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Su(z)12, a novel *Drosophila* Polycomb group gene that is conserved in vertebrates and plants

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SUMMARY

In both *Drosophila* and vertebrates, spatially restricted expression of HOX genes is controlled by the Polycomb group (PcG) repressors. Here we characterize a novel *Drosophila* PcG gene, *Suppressor of zeste 12* (*Su(z)12*). *Su(z)12* mutants exhibit very strong homeotic transformations and *Su(z)12* function is required throughout development to maintain the repressed state of HOX genes. Unlike most other PcG mutations, *Su(z)12* mutations are strong suppressors of position-effect variegation (PEV), suggesting that *Su(z)12* also functions in heterochromatin-mediated repression. Furthermore, *Su(z)12* function is required for germ cell development. The Su(z)12 protein is highly conserved in vertebrates and is related to the *Arabidopsis* proteins EMF2, FIS2 and VRN2. Notably, EMF2 is a repressor of floral homeotic genes. These results suggest that at least some of the regulatory machinery that controls homeotic gene expression is conserved between animals and plants.

Key words: *Su(z)12*, Polycomb group, *Drosophila melanogaster*

INTRODUCTION

In both flies and vertebrates, HOX genes are expressed in spatially restricted patterns to control development of the head, trunk and limbs (Lewis, 1963; Lewis, 1978; McGinnis and Krumlauf, 1992). HOX genes have the potential to be active both within and outside of their correct expression domains, but each HOX gene is selectively silenced in cells where it must remain inactive. This silencing depends on the function of the Polycomb group (PcG) genes (Kennison, 1995; Simon, 1995; Pirrotta, 1998). PcG genes were first identified in *Drosophila* due to mutant phenotypes that suggested that their products function in repressing multiple HOX genes (Lewis, 1978; Struhl, 1981; Duncan, 1982; Ingham, 1984; Jürgens, 1985). Subsequent studies showed that mutations in 11 PcG genes indeed cause misexpression of HOX genes in embryos and in larvae. These genes are *Polycomb* (*Pc*), *Polycomblike* (*Pcl*), *polyhomeotic* (*ph*), *Posterior sex combs* (*Psc*), *Sex combs on midleg* (*Scm*), *Sex combs extra* (*Sce*), *super sex combs* (*sxc*), *pleiohomeotic* (*pho*), *Enhancer of zeste* (*E(z)*), *extra sex combs* (*esc*) and *Additional sex combs* (*Asx*) (Beachy et al., 1985; Ingham, 1985; Struhl and Akam, 1985; White and Wilcox, 1985; Cabrera et al., 1985; McKeon and Brock, 1991; Simon et al., 1992, Fritsch et al., 1999; Beuchle et al., 2001). Most PcG proteins are conserved in both sequence and function in vertebrates (Brunk et al., 1991; van Lohuizen et al., 1991; Pearce et al., 1992; van der Lugt et al., 1994; Müller et al., 1995; Schumacher et al., 1996; reviewed in van Lohuizen, 1998). However, only *E(z)* and *esc* appear to be more widely conserved. In *C. elegans*, sequence homologues of *E(z)* and *esc* function in germline development but, strikingly, they apparently have no role in the regulation of homeotic genes (Holdeman et al., 1998; Korf et al., 1998; Kelly and Fire, 1998). However, in *Arabidopsis*, the E(z)-like protein CURLY LEAF (CLF) functions as a transcriptional repressor of floral homeotic genes in vegetative tissues (Goodrich et al., 1997). This conservation of Polycomb group gene function in plants is particularly striking since HOX genes in animals and homeotic genes in plants are structurally unrelated (McGinnis et al., 1984; Yanofsky et al., 1990).

In *Drosophila*, all PcG genes are expressed in the female germline and maternally deposited wild-type protein often rescues homozygous mutant embryos to a considerable extent (Struhl, 1981; Breen and Duncan, 1986; Soto et al., 1995). Embryos that are doubly homozygous for mutations in two different PcG genes typically show strongly enhanced homeotic transformations, and the phenotype of such embryos is often similar to the null phenotype of the corresponding single mutants (i.e., lacking both maternal and zygotic gene function). Jürgens (1985) used this striking property and generated embryos that were doubly homozygous for PcG mutations and large chromosomal deficiencies. From these tests he estimated that the total number of PcG genes in the *Drosophila* genome would be in the range of 30 to 40 genes

(Jürgens, 1985). Although this number is frequently cited, only two *Drosophila* genes with bona fide PcG mutant phenotypes have been described since Jürgens' original proposal 15 years ago. These are *multi-sex combs* (*mxc*; Santamaria and Randsholt, 1995) and *cramped* (*crm*; Yamamoto et al., 1997).

We report the mutant phenotypes and molecular analysis of a new PcG member, *Suppressor of zeste 12* (*Su(z)12*). *Su(z)12* mutants show very strong homeotic phenotypes caused by widespread misexpression of HOX genes. The phenotypes of *Su(z)12* mutants are comparable to those of the strongest PcG mutants. However, our analyses of *Su(z)12* also reveal some striking properties that distinguish this gene from most other PcG genes; *Su(z)12* function is needed for the development of germ cells and *Su(z)12* loss-of-function mutations suppress PEV. Moreover, *Su(z)12* is not only conserved in vertebrates, but is also related to *Arabidopsis* proteins that function as regulators of floral homeotic genes and other developmental processes.

