Suppressor of zeste 12, a Polycomb group gene in *Drosophila melanogaster;* one piece in the epigenetic puzzle

Anna Birve

Department of Molecular Biology
Umeå University, Sweden
2003

Akademisk avhandling

som med tillstånd av rektorsämbetet vid Umeå Universitet för erhållande av Filosofie doktorsexamen vid Matematisk-Naturvetenskapliga fakulteten, framlägges till offentlig granskning i Major Groove (Molekylärbiologi, byggnad 6L) fredagen den 30 maj, klockan 10.00.

Examinator:	Prof. Anssi Saura
Fakultetsopponent:	Prof. Allen Shearn, Johns Hopkins University, Baltimore, USA

ABSTRACT

***Suppressor of zeste 12*, a Polycomb group gene in *Drosophila melanogaster;* one piece in the epigenetic puzzle**

Anna Birve, Department of Molecular Biology, Umeå University, Sweden

In multicellular organisms all cells in one individual have an identical genotype, and yet their bodies consist of many and very different tissues and thus many different cell types. Somehow there must be a difference in how genes are interpreted. So, there must be signals that tell the genes when and where to be active and inactive, respectively. In some instances a specific an expression pattern (active or inactive) is epigenetic; it is established and maintained throughout multiple rounds of cell divisions. In the developing *Drosophila* embryo, the proper expression pattern of e.g. the homeotic genes *Abd-B* and *Ubx* is to be kept active in the posterior part and silenced in the anterior. Properly silenced homeotic genes are crucial for the correct segmentation pattern of the fly and the Polycomb group (Pc-G) proteins are vital for maintaining this type of stable repression.

As part of this thesis, *Suppressor of zeste 12* (*Su(z)12*) is characterized as a *Drosophila* Pc-G gene. Mutations in the gene cause widespread misexpression of several homeotic genes in embryos and larvae. Results show that the silencing of the homeotic genes *Abd-B* and *Ubx,* probably is mediated via physical binding of SU(Z)12 to Polycomb Response Elements in the BX-C. *Su(z)12* mutations are strong suppressors of position-effect-variegation and the SU(Z)12 protein binds weakly to the heterochromatic centromeric region. These results indicate that SU(Z)12 has a function in heterochromatin-mediated repression, which is an unusual feature for a Pc-G protein. The structure of the *Su(z)12* gene was determined and the deduced protein contains a C2-H2 zinc finger domain, several nuclear localization signals, and a region, the VEFS box, with high homology to mammalian and plant homologues. *Su(z)12* was originally isolated in a screen for modifiers of the *zeste-white* interaction and I present results that suggests that this effect is mediated through an interaction between *Su(z)12* and *zeste.* I also show that *Su(z)12* interact genetically with other Pc-G mutants and that the SU(Z)12 protein binds more than 100 euchromatic bands on polytene chromosomes. I also present results showing that SU(Z)12 is a subunit of two different E(Z)/ESC embryonic silencing complexes, one 1MDa and one 600 kDa complex, where the larger complex also contains PCL and RPD3.

In conclusion, results presented in this thesis show that the recently identified Pc-G gene, *Su(z)12*, is of vital importance for correct maintenance of silencing of the developmentally important homeotic genes.

Keywords: *Drosophila melanogaster*, epigenetic, homeotic genes, Polycomb group, PRE, heterochromatin, *Suppressor of zeste 12,* chromatin silencing

ISBN 91-7305-437-2

Till Grabbsen

Filip och Johan

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ABBREVIATIONS

abd-A	*abdominal-A*
Abd-B	*Abdominal-B*
ANT-C	Antennapedia Complex
Antp	*Antennapedia*
ash1	*Absent and small homeotic discs 1*
ash2	*Absent and small homeotic discs 2*
Asx	*Additional sex combs*
BNLS	bipartite nuclear localization signal
brm	*brahma*
BX-C	Bithorax Complex
Dfd	*Deformed*
Dpp	*decapentapledgic*
E(var)	*Enhancer of variegation*
E(z)	*Enhancer of zeste*
EMF2	Embryonic flower 2
en	*engrailed*
E(Pc)	*Enhancer of Polycomb*
esc	*extra sex comb*
ETP	Enhancers of trithorax and polycomb
Fab-7	Frontabdominal-7
FIS2	Fertilization-independent-seed 2
FLP	yeast 2μm plasmid site-specific recombinase
FRT	FLP recombination target
H3	Histone 3
H4	Histone 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HP1	heterochromatin associated proetein
lab	*labial*
Mcp	Miscadastral pigmentation
NLS	nuclear localization signal
osa	*osa*
PEV	Position effect variegation
pb	*proboscipedia*
Pc	*Polycomb*
Pc-G	Polycomb-Group
Pcl	*Polycomblike*
ph	*polyhomeotic*
pho	*pleiohomeotic*
phol	*pleiohomeotic-like*
PRE	Polycomb Response Element
Psc	*Posterior sex combs*
Rb	Retinoblastoma
Scm	*Sex comb on midleg*
Scr	*Sex combs reduced*
Su(var)	*Suppressor of variegation*
Su(z)	*Suppressor of zeste*
Trl	*Trithoraxlike*
trx	*trithorax*
trx-G	*trithorax-Group*
Ubx	*Ultrabithorax*
VRN2	Vernalization 2
w	*white*
y	*yellow*
z	*zeste*

LIST OF PAPERS

This thesis is based on the following published papers and manuscripts which will be referred to by Roman numerals:

I Birve, A., Sengupta, A.K., Beuchle, D., Larsson, J., Kennison, J. A. Rasmuson-Lestander, Å. and Müller, J. (2001) Su(z)12, a novel *Drosophila* Polycomb group gene that is conserved in vertebrates and plants. Development 128, 3371-3379

II Tie, F., Prasad-Sinha, J., Birve,A., Rasmuson-Lestander, Å., and Harte, P.J. (2003) A 1 MDa ESC/E(Z) complex from *Drosophila* that contains Polycomblike and RPD3. Molecular and Cellular Biology, May 2003, p. 3352–3362*

III Birve, A., Chen, S., and Rasmuson-Lestander, Å. (2003) Suppressor of zeste12 mediates silencing through PREs, interacts genetically with other PcG genes and and has a unique binding pattern on polytene chromosomes. *Manuscript*

IV Birve, A., Chen, S., and Rasmuson-Lestander, Å. (2003) Expression pattern of the *Drosophila* polycomb group gene *Suppressor of zeste 12*. *Manuscript*

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REVIEW OF THE LITERATURE

Epigenetics

Genetics is the study of inheritance, how traits are passed on from one generation to the next. More specifically, genetics is the study of genes, the carriers of heredity, the factors determining the traits we can observe. These observable traits are seen as the phenotype which is the property of an organism that develops through the action of genes and environment. In addition, genetics is also occupied with the elements of the cells in which the genes reside, the chromosomes. For fifty years it has been known that genes carry the necessary information about a trait in the shape of a DNA sequence (Watson and Crick 1953). However, in mulitcellular organisms, like humans and flies, all cells in one individual have the same set of DNA, an identical genotype, and yet their bodies consist of many and very different tissues and thus many different cell types. So, since one set of genes can give rise to a variety of different phenotypes, there must be more to it than just the DNA sequence of a gene to determine a trait. Somehow there must be a difference in how genes are interpreted. One such difference is the way they are expressed; either they are active or inactive. Some genes are turned on and off depending on circumstances and environmental cues. Others are maintained in an active state, which means they are constantly being expressed, while other are kept in an inactive state, *i.e.* they are silenced. This maintained expression pattern is refered to as being epigenetic.

In 1942 C.H Waddington suggested the term epigenetics as ”the study of the processes by which genotype give rise to phenotype”. More than 40 years later Robin Holliday (1987) defined epigenetics as ”the study of the changes in gene expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression” and he also added to this a supplementary definition of epigenetics to include ”transmission of information from one generation to the next, other than the DNA sequence itself” (reviewed in (Morris 2001). Today the term epigenetics has a variety of definitions, one refers to the forms of inheritance that do not follow Mendelian rules, and also disregards gene expression. Another definition, and the one I will use in this thesis is: epigentics – the study of mechanisms behind differences in gene expression that are mitotically heritable and do not involve changes in DNA sequence.

There are several epigenetic examples that are related to each other and these have been studied in many different organisms *e.g.* flies, yeast, plants and mammals. What these phenomena all have in common is the maintenance of a given gene expression profile through cell division and through development. One extensively

studied example in *Drosophila* is Position Effect Variegation (PEV), where a translocation of a gene to a new position in the genome occasionally silences the gene giving rise to a variegated expression of that gene. Another example of maintained gene expression is the regulation of the homeotic genes. The homeotic genes are activated or repressed in a well defined pattern in the early *Drosophila* embryo. If initially activated, the gene is kept in an active state throughout development but if repressed, the gene is maintained in an epigenetically repressed state. An important and by now much studied epigenetic phenomenon in mammals is imprinting, where the parental origin (*i.e.* if the chromosome comes from the mother or the father) of an allele determines if it is going to be expressed or not. In the dosage compensation system, differences in number of sex chromosomes between the sexes is compensated for. In mammals this is done by inactivation of one of the two X-chromosomes in females. Also in these last examples, the specific gene expression pattern is in one way or the other remembered and maintained through generations of cell divisions. In this thesis I shall try to explain some of these phenomena and their underlying mechanisms and also to put our findings of the *Drosophila* gene *Su(z)12* in an epigenetic context.

Position effect variegation

Position effect variegation (PEV) is an epigentic phenomenon, in which the expression pattern of a gene is altered when the gene is transposed from its original position in the genome. A well studied example of PEV in *Drosophila* is *white*^{mottled4} (w^{m4}) where an inversion translocates the *white* gene (required for red eye pigmentation) from its position close to the tip of the X-chromosome to a position close to the centromere. Due to its new genomic position the intact but translocated *white* gene will in some eye precursor cells be turned off and in others turned on and this variegated expression will be maintained throughout development and give an eye phenotype with patches of red and *white* ommatidia (Figure 1) (Muller 1930). So what can explain this phenomenon? Although, in w^{m4}, the *white* gene still reside on the same chromosome there are differences in the environment at the different locations. These differences lie in the configuration of the chromosomal structure, – the chromatin, that consist of DNA and proteins. The original *white* locus is placed in loosely packed chromatin, euchromatin, but in the w^{m4} mutant the *white* gene is placed in the vicinity of tightly packed chromatin, heterochromatin. These are the two types of chromatin in the eukaryotic genome. Heterochromatin is mostly found near centromeres and, when observed cytologically, appears as a highly condensed material throughout the cell cycle (Belyaeva et al. 1997). Euchromatin, on the other

hand, takes up the greatest part of the total genome and appears condensed during mitosis but decondensed during interphase. Euchromatin contains the majority of structural genes and the relaxed appearance during interphase is thought to be necessary for the transcription machinery to be able to access DNA. The tightly packed heterochromatin does not contain many genes but mostly consists of repeated DNA sequences. Other typical features of heterochromatin are crossing-over suppression, late replication in cell cycle and under-replication in polytene chromosomes. Many genes affect PEV when mutated and are thought to be involved in formation of chromatin. These are characterised as either Suppressors or Enhancers of variegation (Figure 1).

