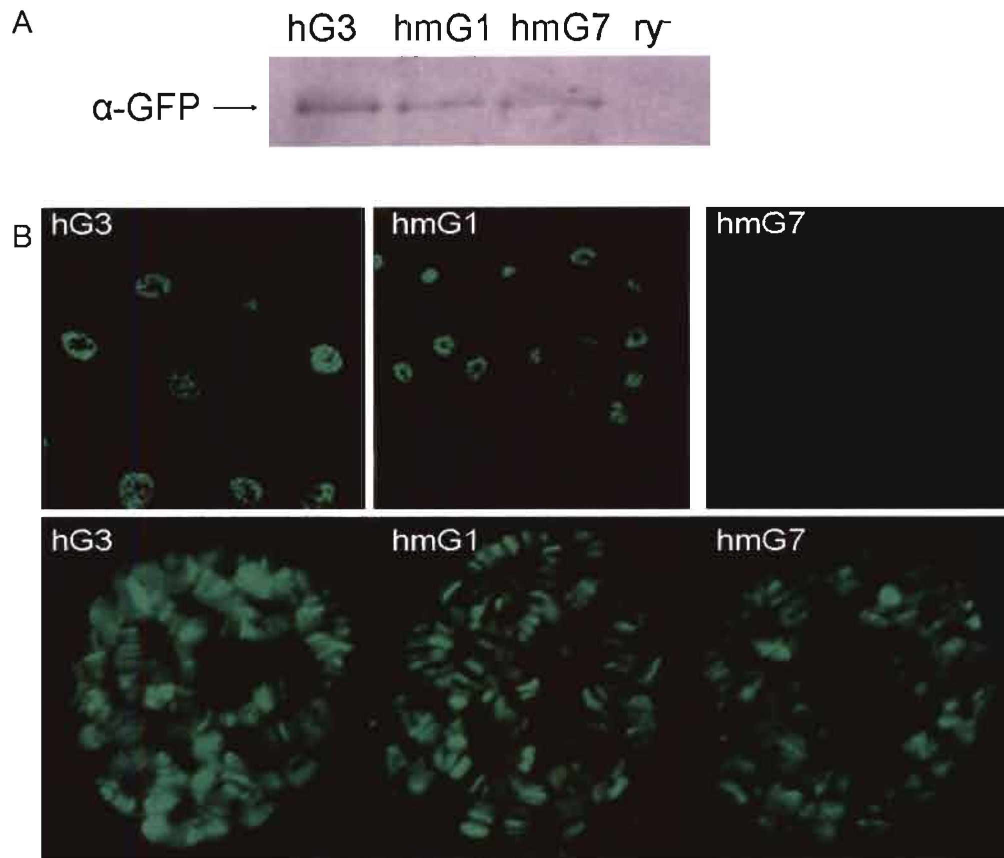


**Figure 6.5 Biological significance of GAGA SUMOylation *in vivo*.** A) Western blot detection of expressed proteins from crude nuclear extracts from homozygous *hG3, hmG1* and *hmG3* transgenic lines. B) Unfixed polytene nuclei from third instar larvae salivary glands of hG3-EGFP, hmG1-EGFP and hmG7-EGFP from homozygous transgenic lines. The upper panel shows that the proteins are localized within the nucleus and not in the cytoplasm. The bottom panel shows magnification image of polytene chromosomes.

segment and thus becomes silenced in some cells (white pigmented cells) (Figure 6.6), but active in others (red pigmented cells), thus giving a mottled eye color (Figure 6.6). Suppressors of PEV are thought to be involved in chromatin condensation, while enhancers of PEV, such as *Trl*, encode chromatin components involved in chromatin decondensation (Reuter and Spierer, 1992). *Trl* mutations have a dosage dependent effect on PEV- it is a haplo-enhancer/ triple-suppressor. That is, one copy enhances PEV giving rise to white eye phenotype, while three copies suppress PEV resulting in more cells with red pigment. To assess whether SUMOylation of GAGA is important for its function in PEV, I assayed the wild type- and mutant transgenes for their effect on PEV of the $w^{m4h}$ marker by crossing transgenic flies to $yw^{m4h}$ line. We assessed these effects either phenotypically (Figure 6.5B) or by extracting eye pigment and measuring its optical density at 480 (Table 6.1). Additional copies of the wild type GAGA encoding gene, hG3 (*hG3/+)* caused suppression of the *white* variegation in $w^{m4h}$ (Figure 6.6) and thus these red eye yielded a higher O.D.480 (Table 6.1). The mutant copies (*hmG1* and *hmG7*) failed to act as triple-suppressors of PEV (Figure 6.5C and Table 6.1), suggesting that SUMOylation of GAGA is needed for its function in PEV.