MATERIALS AND METHODS

Drosophila strains

The four EMS-induced *Su(z)12* alleles, $Su(z)12^3$, $Su(z)12^2$, $Su(z)12^4$ and $Su(z)12^5$ were originally called $l(3)76BDo^1$, $l(3)76BDo^2$, $l(3)76BDo^4$ and $l(3)76BDo^5$, respectively, and their isolation as mutations that fail to complement *Df(3L)kto2* has been described (Kehle et al., 1998). The $Su(z)12^1$ allele was isolated in a P-element mutagenesis screen for mutations that modify the eye color of z^1 mutants. Mutagenized males were mated to z^1 *Dp(1;1)w* $z^{+R61e19}$ virgin females and the male offspring were screened for suppression or enhancement of the yellow eye color. One red-eyed male was isolated and subsequently crossed with balancers to establish a stock. Of several revertants of $Su(z)12^1$, obtained by mobilizing the P element, we isolated five that were viable and fertile in *trans* to $Su(z)12^1$.

Genetic analyses

All *Su(z)12* alleles were recombined onto an FRT2A chromosome to obtain the following strains:

w; Su(z)12^1 *FRT2A/ TM6C, cu Sb e Tb ca*
w; Su(z)12^2 *FRT2A/ TM6C, cu Sb e Tb ca*
w; Su(z)12^3 *FRT2A/ TM6C, cu Sb e Tb ca*
w; Su(z)12^4 *FRT2A/ TM6C, cu Sb e Tb ca*
w; Su(z)12^5 *FRT2A/ TM6C, cu Sb e Tb ca*

Germline clones of each mutant were generated using the standard ovo^D technique. No eggs from germ-line clones were obtained in the case of $Su(z)12^3$ and $Su(z)12^4$. To 'clean' the left arm of the $Su(z)12^3$ and $Su(z)12^4$ chromosomes from other potential lethal mutations, we substituted most of the chromosome arm distal to the *Su(z)12* locus with DNA from a homozygous viable *ru h th st cu sr e ca* chromosome. Four independent ru h th $Su(z)12^3$ *FRT2A* and six independent ru h th $Su(z)12^4$ *FRT2A* recombinant chromosomes were isolated and tested for production of germline clones but no eggs were obtained in either case.

Imaginal disc clones were generated by crossing the appropriate *Su(z)12 FRT2A* mutant strains with either *yw flp122; hs-nGFP FRT2A* or *yw flp122; M(3)*i^{55}*hs-nGFP FRT2A/TM6B* flies and heat-shocking the F_1 larvae. Heat shock treatment to induce clones was done in vials for 1 hour in a 37°C water bath, and the larvae were then allowed to develop for the appropriate time at 25°C. Prior to dissection, larvae were subjected to another 1 hour heat shock followed by a 1 hour recovery period to induce expression of the GFP marker protein.

Effects on PEV were analysed by crossing *Su(z)12* mutant males to $In(1)w^{m4}$ females and comparing the eye phenotypes of the $In(1)w^{m4}$; *Su(z)12/+* and $In(1)w^{m4}$; *Balancer/+* male progeny.

Antibody staining

Antibody staining of embryos with antibody against Ubx protein was done following standard protocols. Imaginal discs were stained with antibodies against Ubx or Abd-B and GFP proteins as described (Beuchle et al., 2001).

Cloning of *Su(z)12*

A LAMBDA library of *Eco*RI digested genomic DNA was generated from $Su(z)12^1$ heterozygotes and screened using P-element sequences as probe. A subclone containing a 2.2 kb insert was isolated; this insert contained P-element sequences and 1.6 kb of flanking genomic DNA. This genomic fragment was used as a probe to isolate a larger genomic fragment from an EMBL 4 library and, with that as a probe, cDNAs for three different transcription units were isolated from an embryonic cDNA library (Clontech). Northern blot analysis revealed that one of these transcripts showed an altered pattern in $Su(z)12^1$ mutants. Two EST clones with 5′ sequences identical to this cDNA were obtained from the Berkeley Drosophila Genome Project (LD13365 and LD02025). LD02025 was sequenced and LD13365 was partially sequenced. Introns were mapped by use of internal primers, PCR amplification and sequencing. The *Su(z)12* gene was mapped to 76D using a digoxigenin-labeled probe for in situ hybridisation on polytene chromosomes from salivary glands.

Sequencing of the EMS-induced *Su(z)12* alleles was done as follows. Genomic DNA was isolated from $Su(z)12^3$ or $Su(z)12^4$ heterozygotes or, in the case of $Su(z)12^2$, from $Su(z)12^2$ homozygous larvae that were identified by the *red* marker mutation on the mutant chromosome. In each case, the genomic DNA spanning the *Su(z)12* open reading frame was amplified by PCR. Three overlapping subfragments covering this interval were amplified, subcloned into bluescript and several independent clones were sequenced. In each mutant, only a single base change was found in several independent clones (Fig. 5). For each mutant allele the identified base changes were confirmed by sequencing clones obtained from a second, independent PCR amplification.