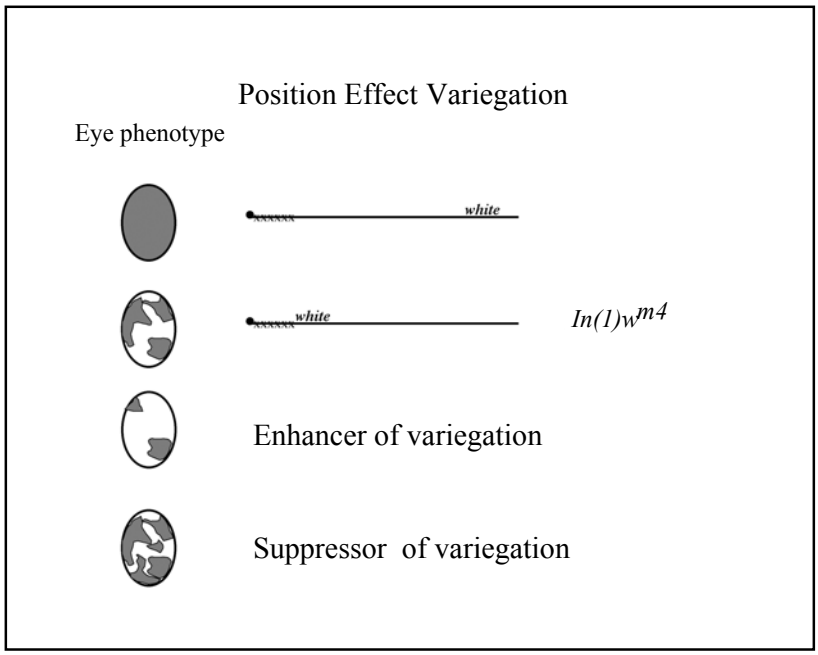

Figure 1. Position effect variegation (PEV)
In *w^{m}4* the *white* gene is translocated by an inversion to centromeric heterochromatin. This gives a variegated expression pattern of the *white* gene and a mottled eye phenotype. Mutations in genes that affect this expression pattern can either enhance the silencing of the *white* gene, enhancers of variegation, or suppress it, suppressors of variegation.

There are several genes affected by PEV in *Drosophila*, for example, *brown*, *rosy* and *hsp70* (Slatis 1955a; Slatis 1955b; Henikoff 1981; Rushlow et al. 1984). Effects on gene expression by neighbouring heterochromatin is also seen in other organisms like yeast and mouse (Festenstein et al. 1996; Milot et al. 1996; Grunstein 1997). Another example of gene silencing associated with heterochromatin is the mammalian dose compensation system where one of the two female X-chromosomes gets inactivated by being totally heterochromatinized into a Barr body. Heterochromatin can be divided into two types; facultative or constitutive. PEV and X-chromosome inactivation are examples of facultative heterochromatin; this chromatin is transiently condensed euchromatin, while pericentromeric and telomeric heterochromatin are examples of constitutive heterochromatin; this chromatin is permanently condensed and silenced. The constitutive heterochromatic regions are shown to be rich in repetitive sequences, like satellite DNA and transposable elements (Gatti and Pimpinelli 1992; Lohe et al. 1993; Pimpinelli et al. 1995). It has been proposed that tandemly repeated inserts of transgenes can induce heterochromatinization and silencing and that this might be a defense mechanism against transposons (Dorer and Henikoff 1994).

There are two major models to explain PEV. The heterochromatinization model and the nuclear organization model. The former model suggests that the dense chromatin structure keeps DNA inaccessible for transcription and in the PEV phenomena this heterochromatin occasionally spreads along the chromosome and into a translocated gene and silences it (reviewed in (Henikoff 1990; Wallrath 1998). It is shown that chromatin close to the centromere is condensed and resistant to nucleases; thus the DNA is probably less accessible to the transcription machinery (Wallrath and Elgin 1995). In the nuclear organization model it is suggested that the silenced heterochromatic regions are localized in certain compartments in the nucleus and that transcription factors don't have access to these compartments (Marcand et al. 1996; Dorer and Henikoff 1997; Marshall et al. 1997). It has also been shown that PEV can spread and inactivate genes in *trans* supported by findings of interactions between topologiacal changes within the nucleus and heterochromatic regions (Slatis 1955a; Slatis 1955b; Dreesen et al. 1991; Belyaeva et al. 1997; Henikoff 1997).

In support of the heterochromatinization model it has been shown that mutations in genes, that in various ways are involved in establishing chromatin structure, affect

PEV. Mutations in the gene *Su(var)2-5*, encoding the heterochromatin associated protein, HP1, (Eissenberg et al. 1990; Eissenberg et al. 1992) and mutations in *Su(var)2-10*, cause abnormal chromosome structure and also suppress PEV (Kellum and Alberts 1995; Hari et al. 2001). Thus the proteins encoded by these genes are needed for proper chromatin structure formation and this is critical for this type of silencing. Mutations in *trithorax* and *zeste*, that encode transcription factors, can enhance PEV. Accordingly, proteins that are involved in activation of gene transcription maintain an open chromatin state (Farkas et al. 1994; Judd 1995). But there are contradicting results regarding the accessibility of the chromatin. Some studies could not reveal any differences in the accessibility of the chromatin fiber to nucleases, between active and inactive genes, suggesting that the chromatin status is not a major transcription regulator in PEV (Schloβherr et al. 1994).

Imprinting

Genomic imprinting is an epigenetic phenomenon, where the expression pattern of a certain allele depends on its parental origin. At imprinted loci, the two parental alleles are treated differently in the zygote; one will be silenced and the other expressed, and at each specific locus it is always the same parental origin of the allele that is silenced, at some loci it is the maternal and at others it is the paternal. This silencing effect is dependent on the sex of the parent, not on the sex of the offspring and therefore the imprint must be reset in the germline for every generation. The new sex-specific imprint is then established during gamete development and maintained in the developing offspring.

Genetic imprinting has been reported in many different organisms for example mammals, zebra fish, insects, yeast and plants (Crouse 1960; Kermicle 1970; Nur 1970; Sharman 1971; Chandra and Brown 1975; Takagi and Sasaki 1975; Mc Grath and Solter 1984; Kuhn and Packert 1988; Martin and McGowan 1995b; Golic et al. 1998; Grossniklaus et al. 1998; Brannan and Bartolomei 1999; Lloyd et al. 1999; Haller and Woodruff 2000; Nakayama et al. 2000). The term imprinting was first used by Helen Crouse 1960 when she described a parent specific silencing of chromosomes in the fungus gnat, Sciara. In *Drosophila* imprinting as a phenomenon was described 60 years ago but under the term parental effects, however this term also included maternal effects, that are caused by RNA deposition by the female into the oocyte. The first description of imprinting in *Drosophila* concernes an X-chromosome inversion that translocates the *scute* gene and thus exposes it to PEV. It was found that if $In(1)sc^{8}$ was paternally inherited there was a greater frequency of

silenced offspring (Lloyd 2000). The imprinted effects in *Drosophila* are seen in both male and female germ line and it is associated with chromosomal rearrangements showing PEV and thus associated with heterochromatin (Singh 1994). So in these imprinting cases in *Drosophila* the wild type expression pattern of the gene in question is not imprinted but when the gene is translocated to heterochromatin and affected by PEV it acquires an imprinted expression pattern. It seems like this pattern spreads from the heterochromatin into the juxtaposed genes. Variegated silencing of imprinted genes is also seen in other organisms like zebra fish, mammals and maize (Kermicle 1970; Martin and McGowan 1995b; Morgan et al. 1999). In *Drosophila* mutations in genes known to modify PEV have been shown to disturb the silencing of the imprinted genes (Lloyd et al. 1999; Lloyd 2000; Joanis and Lloyd 2002). This is also supported by studies in mammals and fish (Bartolomei et al. 1993; Koide et al. 1994; Martin and McGowan 1995a; Hark and Tilghman 1998). Taken together these results imply that heterochromatin may play an important role in creating imprints.

Lloyd et al. (1999) showed that there is a gradient of the imprinting effect which is dependent on the distance of the imprinted gene from heterochromatin, suggesting that the imprinting originates within the heterochromatin and like PEV mediated silencing spreads along the chromosome. This is comparable to imprinting in mammals that spreads from "imprinting centers" (Nicholls et al. 1998; Frevel et al. 1999). Imprinting and heterochromatinization are phenomena that exists in a variety of organisms so it seems likely that there are conserved mechanisms behind the silencing effects. This is supported by studies done by Lyko et al. (1997,1998) who showed that two different mammalian imprinting centers can induce silencing also in *Drosophila* (Lyko et al. 1997; Lyko et al. 1998).