Next we assayed the transgenic lines for rescuing the *E(var)* phenotype caused by the $Trl^{R85}$ mutation, which causes very white eyes (Figure 6.6). We crossed the transgenics to the $yw^{m4h};Trl^{R85}/+$ line and assayed the eyes (Figure 6.6 and Table 6.1). Flies with $yw^{m4h};hG3/\ Trl^{R85}$ genotype showed redder eye phenotype as compared to the white eye color of $yw^{m4h};Trl^{R85}/+$ and they had a

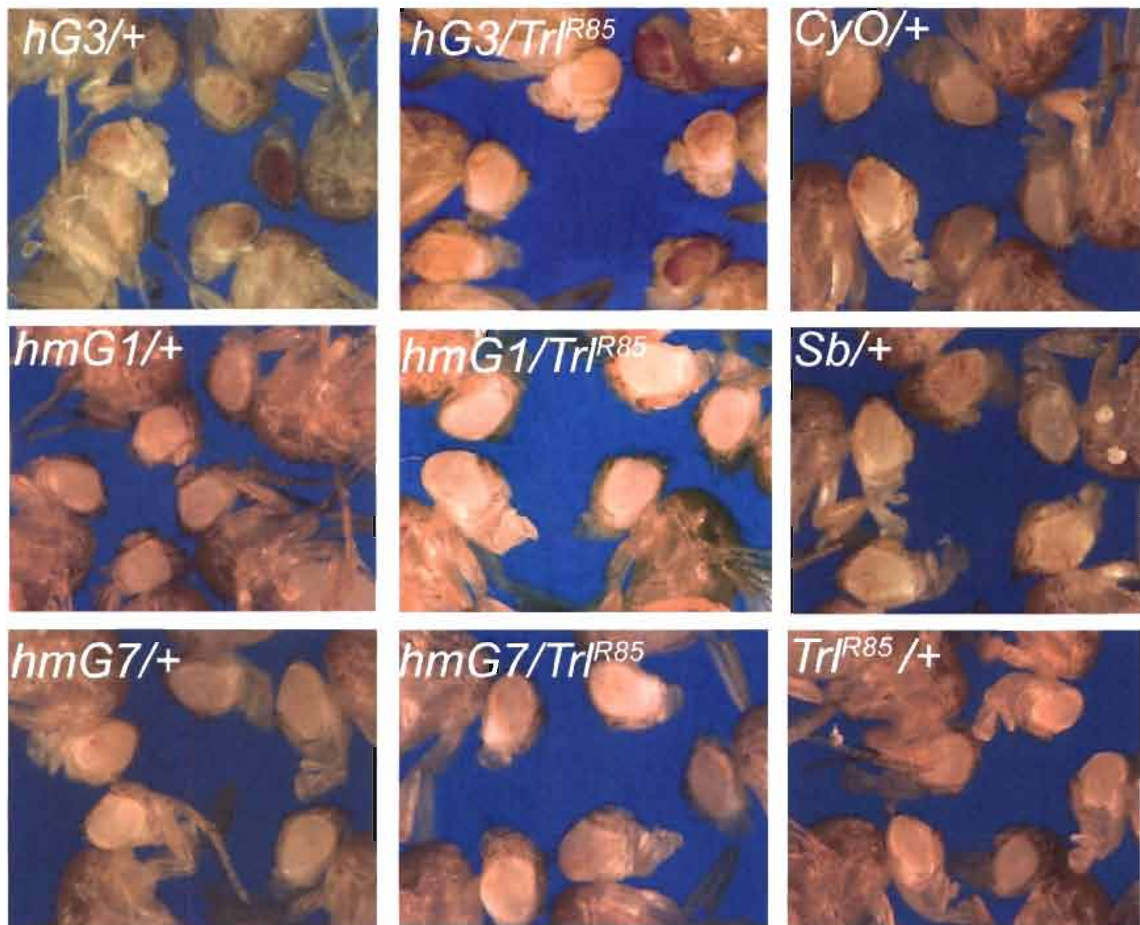


**Figure 6.6 PEV analysis of the GAGA transgenic lines.** *hG3/+* flies have more cells producing red pigment as compared to *Trl*$^{R85}$/+ and *hmG1/+* or *hmG7/+* flies, which indicates the triple-dosage effect of *hG3* and lack of such for the mutant transgenic lines. The middle column of photos shows the rescue experiment- *hG3/Trl*$^{R85}$ flies have more cells with red pigment as compared to *Trl*$^{R85}$/+, while the mutant transgenes fail to rescue the *Trl*$^{R85}$/+ phenotype.