RESULTS

Mutations in the *Su(z)12* locus cause misexpression of HOX genes

In a screen for zygotic-lethal mutations in the 76D region (Kehle et al., 1998), we previously identified a lethal complementation group, *l(3)76BDo*, that turned out to be allelic to $Su(z)12^1$, a P-element-induced mutation that was isolated in a screen for modifiers of the *zeste-white* interaction (see Materials and Methods). Since $Su(z)12^1$ fails to complement any of the four EMS-induced *l(3)76BDo* alleles we have renamed $l(3)76BDo^1$, $l(3)76BDo^2$, $l(3)76BDo^4$ and $l(3)76BDo^5$ (Kehle et al., 1998) as $Su(z)12^3$, $Su(z)12^2$, $Su(z)12^4$ and $Su(z)12^5$, respectively.

Animals that are homozygous or hemizygous for $Su(z)12^1$, $Su(z)12^2$, $Su(z)12^3$ or $Su(z)12^4$ die during the first or second larval instar, whereas several transheterozygous combinations with $Su(z)12^5$ develop into pharate adults with strong posteriorly directed homeotic transformations (Fig. 1). These homeotic transformations are consistent with inappropriate activation of several HOX genes in the *Antennapedia* and *bithorax* complexes. For example, the additional sex combs on meso- and metathoracic legs suggests misexpression of *Sex combs reduced* (*Scr*) in these primordia (Pattatucci and

Kaufman, 1991), whereas the antenna to leg transformation is consistent with inappropriate activation of *Antennapedia* (*Antp*) in the eye-antennal disc (Struhl, 1981) and the wing to haltere transformations most likely reflects misexpression of BXC genes in the wing disc (Cabrera et al., 1985; Fig. 1). This suggests that *Su(z)12* acts as a repressor of several homeotic genes and is a member of the PcG.

Since $Su(z)12^1$, $Su(z)12^2$, $Su(z)12^3$ and $Su(z)12^4$ homozygotes die as larvae without any obvious *Pc*-like phenotypes, we next analyzed *Su(z)12* mutant embryos for misexpression of HOX genes (Fig. 2). Embryos that are homozygous for any of the four EMS-induced *Su(z)12* alleles ($Su(z)12^{2-5}$) show very subtle misexpression of *Ubx*; similar misexpression is observed in embryos that are homozygous for

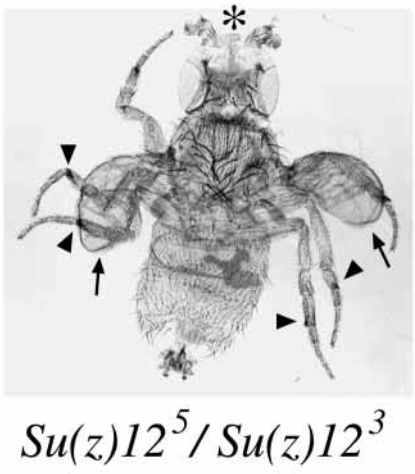


Fig. 1. Homeotic transformations in a *Su(z)12* mutant pharate adult male. Homeotic transformations are evident in several body segments. Sex combs, a structure normally only present on the first leg, are present on the first tarsal segments of all meso- and metathoracic legs (arrowheads); the antennae are partially transformed into legs (asterisk) and wings are much smaller and partially transformed into haltere-like structures (arrows). These homeotic transformations are consistent with inappropriate activation of several ANTC and BXC genes in the imaginal disc primordia of these structures (see text). The genotype of the animal shown is $Su(z)12^5/Su(z)12^3$ but similar homeotic phenotypes are observed in $Su(z)12^5/Su(z)12^4$ pharate adults; these mutant combinations die as pharate adults and never eclose from the pupal case.