The homeotic genes

During development in *Drosophila*, as well as in mammals and other vertebrates, the embryo is segmented along the anterior posterior body axis. Each segment corresponds to certain parts of the adult organism. This spatially restricted patterns of the embryo is regulated by the gap genes, and segmentation genes, followed by homeotic genes (Kaufman 1980; reviewed in McGinnis and Krumlauf 1992; Prince 2002). The function of the homeotic genes are well conserved in evolution and have the same mission in such diverse animals as flies and humans. In *Drosophila* the homeotic genes were first characterized by E.B. Lewis (1978). He described a complex of genes that was needed for proper segmentation of the embryo. In

mammals the homologous hox genes are organised in 4 clusters on different chromosomes with a total of 39 genes (reviewed by McGinnis and Krumlauf 1992). In *Drosophila*. the homeotic complex contains one set of 8 genes located on the same chromosome but split into two clusters, the Antennapedia Complex (ANT-C) and the Bithorax Complex (BX-C). The ANT-C consists of the genes *lab*, *pb*, *Dfd*, *Scr*, *Antp* and controls segment identity in the anterior part of the fly *e.g.* head and thorax structures. The BX-C consists of the genes *Ubx*, *abd-A*, *Abd-B* and controls segment identity in the abdominal part of the fly (Duncan 1987; Kaufman et al. 1990). The homeotic genes are located in roughly the same linear order on the chromosome as the order of their expression domains along the anterior to posterior axis of the embryo (Lewis 1978; Karch et al. 1985; Kondo et al. 1998). So in *Drosophila* the ANT-C gene *lab*, is expressed most anterior in the embryo and *Abd-B* is expressed most posterior (Duboule 1998). See Figure 2 for the expression pattern of the homeotic genes. To make a simplified model of how the homeotic genes accomplishes the unique segment identity one can say that, the combination of a certain set of homeotic genes expressed in a particular parasegment defines the identity of that parasegment.

The expression pattern of the homeotic genes that is established during early embryogenesis must be maintained in an epigenetic manner throughout development. Mutations in the homeotic genes will have consequences for the segment identity, segments where the homeotic genes are misexpressed will posses the identity of segments anterior or posterior. and this will give rise to homeotic transformations in the embryo. If the mutation is not lethal at the early embryonal or larval stages the adult fly will acquire abnormal phenotypes where for example the antennae will be transformed into legs or the halters into wings.

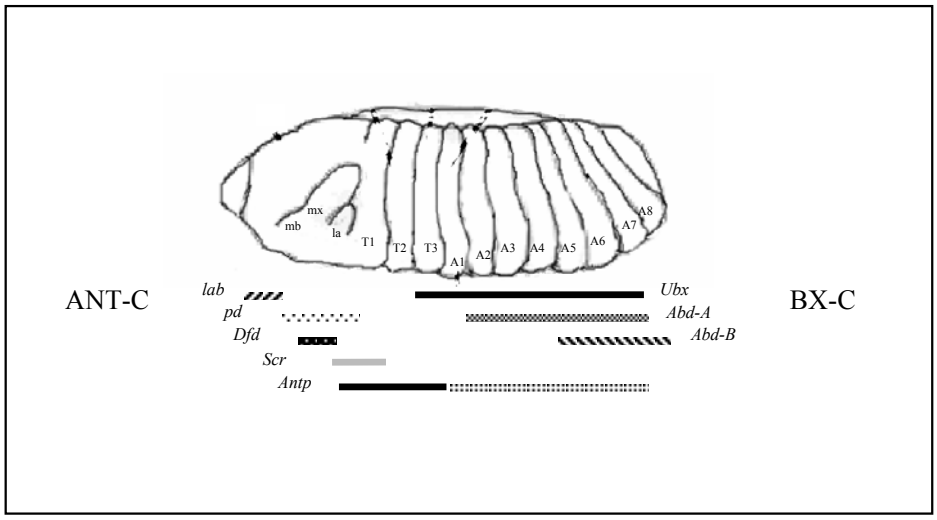

Figure 2. Approximate extents of the expression domains of the homeotic genes (modified from Kaufman et al. 1990). md, mx and la indicate mandibulary, maxillary and labial segments respectivelly. T1, T2 and T3 indicate thoracic segments 1 to 3. A1 to A8 indicate abdominal segments.

Regulation of homeotic genes

The homeotic genes are first transcribed in the blastoderm stage embryos, at around two hours of development. This first expression pattern of the homeotic genes is controlled by the gap gene products like *hunchback*, *knirps* and *Krüppel* (White and Lehmann 1986; Harding and Levine 1988; Irish et al. 1989). However, these cues are transient and after the initial regulation two major groups of genes are responsible for maintaining the right expression patterns of the homeotic genes throughout the life of the fly, the *Polycomb-group* (Pc-G) and the *trithorax-group* (trx-G). The Pc-G and the trx-G are responsible for maintaining the repressed and active status, respectively, of the homeotic genes (Gerd 1985; Simon et al. 1992) (Review in (Bienz and Müller 1995; Kennison 1995; Pirrotta 1997). Mutations in repressive Pc-G genes will cause derepression of the homeotic genes in the anterior part thus giving these segments a more posterior identity. This will give rise to homeotic transformations similar to those of mutations in homeotic genes. Several mutations in Pc-G genes in *Drosophila* will cause transformation of T2 and T3

identity into T1 identity. In male flies this will be visible, since they will have sexcombs not only on the most anterior leg pair but also on the posterior pairs of legs. Because of their characteristic phenotypes the *Drosophila* Pc-G genes have names like *Polycomb*, *extra sex comb*, *Posterior sex combs*, *Sexcombs on midleg*, *Additional sex comb* etc (Lindsley and Zimm 1992). Mutations in the genes belonging to the trx-G counteract the phenotypes caused by mutations in Pc-G genes implying that trx-G proteins promote activation of homeotic genes (reviewed by Pirrotta 1997).

Together the BX-C genes, *Ubx, abd-A* and *Abd-B* are responsible for the identities of the posterior segments of the fly. These, proper identities, are achieved by a specific expression pattern where *Ubx* is expressed in PS 5-13 (corresponding to segment T3-A7), *abd-A* is expressed in PS 7-13 (corresponding to segment A2-A7) and *Abd-B* is expressed in PS 5-15 (segment A5-A8) (Figure 2) (White and Wilcox 1985; Celniker et al. 1989; Karch et al. 1990). To keep the proper developmental expression pattern of these important genes, the BX-C has a large and complex regulatory region of around 300 kb that include nine domains, the infra-abdominal (*iab*) regions, that restrict the expression of the different genes to their right parasegments (Karch et al. 1985; Duncan 1987; Sanchez-Herrero 1991). In this control region there are also several domains that have been shown to be targets for the Pc-G and trx-G and are necessary for maintaining the initiated expression pattern (Gindhart and Kaufman 1995; Hagstrom et al. 1997). Three such domains identified in the BX-C regulatory region are *Ubx* PRE (Polycomb response element), Fab-7 (Frontabdominal-7) and Mcp (Miscadastral pigmentation) (Figure 3)(Busturia and Bienz 1993;Chan et al. 1994; Chiang et al. 1995). These domains are needed for maintaining repression of the homeotic genes in the parasegments where they should not be expressed, therefore they were initially identified as targets for Pc-G mediated repression and thus named Polycomb Response Elements (Busturia and Bienz 1993; Simon et al. 1993; Chiang et al. 1995; Hagstrom et al. 1996; Zhou et al. 1996; Mihaly et al. 1997; Cavalli and Paro 1998).

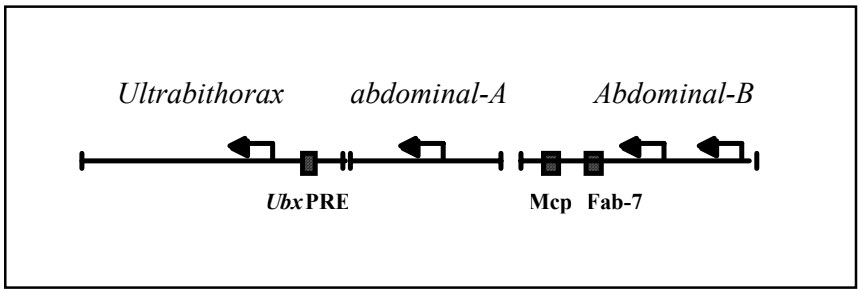

Figure 3. The regulatory region of the BX-C contains Polycomb response elements, PREs, necessary for repression of the homeotic genes.

The Polycomb-Group

Polycomb group (Pc-G) proteins are responsible for the epigenetic silencing of the homeotic genes. The Pc-G genes, just like the homeotic genes, are well conserved in evolution and homologues exist between mammals (Muller et al. 1995; van Lohuizen 1998), flies, and sometimes even plants (paper I; Springer et al. 2002). There are different models for how the Pc-G mediates its repression of the homeotic genes. The proteins encoded by these genes are chromatin binding and it has been suggested that the Pc-G proteins package the chromatin into a tight structure similar to heterochromatin (Zink and Paro 1995; Pirrotta 1997). Pc-G proteins are found to interact genetically and to colocalize on polytene chromosomes (Jürgens 1985; Zink and Paro 1989; van Lohuizen 1998). Therefore, it was early suggested that Pc-G proteins act synergistically to mediate their action by forming multimeric protein complexes (Franke et al. 1992; Alkema et al. 1997a; Gunster et al. 1997; Jones et al. 1998). Indeed two such complexes have now been identified, the PRC1 and the E(Z)/ESC complex (Shao et al. 1999; Ng et al. 2000). The PRC1 (Polycomb repressive complex 1), contains the Pc-G proteins PC, PSC, PH, SCM (Shao et al. 1999), but also the trx-G protein ZESTE (Saurin et al. 2001). The other Pc-G complex, ESC/E(Z) contains the proteins ESC, E(Z), p55, SU(Z)12, PCL and RPD3 (Ng et al. 2000; Tie et al. 2001; Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002; paper II).

Analyses of the genetic interactions concerning the proteins in the different complexes suggests that they may act sequentially. For example it has been shown that ESC is needed early but only transiently for repression (Struhl and Brower

1982; Simon et al. 1995). It has also been shown that an interaction between the two Pc-G complexes early in development is necessary for establishment of Pc-G mediated silencing (Poux et al. 2001b). According to these findings it has been proposed that the ESC-E(Z) complex initiate the Pc-G mediated repression while the PRC1 complex is required continuously to maintain the initiated repression of the target genes (reviewed in Bienz and Müller 1995; van Lohuizen 1999; Francis and Kingston 2001). Some results indicate that Pc-G mediated repression does not involve general changes in the chromatin fiber, which suggests that the mechanisms for Pc-G mediated silencing and heterochromatinization mediated silencing in PEV differ (Schloβherr et al. 1994; McCall and Bender 1996). Two members of the ESC-E(Z) complex, RPD3 and SU(Z)12, are modifiers of PEV (Table 1). It is possible that the ESC-E(Z) complex plays an initiating role in both types of silencing (van Lohuizen 1999). Recently it was shown that the ESC-E(Z) complex exists in two different sizes with slightly different protein compositions, the already characterized 600 kDa complex and another 1 MDa complex also containing PCL (Tie et al. 2001; paper II). Still, several of the known Pc-G members are not identified in either complex so it is probable that further Pc-G complexes will be found. These complexes might interact transiently and might also be flexible in their protein composition.