**Table 6.1 Pigment assays of *GAGA* transgenes**

| Cross # | Genotype | n | O.D. 480 | Ratio |
|---|---|---|---|---|
| 1 | *yw^m4h^; +/+ x hG3/Sb* | | | |
| | *yw^m4h^; hGAGA^3^/+* | 5 | 0.0438 ± 0.009 | 3.37 |
| | *yw^m4h^; Sb/+* | 5 | 0.013 ± 0.002 | |
| 2 | *yw^m4h^; +/+ x hmG1/Sb* | | | |
| | *yw^m4h^; hmGAGA^1^/+* | 3 | 0.03 ± 0.007 | 1.5 |
| | *yw^m4h^; Sb/+* | 4 | 0.017 ± 0.006 | |
| 3 | *yw^m4h^; +/+ x hmG7/CyO* | | | |
| | *yw^m4h^; hmGAGA^7^/+* | 3 | 0.02 ± 0.009 | 1 |
| | *yw^m4h^; CyO/+* | 4 | 0.02 ± 0.009 | |
| 4 | *yw^m4h^; Trl^R85^/Sb x hG3/Sb* | | | |
| | *yw^m4h^; hGAGA^3^/Trl^R85^* | 5 | 0.06 ± 0.003 | 1.3 |
| | *yw^m4h^; hGAGA^3^/+* | 2 | 0.154 ± 0.05 | 3.4 |
| | *yw^m4h^; Trl^R85^/+* | 2 | 0.045 ± 0.0055 | |
| 5 | *yw^m4h^; Trl^R85^/Sb x hmG1/Sb* | | | |
| | *yw^m4h^; hmGAGA^1^/Trl^R85^* | 4 | 0.048 ± 0.004 | 1 |
| | *yw^m4h^; hmGAGA^1^/+* | 2 | 0.064 ± 0.006 | 1.3 |
| | *yw^m4h^; Trl^R85^/+* | 2 | 0.05 ± 0.01 | |
| 6 | *yw^m4h^; Trl^R85^/Sb x hmG7/CyO* | | | |
| | *yw^m4h^; hmGAGA^7^/Trl^R85^* | 5 | 0.01 ± 0.002 | |
| | *yw^m4h^; hmGAGA^7^/+* | 5 | 0.025 ± 0.006 | |

Pigment assays were performed on 50 male flies as described in Materials and Methods. *n* shows the number of assays for each genotype. The O.D.480 values are means ± standard error of the mean on *n* number of assays for each genotype.

correspondingly higher O.D.480, suggesting that the wild type *hG3* transgene was able to rescue the *Trl*$^{R85}$/+ phenotype . Such rescue was not seen in *yw*$^{m4h}$*;hmG1/Trl*$^{R85}$ and *yw*$^{m4h}$*;hmG7/Trl*$^{R85}$ genotypes, as they had the same white eye color as *yw*$^{m4h}$*; Trl*$^{R85}$/+ flies, again pointing to a role for SUMOylation of GAGA for proper PEV function.

## Discussion

Using biochemical approaches, we have shown for the first time that GAGA is SUMO-modified *in vitro* and it interacts strongly with the SUMO conjugating enzyme UBC9 in yeast two-hybrid and GST pull-down assays. We observed that SUMOylation of GAGA does not change its intrinsic DNA-binding activity as is the case with several other transcription factors, namely Ttk69, LEF1, and Sp3 (Lehembre et al., 2000; Sachdev et al., 2001; Sapetschnig et al., 2002).

We have also shown that SUMO modification of GAGA is important for its *in vivo* function in PEV of the *white* gene. The wild type GAGA transgene was able to rescue the silencing of the *white* gene caused by *Trl*$^{R85}$, while the transgenes with mutated SUMO acceptor sites failed to do so.