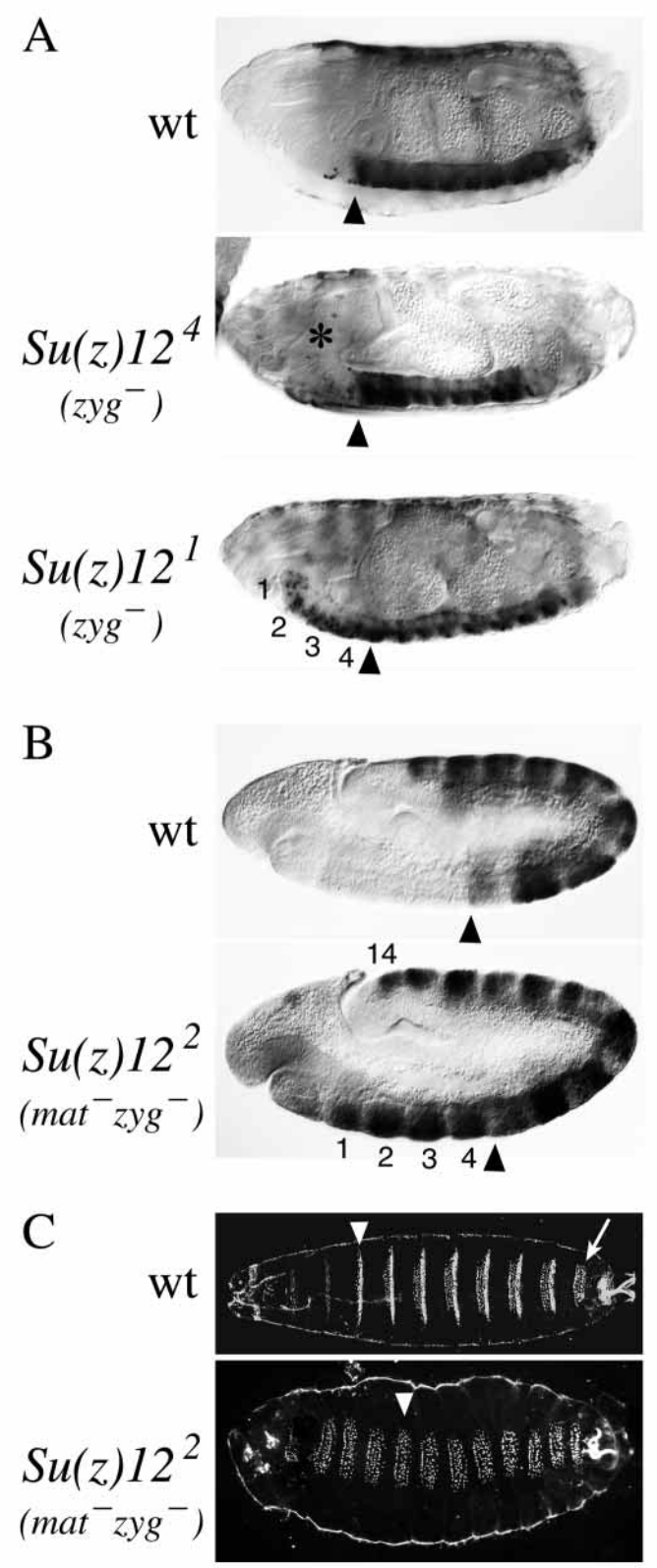


Fig. 2. Misexpression of homeotic genes and homeotic transformations in *Su(z)12* mutant embryos. (A,B) Embryos at stage 16 (A) and stage 11 (B) stained with antibody against Ubx protein. In wild-type (wt) embryos, Ubx is expressed from parasegments (ps) 5 to ps 13 (anterior margin of ps 5 in all cases marked by an arrowhead) and in five midline cells in ps 4 (visible in A). (A) $Su(z)12^4$ homozygous embryos (zyg^-) show misexpression of Ubx anterior to ps 5 but only in a few cells in the CNS and in the brain (asterisk); more extensive misexpression of Ubx is seen in ps 1-4 of $Su(z)12^1$ homozygotes. (B) $Su(z)12^2/Df(3L)kto2$ embryos derived from $Su(z)12^2$ germline clones show strong expression of Ubx from ps 1 to ps 14 already at this earlier stage owing to the lack of both maternal and zygotic ($mat^-\ zyg^-$) wild-type Su(z)12 protein. (C) Cuticles of a wild-type and a *Su(z)12* mutant embryo of the same genotype as in B. In the *Su(z)12* mutant embryo all abdominal, thoracic and several head segments (not all visible in this focal plane) are homeotically transformed into copies of the eighth abdominal segment owing to misexpression of the *Abd-B* gene in every segment (the eighth abdominal segment is marked by an arrow in the wild-type embryo and the first abdominal segment is marked by a white arrowhead). $Su(z)12^5/Df(3L)kto2$ embryos derived from $Su(z)12^5$germline clones show similar misexpression of Ubx and cuticle phenotypes like the embryos shown in B and C (data not shown).

Df(3L)kto2, a deficiency that deletes the *Su(z)12* locus (Fig. 2 and data not shown). Interestingly, *Su(z)12*^{1} homozygotes show substantially more misexpression than embryos that are homozygous for the deficiency or for any of the other alleles (Fig. 2; see below). A likely explanation for these subtle *Pc*-like phenotypes is that maternally deposited wild-type Su(z)12 product rescues these *Su(z)12* mutant embryos.

We therefore attempted to generate embryos from *Su(z)12* mutant germ cells. Females with *Su(z)12* mutant germ cells were crossed to males heterozygous for *Df(3L)kto2*. We found that *Su(z)12* hemizygous embryos derived from *Su(z)12*^{2} or *Su(z)12*^{5} germ cells showed very extensive misexpression of *Ubx* already at the extended germ band stage (Fig. 2 and data not shown). These animals showed severe homeotic phenotypes with all abdominal, thoracic and several head segments transformed into copies of the eight abdominal segment. This phenotype is consistent with *Abd-B* being misexpressed in all segments (Fig. 2 and data not shown). The strong PcG phenotype of these *Su(z)12* mutant embryos is comparable to that of embryos lacking *esc* or *Pc* function (Struhl, 1981; Lawrence et al., 1983). We find that zygotically provided *Su(z)12* function is sufficient to prevent the inappropriate activation of HOX genes; *Su(z)12*^{2}/+ heterozygotes obtained as the progeny of *Su(z)12* mutant germ cells and a wild-type sperm develop into wild-type-looking adults.