In addition to the regulation of homeotic genes, the Pc-G proteins probably also regulates many other genes. In *Drosophila* they bind around 100 sites on polytene chromosomes suggesting several potential target genes (Zink and Paro 1989; van Lohuizen 1998). One such additional target, the INK 4a locus, has been identified for the mouse Pc-G gene, *bmi-1* (Jacobs et al. 1999).

The trithorax-Group

The trx-G of genes were first defined for their counteractions of Pc-G mutant phenotypes (reviewed in Kennison 1995). In contrast to the Pc-G, that mediate repression, they are needed for activation of the homeotic genes and for maintaining of this active state (reviewed in Francis and Kingston 2001). Like the Pc-G proteins, the trx-G proteins also function in protein complexes. It has been shown that several such complexes exist; for example the *Drosophila* trithorax group proteins BRM, ASH1 and ASH2 are subunits of three different protein complexes (Papoulas et al. 1998). The brahma protein in *Drosophila* is homologous to the yeast SWI2/SNF2 gene. The encoded protein is part of a highly conserved protein complex, required for transcription activation, the SWI/SNF complex (Tamkun et al. 1992; Peterson 1996; Kadonaga 1998; Rozenblatt-Rosen et al. 1998; Struhl 1998). In *Drosophila*

Kal et al. (2000) showed that the BRM complex also contains products of the trx-G genes *osa* and *moira* and that the ZESTE can recruit this complex to specific chromosomal target sites (Kal et al. 2000). Another trx-G complex, TAC1, has been shown to containing the TRX and the CBP proteins (Petruk et al. 2001). TRX interacts with components of the BRM complex and with ASH1 (Rozenblatt-Rosen et al. 1998; Rozovskaia et al. 1999). In addition ASH1 interacts with CBP suggesting that sevareal trx-G complexes cooperate in achieving an epigenetic activ transcription state (Bantignies et al. 2000).

It has become evident that the division of the homeotic gene regulators into two distinct groups, Pc-G and trx-G, is a simplification. Some of these genes have both Pc-G an trx-G phenotypes (LaJeunesse and Shearn 1996; Gildea et al. 2000). In a screen for new trx-G genes, several genes already characterised as Pc-G genes, were identified. This suggests that several Pc-G genes are required for both activation and suppression of genes and have therefore been suggest to be called ETPs; enhancers of trithorax and Polycomb (Table 1) (Gildea et al. 2000). As I mentioned earlier, in addition to mediating Pc-G repression, PREs also function in trx-G mediated activation, thus they are simultaneously both PREs and TREs (thrithorax response elements) (Farkas et al. 1994; Strutt et al. 1997). So what factors mediate the contact between these DNA regions and the Pc-G and trx-G complexes? This far only four DNA binding proteins have been identified, the two trx-G proteins, GAGAfactor and ZESTE and the Pc-G proteins PHO and PHOL, encoded by a recently characterized gene that is redundant with *pho* (Benson and Pirrotta 1988; Farkas et al. 1994; Brown et al. 1998; Brown et al. 2003).
It has been shown that the Mcp element need both PHO and GAGA factor for maintaining repression and in vitro studies show that a GAGA containing complex containing binds the *Ubx* PRE (Busturia et al. 2001; Horard et al. 2000 But on the other hand Poux et al. Showed that PHO was not able to recruit any major Pc-G complex (Poux et al. 2001). It is still not clear how the Pc-G complexes are targeted to the PREs. It is possible that this process needs something else than DNA binding proteins *i.e.* certain modifications or marks of the surrounding chromatin (van Lohuizen 1999).

TABLE 1

Gene	Classification modifier of indicated system	Identified in complex	Protein features
Pc	Pc-G	PRC1	Chromodomain
ph	Pc-G	PRC1	Zinc finger
sxm	ETP	PRC1	
Psc	ETP	PRC1	
E(z)	ETP	E(Z)/ESC	SET-domain, HMT
Asx	ETP		
Pcl	Pc-G	E(Z)/ESC	
esc	Pc-G	E(Z)/ESC	
Pho	Pc-G		DNA binding
E(Pc)	ETP/PEV/Imp		
Su(z)12	Pc-G/PEV	E(Z)/ESC	
Su(z)2	ETP		
z	trx-G /PEV	PRC1	DNA binding domain
trx	trx-G	TAC 1	SET-domain, HMT
Trl/GAGA factor	trx-G/PEV/Imp		
ash1	trx-G	Ash1 Complex	SET-domain, HMT
ash2	trx-G	Ash2 Complex	
mod(mdg4)	trx-G/PEV		
brm	trx-G/Imp	BRM	SNF2/SWI2 Bromodomain
mor	trx-G	BRM	
osa	trx-G	BRM	
RPD3	PEV	E(Z)/ESC	HDAC
Su(z)5	PEV		S-adenosyl-methionin synthetase
Su(var)2-5/HP1	PEV/Imp		Chromodomain
Su(var)3-7	PEV		associates with HP1
Su(var)3-9	PEV/Imp		Chromodomain, SET-domain, HMT
Su(var)3-6	PEV		Protein phosphatase 1
modulo	PEV		binds DNA and RNA
E2F	PEV		transcription factor acts on *Su(z)12*
E(var)3-64E	PEV		ubiquitin-specific protease
Su(var)3-3	PEV/Imp		
Su(var)2-1	PEV/Imp		
Su(var)3-8	PEV/Imp		
Su(var)3-10	PEV/Imp		
Sir2	PEV		putative HDAC
CBP		TAC 1	HAT, CREBbinding protein
p55		E(Z)/ESC	

A selection of genes involved in epigenetic phenomena. Their classification as either Pc-G, trx-G or ETP genes is indicated as well as if they are modifiers of PEV or imprinting. If is also indicated if the protein has been identified in any know complex and if it has any particular feature or domain. (Gould 1997; Papoulas et al. 1998; Ng et al. 2000; Saurin et al. 2001; paper II; Czermin et al. 2002; Müller et al. 2002; Kuzmichev et al. 2002; Francis and Kingston 2001; Rozenblatt-Rosen et al. 1998; Brock and van Lohuizen 2001 and references therein)

zeste

One fascinating phenomenon in *Drosophila* and other insects is that the expression of a gene can be influenced by pairing of the homologous chromosomes. This phenomenon of inter-allelic complementation, where one allele can control the expression of the other homologous allele, was first described by E.B. Lewis (1954). He named the phenomenon transvection. Thus enhancers on one gene-homolog can mediate an effect, positive or negative, on the transcription of the encoding sequence on the other homolog. In *Drosophila* transvection effects are found at several loci (Lewis 1954; Lewis 1978; Gelbart 1982; Leiserson et al. 1994; Morris et al. 1999). When studying the transvection effects at the *bithorax* locus Lewis found one gene necessary for bringing about these effects. This was a X-linked gene that he named *enhancer of bithorax, e(bx)*.

Another transvection-like phenomenon in *Drosophila* is the *zeste-white* interaction. This is also pairing dependent phenomenon, first characterized by Madeleine Gans in 1953. She found a X-linked mutation that cause a yellow eye phenotype in homozygous females but not in hemizygous males. Due to the lemon-like yellow eye color of the mutant flies, she named the gene *zeste* (Gans 1953). The mutation represseses the transcription of the *white* gene but only if the there are two copies of the *white* gene, and these must be able to pair (Gans 1953; Jack and Judd 1979; Zachar et al. 1985) (Hazelrigg 1987). Later it was shown that *zeste* and *e(bx)* are alleles, so this gene is involved in chromosome pairing effects at both the *white* and the *bithorax* loci. In addition to the *bithorax* locus, the *zeste* protein is required for transvection at many other loci for instance at *dpp* and *y* (Kaufman et al. 1973; Babu and Bhat 1981; Gelbart and Wu 1982; Geyer et al. 1990).

What properties of the protein encoded by this gene can explain its involvement in gene pairing? The *zeste* gene encodes protein with the ability to bind DNA, to bind other proteins and also to self-aggregate (Pirrotta et al. 1987; Mansukhani et al. 1988; Chen et al. 1992). The protein consists of a N-terminal DNA binding region and C-terminal hydrophobic repeats that are required for the aggregation of proteins, and these have been shown to be necessary for transvection, and *zeste-white* repression (Mansukhani et al. 1988; Bickel and Pirrotta 1990; Chen et al. 1992). It has been shown that the multimerization of ZESTE proteins can crosslink two DNA molecules (Benson and Pirrotta 1988) and that this multimerization actually increases the efficiency of the DNA binding (Chen 1993). These properties may explain how *zeste* mediates its action in this pairing dependent phenomenon. In transvection it is proposed that ZESTE proteins, by binding both homologous genes connect them and make it possible for the enhancer on one of the homologous to act

on the promoter on the other homologue (Bickel and Pirrotta 1990). In *zeste-white* interaction the mutated ZESTE[1] protein, that has a stronger aggregation capacity can connect two *white* alleles and hyperaggregation of the mutated protein causes silencing of the *white* genes (Chen and Pirrotta 1993).

Due to its potential to connect different DNA strands *zeste* probably has a function in normal gene regulation, to bring distant enhancers closer to promotors and activate transcriptipon in *cis* (Benson and Pirrotta 1988). *zeste* has been shown to be involved in normal gene expression of several genes. It is an activating transcription factor involved in *Ubx*, *dpp* and *w* expression (Laney and Biggin 1992) and therefore classified as a trx-G gene. ZESTE binds to specific target sequences in regulatory elements located close to the promoter in regions upstream of the genes *Ubx*, *dpp*, *w*, *Antp* and *en* (Benson and Pirrotta 1988). One could expect that a gene involved in the activation of these developmantally important genes would be of great importance. Therefore the fact that *zeste* is nonessential for viability has been a bit puzzeling (Goldberg et al. 1989). However it has been suggested that there is a redundant control of *Ubx* activation by *zeste* and the GAGA factor (Laney and Biggin 1992; Laney and Biggin 1996).