Interestingly, the major SUMO acceptor sites within GAGA lie outside the defined domains critical for its function, the BTB/POZ, Zn finger and the poly-Q domain (Figure 6.1A). Nevertheless, it will be essential to determine whether SUMOylation of GAGA affects its protein interactions, for example with Pleiohomeotic (*Pho*) and Pipsqueak (*Psq*), both of which are implicated in PcG-

mediated silencing (Faucheux et al., 2003; Schwendemann and Lehmann, 2002). GAGA has been shown to facilitate binding of PHO to chromatinized PREs which enables homeotic gene silencing (Mahmoudi et al., 2003). GAGA also interacts with Tramtrack (*Ttk*) (Pagans et al., 2002), Batman (*Ban*) (Faucheux et al., 2003), Corto (Salvaing et al., 2003), SAP18 (Espinas et al., 2000), NURF301 (Badenhorst et al., 2002; Xiao et al., 2001) and FACT (Orphanides et al., 1998; Shimojima et al., 2003) . Corto and SAP18 are functionally linked with histone deacetylation, and NURF301 and FACT are linked with chromatin remodeling; both processes relate to GAGA's role as an activator of transcription.

In summary we showed that GAGA is SUMOylated and this modification is important for the function of GAGA in PEV. It has to be yet determined whether SUMOylation of GAGA is required its function in homeotic gene silencing.

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# CHAPTER 7

## SUMMARY AND PERSPECTIVES

Homeotic genes specify segment identity in *Drosophila*. Their pattern of expression is set early in the embryo by the transient expression of the segmentation genes. Later in development, when the products of the segmentation genes are no longer present, the Polycomb Group of genes (PcG) silences the homeotic genes within the initially specified segments.

In an effort to determine how *PcG* genes execute silencing of homeotic genes, I carried out a screen for genetic interaction between the $Pc^3$ mutation and a series of mutations that suppress the heterochromatic PEV. In this dissertation, I have shown that a series of PEV modifiers enhance $Pc^3$-induced homeotic transformations, some to a greater extent than others, and the effect was tissue specific. For example, $Su(var)2\text{-}5^5$ induced strong wing-to-haltere and T2-to-T1 transformations, while $Su(var)3\text{-}9^{1,2}$ induced strong wing-to-haltere, T2-to-T1, and A4-to-A5 transformations (Table 2.5). These results are not surprising as it had been shown that the composition of PcG complexes and the silencing mechanisms utilized by them vary in different tissues. Thus, silencing of the *Cbx* gene in wings might require interaction of *Pc* with both *Su(var)2-5* and *Su(var)3-*

*9,* while silencing of Abd-B in the A4 segment requires only *Pc* and *Su(var)3-9.* Also, when assayed for interaction with $Ph^{d}$ and $Scm^{D1}$, $Su(var)2\text{-}10^{03697}$ enhanced only the T3-to-T1 leg transformation.

The molecular identity and function of only few Su(var)s tested here are known (Table 2.1). SU(VAR)2-1 is a HDAC, SU(VAR)3-1 is a histone kinase, SU(VAR)3-9 is a histone methyltransferase and SU(VAR)2-5 is a heterochromatin binding protein (HP1). SU(VAR)3-9 creates a methylation mark on Histone H3 that is recognized and bound by SU(VAR)2-5, which results in recruiting of more heterochromatin proteins and establishing of a silent heterochromatin environment. Our results suggest that mutations in genes from PEV contribute to homeotic gene derepression, probably due to an indirect interaction. It is possible that mutations in some of the *Su(var)* genes lead to derepression of certain downstream genes, which are involved in PcG silencing.

In the beginning our attention was focused on $Su(var)3\text{-}10^{2}$, one of the modifiers that enhanced $Pc^{3}$ phenotype to the greatest extent. *Su(var)3-10*'s has not been cloned, but its cytological location is known and thus we were able to test a few deficiencies that have been shown to map to the same region. Complementation tests revealed that $Su(var)3\text{-}10^{2}$ is sublethal over the tested deficiencies (Table 2.6). Genetic interaction tests between the deficiencies and $Pc^{3}$ showed enhanced $Pc^{3}$-homeotic transformations, which confirmed that the homeotic gene silencing activity also maps within the same region (Table 2.7). Tests for PEV modifications of the $w^{m4h}$ marker gene, revealed that the PEV

suppressing activity of *Su(var)3-10*$^{2}$ also maps within the same region (Figure 2.4).