In contrast to *Su(z)12*^{2} or *Su(z)12*^{5}, we found that germ cells mutant for any of the other three *Su(z)12* alleles failed to develop (*Su(z)12*^{3} and *Su(z)12*^{4}) or developed into highly abnormal eggs (*Su(z)12*^{1}). Two observations suggest that the failure to obtain embryos in the case of *Su(z)12*^{1} and *Su(z)12*^{4} is not caused by second-site mutations on the *Su(z)12* mutant chromosomes but can be attributed to a requirement for *Su(z)12* function in germ-cell development. First, we found that revertants obtained by excision of the P element in the *Su(z)12*^{1} allele are viable and fertile. Second, *Su(z)12*^{4} mutant germ cells still failed to develop even after "cleaning" the chromosome from other potentially lethal mutations by replacing the chromosomal DNA flanking this *Su(z)12* allele with unmutagenized wild-type DNA (see Materials and Methods). Hence, these results suggest that *Su(z)12* function is essential for the development of germ cells. Furthermore, *Su(z)12*^{1}, *Su(z)12*^{3} and *Su(z)12*^{4} are strong alleles and *Su(z)12*^{2} and *Su(z)12*^{5} are weaker alleles (see below). The

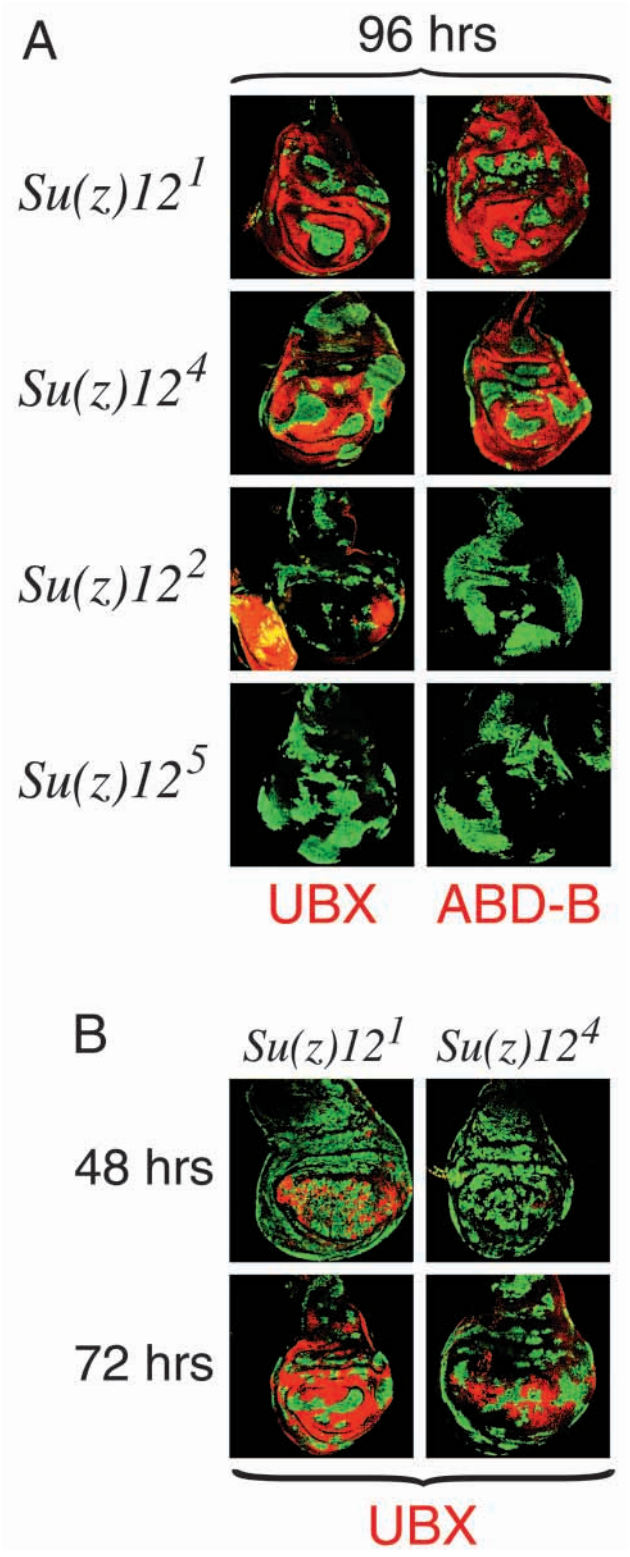


Fig. 3. *Su(z)12* function is required throughout development to repress HOX genes. Wing imaginal discs with clones of cells that are homozygous for the indicated *Su(z)12* allele were stained with antibodies against GFP (green) and Ubx or Abd-B protein (red) as indicated. Neither Ubx nor Abd-B proteins are normally expressed in the wing imaginal disc. In each case, homozygous *Su(z)12* mutant cells are marked by the absence of GFP protein (green) and in all experiments the Minute technique was used (see text). (A) Clones were induced 96 hours before analysis. Strong misexpression of Ubx and Abd-B protein is detected in clones that are homozygous for the strong alleles *Su(z)12*^{1} and *Su(z)12*^{4}; note that both homeotic genes are derepressed in almost all clones, throughout the disc. In clones homozygous for the hypomorphic allele *Su(z)12*^{2}, misexpression of *Ubx* occurs only in clones in central regions of the wing pouch and in the hinge region; *Abd-B* is not derepressed in these clones. No derepression of *Ubx* or *Abd-B* is detected in *Su(z)12*^{5} mutant clones, consistent with the genetic data that this allele is a weaker hypomorph than *Su(z)12*^{2}. (B) Kinetics of derepression of *Ubx* and *Abd-B* in *Su(z)12*^{1} and *Su(z)12*^{4} mutant clones. 48 hours after clone induction *Ubx* is still repressed in almost all *Su(z)12*^{4} mutant clones but *Su(z)12*^{1} mutant clones in the wing pouch already show strong misexpression of *Ubx*. 72 hours after clone induction, most *Su(z)12*^{1} mutant clones show strong misexpression of *Ubx*, whereas only *Su(z)12*^{4} mutant clones in the pouch show strong Ubx signal and *Ubx* is apparently still repressed in other regions of the disc.

fact that *Su(z)12*^{1} homozygous embryos show more severe misexpression than *Df(3L)kto2* homozygotes suggests 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 protein. We note that *Su(z)12*^{1}/+ embryos show no misexpression of homeotic genes in the embryo (not shown).