Although *zeste* has a role in activating gene expression it is also suggested to have a function in epigenetic repression of genes. There is a great overlap of polytene chromosomes binding sites between *zeste* and Pc-G proteins and it has been shown that *zeste* is needed in Polycomb maintained repression of *Ubx* (Rastelli et al. 1993; Hur et al. 2002). It is also interesting to note that ZESTE interacts with the activating brahma complex and is also part of the repressing *Polycomb* complex, PRC1 (Kal et al.2000; Saurin et al. 2001). As discussed above, the border between the activating trx-G factors and repressing Pc-G factors is not sharp.

Many modifiers of *zeste*, for example *Su(z)2*, *E(z*), *Psc*, and *scm* are involved in various types of epigenetic phenomena. They have been characterized as either Pc-G genes, trx-G genes or modifiers of PEV (Wu et al. 1989; Judd 1995; Bornemann 1996). Therefore the *zeste-white* system can be used as a model system, suitable for identifying genes important in epigenetic gene regulation. *Suppressor of zeste 12* was isolated in such a P-element mutagenesis screen for modifiers of *zeste*.

Mechanisms for epigenetic silencing

Gene expression is regulated at many levels. In the previous sections I have reviewed different epigenetic phenomena were genes are silenced in a manner inheritable through mitosis. So what are the mechanisms that accomplish this? Epigenetic silencing is thought to be established and regulated by alterations in chromatin structure but it has also been suggested that the spatial organisation in the nucleus has an impact on gene activity.

The eukaryotic nucleus appears to be strictly ordered with sub-nuclear compartments, where factors involved in common pathways are concentrated. One well known example is the nucleolus, a visible compartment in the nucleus, where rRNA transcription is intense. There is also a compartmentalization of chromatin in the nuclues. Chromosomes with low gene density, and silenced regions are localized to the pheriphery of the nucleus while active chromatin and chromosomes with high density of genes, reside in the interior (Mahy et al. 2002a; Mahy et al. 2002b; Parada and Misteli 2002). It is not yet clear how significant these nuclear compartments are for nuclear functions. It has been shown that nuclear reactions can proceed in the absence of their compartments and there are some results suggesting that it is actually the nuclear functions that establishes the compartment (Gang Wang et al. 1998; Hediger et al. 2002; Sirri et al. 2002; Chubb and Bickmore 2003). Even if the chromatin compartments are an effect of transcription, still, it is possible that the local concentration of different factors may have great impact on the efficiency of a process. Localization of a gene at the nuclear periphery or proximal to heterochromatin might expose it to elevated concentrations of silencing proteins or to decreased concentration of transcription activators. Chubb and Bickmore (2003) suggests that "Transcription may drive the establishment of nuclear order but the order itself may facilitate the control of transcription".

Even if nuclear localization has an influence on gene expression and on chromatin structure, there is clearly a connection between the state of chromatin and the expression pattern of the genes residing in it. In the eukaryotic cell the large amount of DNA is packed into a small volume by wrapping the DNA double helix around proteins. Approximately 200bp of DNA associates with a histone octamer consisting of two copies of each of the histones H2A, H2B, H3 and H4. Together this organisation of DNA and proteins forms the basic unit of the chromosome, the nucleosome. Nucleosomes are differently packed in euchromatin and in heterochromatin. In heterochromatin it is packed in regular arrays while in euchromatin it can exhibit an irregular spacing. This wrapping of DNA around nucleosomes compacts and protects the DNA, but it also has repressive effects on

transcription of genes, since the DNA becomes less accessible for the transcription machinery (Cremer and Cremer 2001). As discussed in the previous sections, many genes involved in epigenetic systems, encode proteins that affect chromatin structure (Table 1). Among these proteins we can find; SET domain proteins, shown to be histone methyltransferases (HMTs), encoded by *Su(var)3-9*, *E(z)* and *trithorax* (from which this domain got its name) (Briggs et al. 2001; Roguev et al. 2001; Bannister et al. 2002), *Su(var)3-64E* encoding an ubiquitin specific protease, and *RPD3* a histone deacetylase (Henchoz et al. 1996; Kadosh and Struhl 1998). So what are the molecular mechanisms behind these acetyl, ubiquitin and methyl associated proteins, how can they affect chromatin structure and regulation of gene expression? It has recently become clear that histones have a function, not only to protect and compact DNA but also in epigentic gene regulation.

The N-terminal tails of histones are external to the core structure and are therefore accessible for protein-protein interaction (Luger et al. 97?). They can thus be exposed to different covalent modifications, *e.g.* acetylation, methylation, phoshorylation and ubiquitination. These modifications can modulate the compaction of chromatin and thus the accessability of DNA (Strahl and Allis 2000). It has been proposed that these histone tail modifications serve as a code so that chromatin proteins can interpret the expression status of the particular chromatin sequence (Jenuwein and Allis 2001; Li et al. 2003). Acetylation and phosphorylation of histones are reversible processes but since no histone-demethylases have been identified yet, it is possible that histone metylation could be a stable mark for an epigenetic status. On the histone tails of each nucleosome there is 18 residues that are possible to methylate, on H3; there are three arginine residues (R2, R17, R26) and four lysine residues (K4, K9, K27, K36); on H4; there is one arginine residue (R3) and one lysine residue (K20). lysine can be mono-, di, or tri methylated and arginine can be mono-, or dimethylated (reviewed in (Bannister et al. 2002). Taken together this will give $15x10^6$ different combinations on one nucleosome, which gives a great potential of specifying the transcriptional state, of a particular area of chromatin.

Connections between acetylation and methylation of histones and transcriptional activity have indeed been found. Several studies in various organisms show a positiv correlation between acetylation of H3 and H4, in promoter regions, and transcriptional activity. It is also found that these histones are methylated in the coding regions of active genes (Bernstein et al. 2002). This is consistent with other findings showing that heterochromatin, that is transcriptionally inactive, is both

hypoacetylated and hypomethylated (Braunstein et al. 1993; reviewed by Jenuwein and Allis 2001, and Richards and Elgin 2002; Bernstein et al. 2002).

In PEV, where silencing is mediated by heterochromatin, histone methylation has been shown to have clear impact on the silencing effects. The heterochromatin associated protein HP1 encoded by *Su(var)205* (Table 1), has been found to associate with heterochromatin on polytene chromosomes and is needed for heterochromatin formation (Eissenberg and Elgin 2000). Li et al. (2003) found that tethering of HP1 to euchromatic regions causes silencing in that region and induces formations of ectopic fibers between the site of tethered HP1 and other chromosomal locations. This might bring distal chromosomal regions together and in this way influence the packing of chromatin (Li et al. 2003). It has been shown that the chromatin association of HP1 is due to an interaction of its chromodomain with methylated K9 on H3 (Bannister et al. 2001; Lachner et al. 2001; Jacobs and Khorasanizadeh 2002). Among the modifiers of PEV we find *Su(var)3-9* (Table 1), that encodes a SET domain protein, and this HMT protein, is responsible for the methylation of K9 on H3 (Lachner et al. 2001; Nielsen et al. 2001; Schotta et al. 2002). In mammals loss of *Su(var)3-9* function disrupts heterochromatin formation (Peters et al. 2001). So, it can be concluded that methylation of H3 has an important role in heterochromatin mediated silencing.

In addition to its implications in PEV, histone methylation is also important in Pc-G mediated silencing. The HMT protein E(Z) is found in the E(Z)/ESC Pc-G complex, and the histone methylation mediated by this complex influences the transcriptional state of certain genes and also the maintenance of both the repressed and active expression states (Beisel et al. 2002; Cao et al. 2002; Czermin et al. 2002; Müller et al. 2002). It has been shown that the E(Z)/ESC complex possesses HMT activity with specificity for K9 and K27 on H3, to be more specific, E(Z) tri-methylates K9 and K27 (Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002). PC a member of the other Pc-G complex, PRC1, binds chromatin and has a high affinity for H3 methylated at K27 (Cao et al. 2002; Czermin et al. 2002; Müller et al. 2002). It is postulated that the E(Z)/ESC complex makes a methyl mark on the K27 of histone H3 and this mark is recognised by PRC1. This all fits in with a model were the E(Z)/ESC complex initiates the silencing and a subsequent interaction between the two complexes is needed for PRC1 to be able to maintain the silenced state (Poux et al. 2001b; Petruk et al. 2001; Breiling and Orlando 2002)

Histone methylation is also correlated to trx-G mediated activation. The HMT protein, TRX, is found in the TAC1 complex (Petruk et al. 2001). It has been shown

that TRX interacts with components of the BRM complex and with ASH1 (Rozenblatt-Rosen et al. 1998; Rozovskaia et al. 1999). ASH 1, yet another SET domain protein (Table 1), which is found to methylate H3 at K4 and K9, and H4 at K20 and this action has been shown to be necessary for trx-G activation (Beisel et al. 2002). ASH1 also interacts with another component in the TAC1 complex, the protein CBP (Bantignies et al. 2000). Together, this implies that ASH1 makes a methylation mark that recruits other trx-G complexes *e.g.* BRM, ASH2 or TAC1 and that several trx-G complexes cooperate in initiating and maintaining an active chromatin status.

In yeast, active genes have been found to be marked by a tri-methylated K4 of H3 (Santos-Rosa et al. 2002). Both ASH1 and TRX methylates K4 of H3 (Rozenblatt-Rosen et al. 1998), thus it is possible that both ASH 1 and TAC1 are needed for accomplishing a tri-methylated K4 mark in epigenetic activation. It has also been shown that tri-methylated K9 of H3 overlaps with Pc-G binding in *Drosophila,* while dimethylated K9 does not (Czermin et al. 2002). Therefore, it has been suggested that tri-methylated K4 of H3 marks an active transcription state while a tri-methylated K9 of H3 marks a repressed state (Breiling and Orlando 2002).