*Su(var)2-10* is one of the genes that greatly enhanced the *Pc*$^{3}$-induced homeotic transformations in the preliminary genetic screen. *Su(var)2-10* alleles enhanced both *Pc*$^{3}$- and *Pc*$^{4}$- induced adult homeotic phenotypes very strongly, and they also interacted with two other PcG members, *Ph*$^{d}$ and *Scm*$^{D1}$, resulting in adult homeotic transformations. That these interactions are due to mutations in the *Su(var)2-10* alleles and not the genetic background was confirmed when we used a revertant allele, *Su(var)2-10*$^{PEX2C}$, which did not enhance any PcG-induced phenotypes. Thus, all our genetic interaction data presented in Chapter 2, suggest not only that *Su(var)2-10* does contribute to the PcG-mediated silencing but that it also is a novel PcG member. I have shown that mutations in *Su(var)2-10* enhance the *Pc*$^{3}$-induced derepression of an endogenous homeotic gene, *Ubx* (Figure 3.2), which also confirms that the genetic interaction with *Pc*$^{3}$ seen in adults is indeed due to *Su(var)2-10*'s contribution to homeotic gene silencing. I have also demonstrated that *Su(var)2-10* acts on homeotic gene silencing via a PRE-dependent mechanism, which is suggested from the fact that *Su(var)2-10* derepresses PRE-dependent reporter genes on its own (Figure 3.3A, B).

SU(VAR)2-10 shares a high degree of sequence identity to the mammalian family of Protein Inhibitor of Activated STATs (PIAS) proteins (Hari et al., 2001). In mammalian systems, PIAS proteins have been reported to function as SUMO E3 ligases in a protein modification pathway, called SUMO pathway.

Using genetic approaches we were unable to detect that SUMO pathway contributes to PcG-mediated silencing of homeotic genes. Mutations in *lwr* did not enhance $Pc^3$-induced homeotic transformations. We saw weak genetic interaction between *smt3* mutation and $Pc^3$; the penetrance of homeotic transformation was indeed enhanced in double heterozygotes for *smt3* and $Pc^3$, but the expressivity of the observed transformations was relatively weak. We did not see derepression of PRE-dependent transgenes by both *smt3* and *lwr*. Double recombinants for either *lwr* or *smt3* with *Su(var)2-10* did not enhance $Pc^3$-induced homeotic transformations more than *Su(var)2-10* does alone (Table 4.5) but they enhanced the $ph^D$-induced A4-to A5 transformation. We were not able to detect derepression of homeotic genes in embryos homozygous for $lwr^{05486}$ $Su(var)2\text{-}10^{03697}$ and $smt3^{04493}Su(var)2\text{-}10^{03697}$ (Figure 4.2). Thus, we do not have definitive data that the SUMOylation pathway contributes to PcG silencing and that *Su(var)2-10* interacts with SUMOylation pathway mutations to compromise homeotic gene silencing. It is also possible that our genetic approaches are not sensitive enough to detect such contributions. Another possibility might be the existence of redundant genes coding for either SUMO-1 or UBC9. In *Drosophila* there is only one gene reported to encode SUMO-1 and one gene for UBC-9 while in mammalian systems more than one SUMO peptide is reported (Hoege et al., 2002; Matunis et al., 1996).

The definition for a SUMO E3 ligase is that E3 binds to the E2 conjugating enzyme, it binds to a substrate and it enhances the conjugation of SUMO peptide to the substrate. The experiments described above showed that SU(VAR)2-10

binds to GAGA factor, which suggest the latter to be a potential target. I was not able to detect interaction between SU(VAR)2-10 and the E2 conjugase in the GST pull-down assay. This suggests that either SU(VAR)2-10 is not an E3 ligase or it is possible that SU(VAR)2-10 is a part of a multiprotein E3 complex similar to some Ubiquitin E3 complexes, and it is the interacting partner of SU(VAR)2-10 that interacts with UBC9, while SU(VAR)2-10 binds only the substrate.