We next tested the requirement for *Su(z)12* at later developmental stages by generating *Su(z)12* mutant clones in imaginal discs. We assayed for HOX gene silencing in such clones by monitoring the expression of the HOX genes *Ubx* and *Abd-B* in the imaginal wing disc (where they are normally stably repressed) using antisera against their protein products. In these experiments, the *Su(z)12* mutant cells were identified by the absence of a GFP-expressing marker gene (see Materials and Methods). In addition, we used the Minute technique to generate *Su(z)12*$^{-}$/*Su(z)12*$^{-}$clones that carry two copies of a wild-type Minute allele (i.e., *Su(z)12*$^{-}$ *M*$^{+}$/ *Su(z)12*$^{-}$ *M*$^{+}$), which gives them a growth advantage relative to their *Su(z)12*$^{-}$ *M*$^{+}$/ *Su(z)12*$^{+}$ *M*$^{-}$ neighbours.

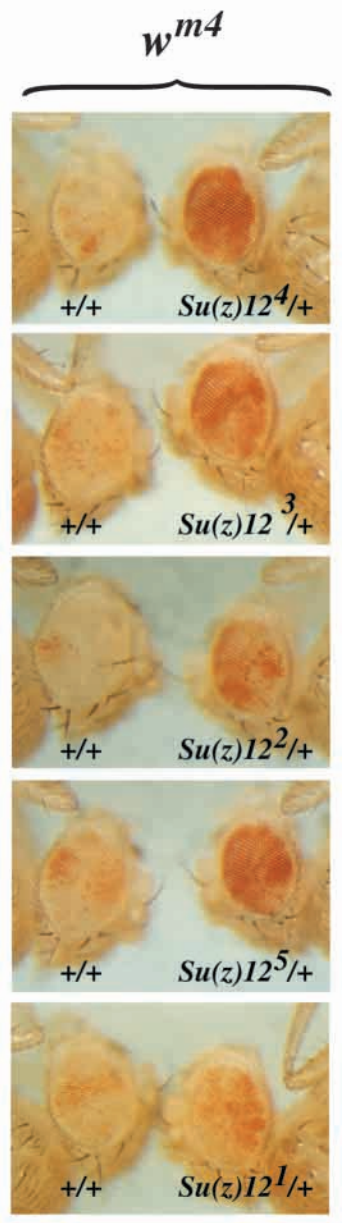
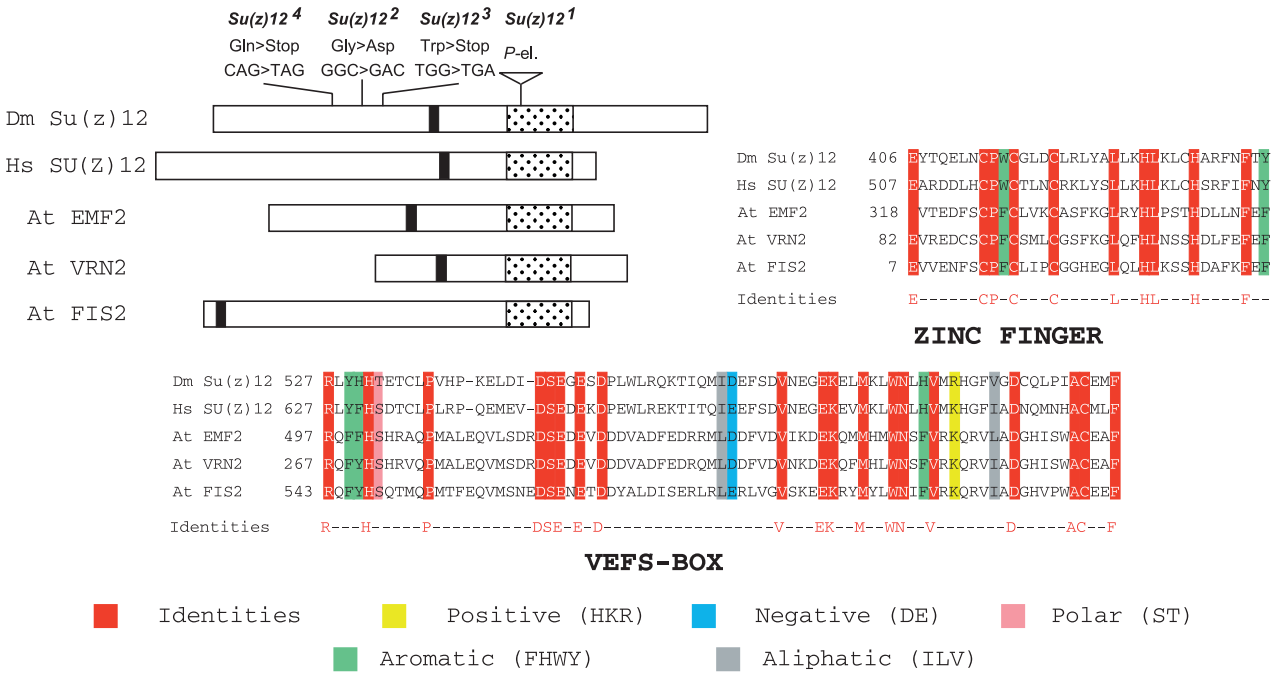