In addition to methylation of histones also acetylation of these seem to be linked to Pc-G and trx-G function (van der Vlag and Otte 1999; Tie et al. 2001). One member of the E(Z)/ESC complex in *Drosophila*, RPD3 is a histone deacetylase (HDAC) and in the TAC 1 complex we find CBP, a histone acetyltrasferase (HAT) (se Table 1). Even though the results concerning the potential HDAC activity of the E(Z)/ESC complex are contradicting (van der Vlag and Otte 1999; Tie et al. 2001; Cao et al. 2002; Czermin et al. 2002; Müller et al. 2002), the finding of HDAC in a Pc-G complex and a HAT in the a Trx-G complex implies that histone acetylation may play an important role in epigenetic gene regulation (Santos-Rosa et al. 2002)

There is also a correlation with histone deacetylation and silenced heterochromatin. For example *Drosophila* mutants in *Rpd3*, a gene encoding a HDAC in yeast, enhance PEV, but, on the other hand, mutations in *Sir2*, encoding a putative HDAC, are suppressors of PEV (De Rubertis et al. 1996; Newman et al. 2002; Astrom et al. 2003). In yeast, both histone acetylation and deacetylation are required for proper heterochromatin formation and transcriptional silencing (Braunstein et al. 1996). Heterochromatin is shown to be relatively hypoacetylated and histone deacetylation has been correlated with regions of the chromatin that are transcriptionally inactive (Laurenson and Rine 1992; Turner et al. 1992; Braunstein et al. 1993; Braunstein et al. 1996). It has been proposed that histone acetylation might open up the chromatin

structure. In this model neutrally charged acetyl groups sitting on histone tails are predicted to have lower affinity for the negatively charged DNA causing chromatin to relax and thus making it more accessible for the transcription machinery (Pennisi 1997).

Acetylation is a reversible processes while several studies conclude that methylation is not (Byvoet 1972; Zhang and Reinberg 2001). No histone-demethylases have been identified yet and as discussed earlier this would nominate histone metylation as a good candidate for a stable mark stating a long term epigenetic status (Eissenberg and Elgin 2000). But on the other hand there are studies proposing that the methylated pattern of histones can be reversed. For example studies in yeast shown that when promoters are derepressed their methylation pattern changes from tri-methylated to di-methylated K4 on histone 3 (Santos-Rosa et al. 2002). In flies silencing mediated by the Pc-G can be derepressed (Beuchle et al. 2001; Breiling et al. 2001), so if this silencing is marked by a methylated pattern there must be a mechanism that can remove this mark. In addition to a histone-demethylase mechanism, alternative models are suggested for demethylation. Two models that have been suggested are; histone replacement, where a methylated H3 is replaced by an unmetylated variant and clipping, where the tail on histone 3 is proteolytically cleaved and the metylated K4 removed (Bannister et al. 2002).

Apart from histone modifications there is yet another player causing epigentic molecular marks, namely DNA methylation. In mammals and plants it is known that methylation of the cytosine residues in DNA plays an important role in imprinting and X-chromosome inactivation (Jaenisch 1997; Feil and Khosla 1999; Holliday and Ho 2002). Interestingly it has now been shown that cytosine methylation and cytosine methyltransferases interact with HDAC complexes and results from studies in *Arabidopsis* and *Neurospora* show that there is a connection between DNA methylation and HMTs (Dobosy and Selker 2001; Tamaru and Selker 2001; Jackson et al. 2002; Malagnac et al. 2002). In studies of X-chromosome inactivation in mice, H3 methylation of K9 preceeds cytosine methylation suggesting that methylated H3 acts upstream of DNA methylation in creating an epigenetic mark (Heard et al. 2001; reviewed in Richards 2002).

Until a few years ago DNA methylation was not believed to occur in *Drosophila.* Today we know that methylation of DNA exists in the early *Drosophila* embryo (Lyko 2001). However, it is not known if it has any implications on epigenetic gene regulation in the fly. It is interesting to note that *Su(var)3-9* that affects methylation in *Neurospora crassa* and *Arabidopsis* (Tamaru and Selker 2001; Malagnac et al.

2002), also affects DNA methylation in flies (F. Lyko personal communication). In mammals there is a tight connection between imprinting and DNA methylation. Even if there are evident differences between imprinting in mammals and in *Drosophila* it is appealing to speculate about a common mechanism in these two systems. DNA methylation in *Drosophila* seems to be restricted to the centromeres (F Lyko personal communication) and *Drosophila* imprinting is exclusively associated with centromeric heterochromatin. In addition, *Su(var)3-9*, affects both methylation and imprinting in *Drosophila* (Joanis and Lloyd 2002). Taken together, this might imply a role for DNA methylation also in imprinting in flies.

Finally, RNA seems to play a role in regulation of gene expression and chromatin status. It has been shown that RNA is involved in the formation of heterochromatin (Maison et al. 2002). The Pc-G protein PC and the heterochromatin protein HP1 both contain chromodomains (Table 1), that are found to be protein–RNA interaction modules (Akhtar et al. 2000). It has been known for quite a few years that non-coding RNAs play important roles in imprinting and X-chromosome inactivation in mammals (Panning et al. 1997; Sleutels et al. 2002), and the *Drosophila* dosage compensation system also involves RNA components (Meller et al. 1997; Franke and Baker 1999). Thus, RNA could play an important role in heterchromatin silencing as well as Pc-G mediated silencing.

Epigenetic genes and disease

The fruit fly, *Drosophila melanogaster* have been studied for almost a hundred years and is, therefore, very well characterised genetically (reviewed by Rubin 2000). Three years ago the genome project also added the complete *Drosophila* genome sequence to the previous knowledge (Adams et al. 2000). Thus the fly provides us with a good model organism for understanding genetic and basic biological functions as well as molecular mechanisms. The comparative analysis during the past decades has curiously shown that many genes and basic cellular mechanisms are conserved between organisms as diverse as flies, worms and mammals. Therefore, studies in *Drosophila* can contribute to understanding human diseases. Actually, of around one thousand studied genes implicated in human diseases, approximately 77% have one or more *Drosophila* homologue (Reiter et al. 2001). The great bulk of information about *Drosophila* genes and many useful molecular methods provides excellent tools for studies of biological phenomena or diseases. By inducing mutations, that affect a particular disease or phenomenon, new genes involved in the system can be identified. If genes, already identified and

characterised in certain diseases in human, have homologues in *Drosophila*, these can be mutated and their function studied. Examples of phenomena or "diseases" studied in flies are ageing, neurodegeneration, immune response, and control of behaviour and physiology (O'Kane 2003).

As mentioned earlier the homeotic genes and their function to control the body plan are well conserved between *Drosophila* and humans (reviewed by Gellon 1998 and Prince 2002). Misexpression of these genes and the genes controlling them, (like the Pc-G and trx-G genes), are associated with disease and malformations. For instance mutations in Pc-G or homeotic genes in mice and humans can give rise to skeletal defects (Muragaki et al. 1996). Like homeotic transformations in *Drosophila* these effects are characteristic for faulty segmentation identity (Gould 1997; Schumacher and Magnuson 1997; van Lohuizen 1998). These mutations can also cause neurological defects and affect the sex-determining pathway (Yuko Katoh-Fukui 1998). In humans misregulation of Hox genes can also cause various forms of leukaemia (Borrow et al. 1996).

Drosophila has homologues of many human oncogenes and tumour suppressor genes and studies of these have contributed to understanding the basic function of many of these genes (Simon et al. 1991; Bilder et al. 2000). The normal function of tumour suppressor genes is to control the cell cycle by suppressing cell division. Loss of function mutations in such genes would cause a loss of suppressing control of cell division leading to uncontrolled cellproliferation. Proto-oncogenes, has a normal function in cell cycle control by promoting cell division. Mutations that cause a permanent expression of these genes are called oncogenes since this leads to uncontrolled cell division.

Several of the positive regulators of homeotic genes the, trx-G, are associated with cancer. The human homologue of the *Drosophila* trx gene, MLL is frequently associated with translocations in leukaemia's (Javier Corral 1996; Look 1997). The two human homologues of *Drosophila brahma*, *brm* and *BRG-1* encode proteins that can bind Rb (retinoblastoma) protein (Dunaief et al. 1994) and reduction of brm is associated with facilitated transformation of rodent fibroblast by the *ras* oncogene (Muchardt et al. 1998). Another trx-G gene, little imaginal discs, is the *Drosophila* homologue of the human Retinoblastoma Binding Protein 2 (Gildea et al. 2000).

Also genes in the repressive Pc-G are associated with cancer. The murine *bmi-1* gene, that encodes a protein with homology to *Drosophila* PSC and SU(Z)2, is a

proto-oncogene (Brunk et al. 1991). Apart from the normal targets, the homeotic genes, there is an additional target locus for Bmi-1 identified in mice, the INK 4a locus. This locus encodes two tumour suppressor proteins p16 and p19Arf and these are shown to be overexpressed in Pc-G mutant cells (Jacobs et al. 1999). Over expression of *bmi-1* in transgenic mice, induces lymphomas (Alkema et al. 1997b) possibly by downregulation of p16 and p19Arf. Taken together these results indicate that there is a connection between Pc-G genes and control of cell proliferation. Recently it has been shown that two members of the E(Z)/ESC complex are upregulated in different cancers. The human homologue of E(Z), EZH2, is upregulated in metastatic prostate cancer (Varambally et al. 2002) and the human homologue to *Su(z)12* is overexpressed in colon and liver tumours (Weinmann et al. 2001). Furthermore, aberrations in *hSu(z)12* is found in endometrial stromal tumours (Koontz et al. 2001). This might suggest that *Su(z)12* is a proto-oncogene that have a function in cell cycle regulation. *Su(z)12* has therefore been suggested to be a potential target for an antitumour agent in cancer therapy (Kirmizis et al. 2003).

AIMS OF THIS STUDY

The overall aim of my PhD studies has been to clone and characterize the gene *Suppressor of zeste 12* in *Drosophila melanogaster* and to gather some understanding about its function.

More specifically, my aims have been;

- To clone and characterize the *Suppressor of zeste 12* gene (paper I and IV).
- To analyse the expression pattern of *Su(z)12* (paper IV).
- To phenotypically and molecularly characterize the *Su(z)12* mutations (paper I and III).
- To characterize the SU(Z)12 protein (paper II and III).

RESULTS AND DISCUSSION

Isolation of *Suppressor of zeste 12*

Su(z)12 was identified in a P-element screen for modifiers of *zeste-white* interaction. The induced mutation caused a dominant suppression of the $zeste^{1}$ mediated repression of *white*. In a parallel screen for zygotic lethal mutations in the 76D region, a complementation group with four lethal alleles was identified: $l(3)76BDo^{1}$, $l(3)76BDo^{2}$, $l(3)76BDo^{4}$ and $l(3)76BDo^{5}$ (Kehle et al. 1998). Complementation tests showed that these were alleles to the P-element mutation *Su(z)12*. We therefore decided to call the locus *Su(z)12*, the four EMS-induced alleles $Su(z)12^{2}$, $Su(z)12^{3}$, $Su(z)12^{4}$, and $Su(z)12^{5}$, respectively and the P-element induced mutation $Su(z)12^{1}$ (paper I).