Using *in vitro* SUMOylation systems we did not detect any ligase activity of SU(VAR)2-10 towards GAGA. There might be a few possible explanations for this. Either SU(VAR)2-10 does not have an E3 activity, or GAGA is not the right substrate for SU(VAR)2-10. As it was mentioned earlier it is possible that SU(VAR)2-10 is a part of a bigger multiprotein E3 ligase complex ant the other components are not present in the *in vitro* systems we have used. One might raise the question as to whether the *Drosophila* SU(VAR)2-10 is able to cooperate with the human UBC9 that we have used. The RING domain of SU(VAR)2-10 that mediates the interaction with UBC9 is mediated has 84 % sequence identity with the RING domains of the human PIAS proteins. In addition, it has been reported that SUMO modification of many substrates in mammalian systems can be stimulated by several different PIASes, both *in vitro* and in cell cultures. For example, SUMOylation of p53 is enhanced by three different PIASEs: PIAS1, PIAS3 and PIASy (Kahyo et al., 2001; Schmidt and Muller, 2002). In yeast, the two Siz/PIAS SUMO ligases, Siz1 and Siz2, enhance SUMOylation of several proteins, such as PCNA, Cdc3, Cdc11 and Shs/Sep7, (although each ligase has preferences toward a unique substrate *in vivo*)

(Reindle et al., 2006). More importantly, Lehembre et al. have shown that *Drosophila* SUMO-1 can be processed and conjugated to its substrates in human cell lines and that human PML protein can be modified in *Drosophila* cells (Lehembre et al., 2000). Thus, the SUMO modification pathway is evolutionarily conserved and SU(VAR)2-10 should be able to work with the human proteins used in our *in vitro* assays.

It is interesting to speculate why adding increasing amount of SU(VAR)2-10 leads to lessening the SUMOylation of GAGA. SU(VAR)2-10 has several SUMO acceptor sites and thus might be competing with GAGA for the SUMO peptide. This can be argued against, simply because SUMO-1 was added in excess at two time points-first in the beginning of the incubation and 45 min later. Another possible explanation is that SU(VAR)2-10 binds to GAGA in a way that makes its acceptor sites inaccessible. Although, the GST pull-down assays did not detect any interaction between SU(VAR)2-10 and UBC9, one might argue that SU(VAR)2-10 might compete with GAGA for binding to UBC9, which was added in suboptimal concentrations.

Our attempt to characterize *in vivo* the mode of action of SU(VAR)2-10 was not successful, because the wild type transgene failed to be expressed. Unfortunately, no transgenic flies carrying mutant versions of *Su(var)2-10* we obtained due to sterility of the transgenic clones.

To resolve some of the questions arising from the biochemical studies, one should identify the interacting partners of SU(VAR)2-10. Thus far, it has been reported that SU(VAR)2-10 physically interacts with STAT92E as a part of

the JAK/STAT pathway involved in *Drosophila* eye formation and determination (Betz et al., 2001). John Lis's lab reported that SU(VAR)2-10 binds to GAGA in yeast-two hybrid screen, something that I have confirmed with in a GST pull-down experiment. Identifying proteins that bind SU(VAR)2-10 would reveal either potential substrates for ligase activity or that SU(VAR)2-10 is a part of a bigger SUMO E3 ligase complex.

We propose two possible models how *Su(var)2-10* contributes to homeotic gene silencing. First, SU(VAR)2-10 may contribute to SUMOylation of GAGA or other PcG proteins. Second, it has been shown that SU(VAR)2-10 localizes to silencing compartments such as the nuclear lamina and nuclear bodies (Hari et al., 2001). Thus, the other model is that SU(VAR)2-10 may recruit PcG proteins and their homeotic targets to silencing compartments such as nuclear lamina or nuclear bodies.

Using biochemical approaches, we have showed for the first time that GAGA is SUMO modified *in vitro* and it interacts strongly with the SUMO conjugating enzyme UBC9 in yeast two-hybrid and GST pull-down assays. We observed that SUMOylation of GAGA does not change its binding to DNA, as has been seen with several other transcription factors, namely Ttk69, LEF1, and Sp3 (Lehembre et al., 2000; Sachdev et al., 2001; Sapetschnig et al., 2002).

We have also shown that SUMO modification of GAGA is important for its *in vivo* function in PEV of the *white* gene. A wild type GAGA transgene was able to rescue the silencing of *white* gene caused by $Trl^{R85}$, while the transgenes with mutated SUMO acceptor sites failed to do so. These results suggest that

SUMOylation of GAGA is important for its function in PEV. It is yet to be determined whether SUMOylation of GAGA is required for homeotic gene silencing. In addition, it is essential to study whether SUMOylation of GAGA affects its protein interactions, for example with Pleiohomeotic (*Pho*) and Pipsqueak (*Psq*), both of which are implicated in PcG-mediated silencing (Faucheux et al., 2003; Schwendemann and Lehmann, 2002).

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