Fig. 4. *Su(z)12* mutations suppress position-effect variegation. Heads of adult flies that are hemizygous for the *w*^{m4} rearrangement. In wild-type flies (left), pigmentation in eyes is drastically reduced owing to silencing of the *white* (*w*) gene in most ommatidia. In animals that are heterozygous for any of the *Su(z)12* alleles (right), there is a partial release from silencing and the *w* gene is expressed in most ommatidia; the loss of silencing is stronger in case of the EMS-induced *Su(z)12* alleles. In each case, the wild-type (+/+) control flies on the left carry the *TM3* balancer chromosome and are the siblings of the corresponding *Su(z)12* mutants.

In a first set of experiments, we analyzed cell clones of the different *Su(z)12* alleles 96 hours after clone induction. We found that *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). *Su(z)12*^{2} mutant clones also showed misexpression of *Ubx* 96 hours after clone induction but misexpression is confined to the pouch and hinge region in the posterior compartment of the wing disc (Fig. 3A). No misexpression of *Abd-B* was detected in *Su(z)12* ^{2} mutant clones and neither *Ubx* nor *Abd-B* were misexpressed in *Su(z)12*^{5} mutant clones (Fig. 3A). We also found no misexpression in *Su(z)12*^{3} mutant clones but we found that these clones were much smaller than those obtained with the other *Su(z)12* alleles (data not shown). We do not know whether the cell proliferation/survival defect associated with the *Su(z)12*^{3} chromosome is caused by a second mutation in a closely linked gene (see Materials and Methods) or is a unique property of this particular allele. In summary, the PcG phenotypes observed with several *Su(z)12* alleles suggest that *Su(z)12* is needed throughout development to keep HOX genes repressed. Moreover, these results support the allele classification obtained by the analysis of germ-line clones; namely, that *Su(z)12*^{2} and *Su(z)12*^{5} are hypomorphic alleles whereas *Su(z)12*^{1} and *Su(z)12*^{4} appear to be stronger alleles.

We next examined the kinetics of HOX gene derepression in *Su(z)12*^{1} and *Su(z)12*^{4} mutant clones by analyzing *Ubx* expression 24, 48 and 72 hours after clone induction. We again used the Minute technique in these experiments. 24 hours after clone induction, *Ubx* is still tightly repressed. 48 hours after clone induction, *Su(z)12*^{1} mutant clones show misexpression of Ubx protein in the wing pouch but *Ubx* is still stably silenced in other parts of the wing disc (Fig. 3B). In *Su(z)12*^{4} mutant clones, *Ubx* is still stably silenced 48 hours after clone induction except in a few clones in the center of the pouch where we detect weak *Ubx* signal (Fig. 3B). Finally, 72 hours after clone induction, repression of *Ubx* is lost in most *Su(z)12*^{1} and *Su(z)12*^{4} mutant clones in the pouch, in the latter case *Ubx* is still silenced in some parts of the disc (Fig. 3B). This slow and gradual loss of silencing is comparable to the kinetics of HOX gene derepression in *Pc*, *Pcl*, *Scm* or *Sce* mutant clones (Beuchle et al., 2001).

We note that the loss of silencing occurs more rapidly in *Su(z)12*^{1} clones than in *Su(z)12*^{4} clones (Fig. 3). The molecular characterization of *Su(z)12*^{4} suggests that this is most likely a null allele (see below). Our analysis of *Su(z)12*^{1} homozygous embryos suggested that *Su(z)12*^{1} is not a simple loss-of-function allele but is an antimorphic allele (see above). It is possible that the more rapid loss of silencing in *Su(z)12*^{1} mutant clones again reflects an interference of the mutant Su(z)12^{1} product with wild-type Su(z)12 molecules (i.e., during the depletion of persisting wild-type Su(z)12 protein after clone induction).