Su(z)12 mutations are dominant suppressors of *zeste*

Su(z)12 was isolated as a dominant suppressor of the $zeste^{1}$. All *Su(z)12* mutations have a more or less strong suppressing effect on the *zeste-white* interaction. Analysis of genetic interaction with *Su(z)12* and different alleles of *white* and *zeste* suggests that *Su(z)12* mediates this effect by interaction with *zeste* and not with *white* (Table 2; paper III) and that the suppressing effect of $zeste^{1}$ on the expression of the *white* gene is dependent on the $Su(z)12^{+}$ gene product. However, as shown below, there is no binding of SU(Z)12 protein to the *white* locus on polytene chromosomes in salivary glands.

Su(z)12 mutations are homozygous lethals

The four EMS-induced alleles $Su(z)12^{2\text{-}5}$ were isolated as recessive lethals and $Su(z)12^{1}$ is also a homozygous lethal mutation. Animals that are homozygous or hemizygous for alleles 1, 3, 4 or 5 die during embryogenesis or the first larval instar. This suggested that *Su(z)12* is important during embryonic development. However, several transheterozygous combinations with $Su(z)12^{5}$ can develop into pharate adults with strong homeotic transformations (Fig. 1; paper I). No mutation has been identified in the open reading frame of $Su(z)12^{5}$, it is therefore tempting to speculate about interallelic complementation, like transvection or trans-splicing.

Cloning of *Suppressor of zeste 12*

To verify that the phenotypes of $Su(z)12^{1}$ were due to the insertion of the P-element, revertants were induced by secondary P-element mutagenesis and the loss of the P-element was confirmed with Southern blot analysis. The obtained revertants lost the suppression phenotype and were also viable with $Su(z)12^{1}$. This proved that the phenotype was due to the P-element insertion, so I went ahead and cloned the gene

using the P-element as a tag. I then mapped *Su(z)12* to 76E-77A by *in situ* hybridisation using a cloned genomic fragment as a probe (see picture on the cover). According to Flybase, where the location is computationally determined from the genome sequence, the cytological position is at 76D4.

The *Su(z)12* gene spans approximately 5 kb and encodes at least four different transcripts (Fig. 1C; paper IV). Two of the four *Su(z)12* transcripts have been sequenced, one cDNA, is 4,041 nucleotides long and the other cDNA, LD02025 is 3,637 bases long (Fig.2; paper IV). The 4.0 kb transcript has an extra exon, and due to a stop codon in this exon, the deduced protein is shorter than the one encoded by the 3.6 kb transcript. The other two transcripts have not been cloned. The 3.6 kb transcript encodes a protein that contains a C2-H2 zinc finger domain, two bipartite nuclear localization signals (BNLS), several nuclear localization (NLS) signals, and a region, the VEFS box (VRN2, EMF2, FIS2 and SU(Z)12) with high homology to the human and *Arabidopsis* homologues, (Fig. 5; paper I). The 4.0 kb transcript encodes a 95 kDa protein that differs in content in the C terminal region compared with the 100 kDa protein, where it lacks the second BNLS (Fig. 2; paper IV). Both transcripts also encode an aspargine rich region, located between the zinc finger and the VEFS box, and a serine rich region, located C-terminally to the VEFS box.

Molecular Characterization of mutant alleles

Of the EMS induced mutations, allele *2, 3* and *4*, have a single base substitutions in the open reading frame at positions corresponding to codons 274, 298 and 218, respectively. The first leads to an amino acid substitution and the two latter to stop codons. No lesion has yet been identified in the $Su(z)12^5$ allele. The P-element in $Su(z)12^1$ is inserted in the VEFS box encoding region (Fig. 5; paper I). The predicted protein products encoded by $Su(z)12^3$ and $Su(z)12^4$ will be truncated, lacking both the zinc finger and the VEFS box. These probably represent null alleles. $Su(z)12^1$ on the other hand encodes a 560 amino acid protein contain the zinc finger and one BNLS but lack the major part of the VEFS box. This potential protein might have a dominant negative effect, which could explain its sometimes differing properties. In $Su(z)12^2$ a single amino-acid substitution, from a neutrally charged Gly to a negatively charged Asp, at codon 274, N-terminal to both the VEFS box and the zinc finger, causes lethality at the pupal stage, which implies that this part of the protein also is of major importance for its function.

Expression pattern of *Su(z)12*

Northern blot analyses reveal that *Su(z)12* is expressed throughout the entire life of the fly. The mRNA is highly expressed in oocytes and during early development

and the transcripts found during these stages are of four different sizes, determined to be approximately; 4.0, 3.7, 3.4 and 3.2 kb (Fig. 1A-C; paper IV). All four transcripts are expressed in ovaries and sustain for 2 hours of embryonic development until stage 4 of embryogenesis. After this, only the largest transcript continues to be expressed. The SU(Z)12 protein is expressed ubiquitously in the embryo (Fig. 4A; paper IV) and also in wing imaginal discs and larval brain. SU(Z)12 was found in nuclei of ovaries and salivary glands, which is not surprising, due to the NLS and BNLS found the protein (Fig. 4 and C; paper IV). Developmental Western blots have corroborated the existence of the 100 and 95kDa proteins (not shown).

***Su(z)12* mutations are dominant suppressors of PEV**

All mutant *Su(z)12* alleles have been shown to be dominant suppressors of the PEV mediated silencing on the *white*$^{+}$ gene in w^{m4} (Fig.4; paper I). *Su(*z*)12*^{1} is a weaker suppressor than the other mutant alleles, suggesting *Su(z)12*^{1} has a different effect from the other mutant alleles. However, these results imply that *Su(z)12*$^{+}$ also has a function in heterochromatin mediated repression which is an unusual characteristic for a Pc-G gene. The Pc-G repressors usually do not affect PEV, with the exceptions of *E(Pc*), that is a suppressor of PEV, and *Asx* and *E(z),* in which mutations have been reported to be weak modifiers of PEV (Laible et al. 1997; Sinclair et al.1998a; Sinclair et al. 1998b). However, neither E(PC) nor ASX have so far been included in any of the purified Pc-G complexes. Intriguingly, *Su(*z*)12*^{1} is a weaker suppressor of PEV than the other mutant alleles while it has a stronger effect as a Pc-G mutant (see below). This might suggest that loss of function and dominant-negative mutations have different effects in the two silencing systems; repression of homeotic genes and repression via heterochromatin.

***Su(z)12*^{1} interacts genetically with other Pc-G genes**

Su(z)12^{1} has in itself a weak extra sex comb phenotype. After out-crossing the balanced strain to wild-type, around 30% of the male offspring will have a T2 leg with a sex comb consisting of 1-2 teeth. These transformations are not seen in the other *Su(z)1*2 alleles. This phenotype indicates a weak haploinsufficiency, which is not overcome by the maternally deposited gene products. We screened for genetic interactions with *Su(z)12*^{1} and *Su(z)12*^{4} with other Pc-G mutations and showed significant enhancement of the sex comb trait with *Pc*^{11}, *Pcl*^{7}, *esc*^{9}, and *E(Pc)*^{1} (Table 1; paper III). The potential null allele *Su(z)12*^{4} did not give these effects, supporting the hypothesis that *Su(z)12*^{1} is a dominant negative protein that can interfere with the wild-type protein and its function.

***Su(z)12* mutations cause misexpression of homeotic genes**

The early embryonic lethality of strong *Su(z)12* alleles suggests that the gene has a function in development. Still the embryos die without any obvious phenotypes, such as homeotic transformations. However, the genetic interaction with other Pc-G genes prompted us to investigate if *Su(z)12* could be a Pc-G gene and thus affect regulation of the homeotic genes. Therefore *Su(z)12* mutant embryos were analyzed for misexpression of the homeotic genes *Abd-B* and *Ubx* (Fig. 2; paper I). Embryos, homozygous for any of the four EMS-induced *Su(z)12* alleles 2-5 show very subtle misexpression of *Ubx*. However $Su(z)12^{1}$ homozygotes show substantially more misexpression, again suggesting that $Su(z)12^{1}$ is not a simple loss-of-function allele but is an antimorphic allele that encodes a product that interferes with the function of maternally deposited, wild-type SU(Z)12.

A likely explanation for the lack of derepression of homeotic genes would be that maternally deposited wild-type *Su(z)12* product partly rescues these mutant embryos. We therefore generated *Su(z)12* mutant germ cells by using FLP/FRT recombinase. Females with clones of *Su(z)12* mutant germ cells were crossed to males heterozygous for *Df(3L)kto2*. Embryos derived from $Su(z)12^{2}$ *and* $Su(z)12^{5}$, lacking both maternal and zygotic $Su(z)12^{+}$ showed extensive misexpression of *Ubx* (Fig. 2B; paper I and data not shown). When developed further the early larvae showed severe homeotic transformations with phenotypes where all abdominal, thoracic and several head segments were transformed into copies of the eight abdominal segment (Fig. 2C; paper I). Furthermore, germ line mutants for any of the other three *Su(z)12* alleles (1,3,4) failed to develop or developed into highly abnormal eggs (paper I). These results propose a role for *Su(z)12* in germ line development. In contrast, germ cells mutant for many other Pc-G members are able to complete oogenesis (Breen and Duncan, 1986; Soto et al. 1995) although *E(z)*, *crm* and *mxc* seem to be required for germ cell development (Phillips and Shearn, 1990; Yamamoto et al.1997; Saget et al. 1998).

We also assayed for silencing of the homeotic genes *Ubx* and *Abd-B* in wing imaginal disc clones, made homozygous for the different *Su(z)12* mutations. $Su(z)12^{1}$ *and* $Su(z)12^{4}$ mutant clones showed strong misexpression of both *Ubx* and *Abd-B* in most mutant cells (Fig.3A; paper I). $Su(z)12^{3}$ mutant clones don't show this misexpression but we found that these clones were much smaller than those obtained with the other *Su(z)12* alleles (data not shown) implying a role for $Su(z)12^{+}$ in cell proliferation. However, it is puzzling that the two potential null alleles, $Su(z)12^{3}$ *and* $Su(z)12^{4}$, show such differences in this aspect. One can speculate that the 80 amino acid difference in the respective proteins has an important function in

the wild-type protein and that this extra motif in the SU(Z)12^{3} can exhibit antimorphic interfering functions with its targets or co-workers, compared to the SU(Z)12^{4} protein. The position of the lesion in the *Su(z)12*^{2} allele right in this region supports its importance. However, the differences in phenotype could also be due to a second site mutation.

The strong misexpression of homeotic genes in embryos and in larval tissues homozygous for *Su(z)12* mutations clearly classifies *Su(z)12* as a Pc-G gene.

***Su(z)12* mediates silencing via PREs**

We found that *Su(z)12*^{1} dominantly suppresses the PRE mediated silencing of a *mini-white* gene in four different transgenic lines. The PREs used in these lines where either *Fab-7* or *Mcp* originating from the BX-C (Fig.1; paper III). Some of the constructs used had previously been shown to be sensitive either to Pc-G mutants, PEV modifiers or in their pairing requirements (M. Muller, personal communication; Muller et al. 1999; Hagstrom et al. 1997). *Su(z)12*^{1} modifies all these transgenes, which supports our previous findings that *Su(z)12* acts both as a Pc-G gene and as a suppressor of PEV (paper I).

Anti-SU(Z)12 stainings on polytene chromosomes show that SU(Z)12 physically binds to PREs in vivo. Binding is shown to both Mcp (Fig. 2; paper III) and *Ubx*PRE (Fig. 6C and D; paper II). SU(Z)12 also binds to the original BX-C locus at 89D/E (Table 4; paper III). Together, these results strongly indicate that wild-type SU(Z)12 protein physically binds to the regulatory regions of *Abd-B* and *Ubx* and mediates its silencing function on these target genes through PREs.

SU(Z)12 has a unique binding pattern on polytene chromosomes

Given that SU(Z)12 is a nuclear protein and interacts with Pc-G and PEV we wanted to investigate if this was a chromatin binding protein. Anti-SU(Z)12 stainings of polytene chromosomes show that SU(Z)12 like other Pc-G proteins binds to more than 100 euchromatic bands and around 50% of these overlap with other Pc-G proteins, or the trx-G proteins, ZESTE or TRX (Table 4; paper III). We also find that SU(Z)12 binds weakly to the heterochromatic centromeric region (Fig. 3; paper III) consistent with the suppressor of PEV phenotype. Furthermore, we found that SU(Z)12 binds to the BX-C at 89 D/E, which is consistent with the fact that *Su(z)12* is needed for silencing of *Ubx* and *Abd-B*.

These stainings where carried out with four different antibodies, and these show big differences in target site recognition. This suggests that SU(Z)12 is present in

several silencing complexes with different protein compositions resulting in divergent affinities for the different antibodies.

SU(Z)12 is part of Pc-G complexes

SU(Z)12 has been identified in a 600kDa ESC/E(Z) Pc-G complex (Ng et al. 2000; Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002; Tie et al. 2003). Recently, we have shown that it is also a subunit of a 1MDa ESC/E(Z) complex distinguished from the 600 kDa complex by the presence of the Pc-G protein PCL and the histone deacetylase RPD3 (Fig.2; paper II). This is consistent with the observed genetic interactions between *Su(z)12* and *Pcl* and *esc*. Surprisingly, no interaction was found with either *E(z)* or *Rpd3* (Table 1; paper III) in spite of the biochemical proofs of protein interactions. The fact that SU(Z)12 is found in an embryonic silencing complex with E(Z)/ESC and PCL, but also show interactions with *Pc* and *E(Pc)* supports an hypothesis that the SU(Z)12 protein is present in several silencing complexes in different temporally and spatially regulated patterns.

***Su(z)12* in other organisms**

As discussed previously SU(Z)12 has protein homologues in humans and plants, but also other organisms, for example *Anopheles gambiae*, *Fugu rubripes* and mouse have homologues. However, the gene is not found in *C. elegans* or yeast.

The zinc finger motif and the VEFS box are well conserved in the proteins in humans and *Arabidopsis* (Nagase et al. 1995; Grossniklaus et al. 1998; Gendall et al. 2001; Yoshida, 2001) (Fig. 5; paper I). The three SU(Z)12-related proteins in *Arabidopsis*, have been identified as regulators in plant development (Grossniklaus et al. 1998; Gendall et al. 2001; Yoshida 2001). Of the other *Drosophila* Pc-G genes, only *E(z)* and *esc* are also conserved in plants and the E(Z) homologue is needed for repression of floral homeotic genes in leaves (Goodrich et al. 1997). Therefore it seems likely that at least some of the regulatory machinery that controls homeotic gene expression is conserved between animals and plants. It is interesting to note that, the partners in the ESC/E(Z) Pc-G complex; SU(Z)12, E(Z) and ESC, all have homologues in plants while most other Pc-G proteins do not. This suggests that ESC/E(Z) might represent a more ancient repressor system.

Many *Drosophila* Pc-G genes have homologues in mice and these have been shown to be needed for repression of homeotic genes (reviewed by van Lohuizen, 1998) and it seems likely that vertebrate SU(Z)12 also have this function. However, recent publications concerning the human homologue of *Su(z)12* have shown that the gene is associated to various tumours suggesting it might be involved in regulation of cell

proliferation. A certain type of translocation with a breakpoint in the human *Su(z)12* gene is found in endometrial stromal tumours and it has also been shown to be over-expressed in various tumours (Weinmann et al. 2001; Koontz et al. 2001). This suggests that *Su(z)12* is a proto-oncogene that might have a function in cell cycle regulation and therefore *Su(z)12* has been suggested to be a potential target for an antitumour agent in cancer therapy (Kirmizis et al. 2003).

CONCLUSIONS

The role of *Suppressor of zeste 12* in epigenetics;

- ***Su(z)12* is a Polycomb group gene**, since it is needed for silencing of the homeotic genes *Abd-B* and *Ubx*.

- ***Su(z)12* mediates this silencing via PREs.** SU(Z)12 binds to the BX-C locus and also to at least two transgenic PREs *in vivo*.

- ***Su(z)12* is required for germ cell development**. Mutations in *Su(z)12* causes abnormal eggs or absence of egg development.

- ***Su(z)12* affects heterochromatin.** Mutations in *Su(z)12* are suppressors of PEV and SU(Z)12 binds to heterochromatic regions in the chromocenter of polytene chromosomes.

- **SU(Z)12 is found in E(Z)/ESC embryonic silencing complexes**. Co-immunoprecipitations and protein fractionations have shown that SU(Z)12 is a subunit in two ESC/E(Z) complexes.

- ***Su(z)12* interact genetically with other Pc-G genes.** Significant enhancement of an extra sex comb phenotype was shown in combination with *Pc*, *Pcl*, *esc*, and *E(Pc)*.

- **SU(Z)12 has a unique binding pattern on polytene chromosomes.** SU(Z)12 binds to 145 specific sites on polytene chromosomes, of these 50% are unique for SU(Z)12 when compared to a group of Pc-G or trx-G proteins. Different antibodies directed against separate parts of SU(Z)12 give diverse patterns.

In summary, SU(Z)12 is part of two different E(Z)/ESC silencing complexes, interacts with PC and E(PC), effects heterochromatin and has a unique binding pattern on polytene chromosomes. Taken together these results support an hypothesis that the SU(Z)12 protein is present in several silencing complexes and

has functions both in silencing of the homeotic genes and in formation of heterochromatin.

Acknowledgments

I wish to express my sincere appreciation to all those who in different ways have helped me to finally put together this thesis. In particular **I would like to thank;**
My supervisor **Åsa Rasmuson-Lestander**, for support and encouragement and excellent guidance in the scientific world, for always having time for my questions and thus sharing her knowledge about *zeste*, *white*, *polycomb* and all the others.
Jan Larsson, for great support and excellent supervision in lab and theory, for valuable advise concerning this thesis and for understanding the absolute beauty of polytene chromosomes.
Stefan Andersson Escher, who talked me into starting these studies and with his enthusiasm made me believe I could do it, thanks for support and ”Lingon” friendship.
Anssi Saura, for his great knowledge in Genetics and geneticists, and in all historical and religious genres, and for support (especially the warm coffee bread) and advise concerning this thesis.
Anja Saura, for her excellent guidance in mapping polytene chromosomes.
David Roberts, for honouring our Department with his visits and always being encouraging and inspiring.
Helena Östbye, for always being helpful with all administrative arrangements and not being angry with me when I lose my keys or entrance card for the Xth time.
Kerstin Kristiansson, for excellent help with the lab work.
Karin Ekström, for her knowledge about flies and fly stocks.
Marianne Rasmuson, for good statistical advise.
Anna-Sara, **Gunilla**, **Maggan**, for countless tubes and bottles with fly food.

My PhD student colleagues;
Sa , for being a great and helpful friend and ambitious and skilled co-worker, I am really glad she joined me in the Su(z)12 project.
Jesper, for good company in the fly room and for opening my ears to radio P1.
Elisabeth, for being a great room mate and for good advise concerning life and health.
Per, for introducing movie making, translocating and partying at the department.
Magnus, for being very helpful and kind in spite of his efforts to be evil.
Marcus, for believing I could understand *some clatsdisdtics*.
Mikael, for not abandoning his old colleagues when they need his acting skills.
Malin, for appreciating good British music.
Ammie, for nice interesting chats during her, too few, visits to Umeå.

Thanks to all of you, the (now previous) Department of Genetics have been a joyful and happy place and it has always (well, maybe a few exceptions) been a pleasure to go to work.

I am also grateful to **Jurg Müller** and **Peter Harte** and their co-workers. Thanks for good collaboration !

I would also like to thank all the helpful people at BMG that make teaching smooth and pleasant. In particular I would like to thank **Birgitta**, who has full control of all administrative stuff and always helps out when things don't work as you expected, **Gun** and **Carola** who even at short notice prepare solutions and arrange for the student labs and **Björn** for taking care of the flies.

Many people outside the University have also supported and encouraged me during these yaers, theefore I would also like to thank;

my dear dear family, my parents Ewa and John, my sister Linda, my cousin Johan, my grandmother Birgit and all the others. Thanks for all love and support and for always being there for me. A special thank to my mother, who has done a great job taking care of my sons and my home when I wrote this thesis. A special thank also to my sons Filip and Johan for having confidence in their mum who "doesn't come home until it's night" since "she is sort of building a moon rocket," and to their father Simon for carrying the heavier burden of child care during these "thesis writing" months.

all my wonderful friends. Thanks for all support and care, for feeding me excellent food and home baked bread, for moral advise and support, for spelling and grammar suggestions, for listening to my complains, and lots lots more…..

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