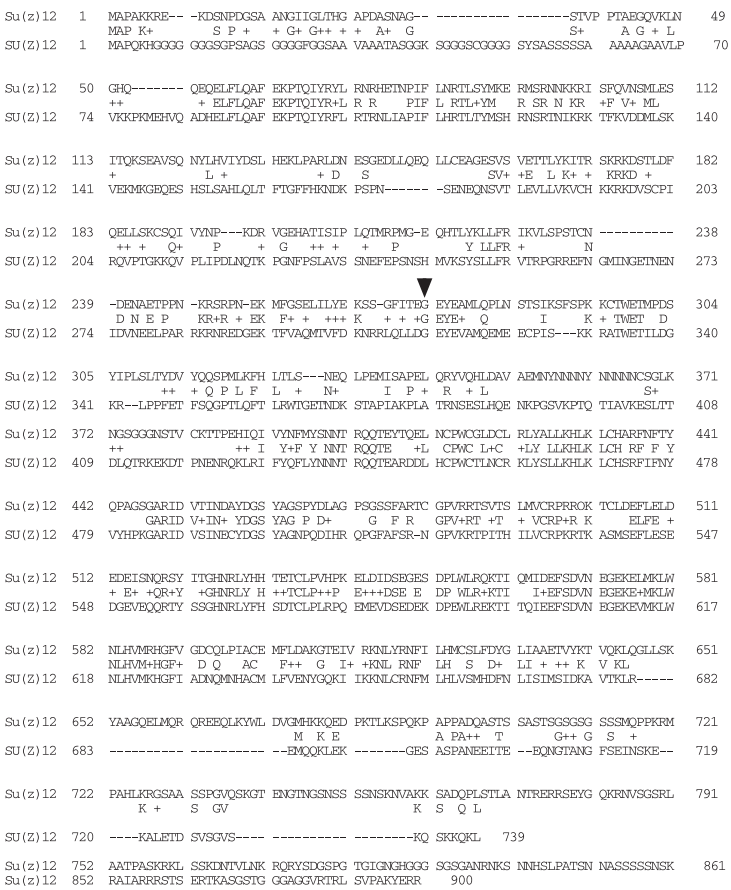


Fig. 5. Lesions in mutant *Su(z)12* alleles and comparison between Su(z)12 and related proteins in humans and in *Arabidopsis*. (Top) Schematic representation of the *Drosophila malnogaster* Su(z)12 (Dm Su(z)12), *Homo sapiens* Su(z)12 (Hs SU(Z)12, KIAA0160) and *Arabidopsis thaliana* (At) EMF2 (N. Yoshida, personal communication), VRN2 (A. Gendall, personal communication) and FIS2 (Luo et al., 1999) proteins (white rectangles) with zinc finger (black box) and VEFS box (stippled box). The locations of the molecular lesions in four different *Su(z)12* alleles are indicated (see text). Alignment of the zinc fingers (top right) and the VEFS box (below). Identical amino acids that are found in all five proteins are boxed in red and listed below the alignments, similarities are boxed in colors using the code listed below. Bottom: alignment of *Drosophila* Su(z)12 and human SU(Z)12 amino acid sequences. Note that the two proteins are conserved over the whole length of the protein. The conserved Gly mutated in *Su(z)12*^{2} is indicated by an arrowhead.

Su(z)12 mutations suppress position-effect variegation

To test whether *Su(z)12* may also participate in other processes of transcriptional silencing, we tested whether *Su(z)12* mutations suppress position-effect variegation (PEV). PEV is observed in chromosomal rearrangements in which a euchromatic gene is placed near heterochromatin. The translocated gene may then become inactivated in a fraction of cells, presumably because transcription of the gene is silenced by heterochromatin-associated proteins. A number of mutations have been identified that suppress or enhance PEV in a dosage-dependent fashion (reviewed by Wakimoto, 1998). Mutations that suppress PEV are generally referred to as *Su(var)s*; some *Su(var)* gene products have indeed been shown to be components of heterochromatin (Eissenberg et al., 1990). One well-studied reporter for PEV is w^{m4}, a chromosomal inversion juxtaposing the *white* gene to centromeric heterochromatin (Muller, 1930). As illustrated in Fig. 4, mutations in *Su(z)12* strongly suppress PEV at the w^{m4} locus; in animals that are heterozygous for any of the five *Su(z)12* alleles, the *white* locus is transcriptionally active in a higher proportion of ommatidia than in control animals. We note that suppression of PEV was observed with four different EMS-induced *Su(z)12* alleles but not with most other mutations that were isolated in the same EMS-mutagenesis experiments, suggesting that suppression of PEV is indeed due to the mutations at the *Su(z)12* locus and not due to other PEV modifiers on the mutagenized chromosomes (data not shown). These results suggest that *Su(z)12* can be classified as a suppressor of PEV.

The Su(z)12 protein is conserved in vertebrates and plants

To identify the *Su(z)12* gene we isolated and cloned the chromosomal DNA flanking the P-element insertion in the $Su(z)12^1$ allele (see Materials and Methods). Using this genomic DNA as probe we isolated several cDNAs by screening a cDNA library and by searching an EST database for cDNAs that match this genomic DNA. Sequence analysis of the longest cDNA (LD 02025) revealed a single open reading frame of 900 amino acids. The P-element in $Su(z)12^1$ is inserted into codon 564 of this open reading frame and would therefore result in a C-terminal truncation of the predicted protein (Fig. 5). Further proof that the identified open reading frame encodes the Su(z)12 protein was obtained by sequencing the coding region of three EMS-induced *Su(z)12* alleles. $Su(z)12^2$, $Su(z)12^3$ and $Su(z)12^4$ each show single base changes in this open reading frame; $Su(z)12^3$ and $Su(z)12^4$ both show base substitutions that result in predicted premature termination codons after codon 218 and codon 298, respectively, and in $Su(z)12^2$, a base substitution changes the codon for Gly^{274} into a codon for Asp (Fig. 5; this glycine is conserved in the human Su(z)12 homologue described below). The molecular characterizations of these lesions prove that we identified the *Su(z)12* open reading frame. Furthermore, these lesions support our classification of the different alleles based on phenotypic criteria; $Su(z)12^2$ and $Su(z)12^4$ both have stop codons in the N-terminal third of the protein and therefore may represent null (or at least strong loss-of-function) alleles whereas the amino-acid substitution in $Su(z)12^2$ is consistent with the idea that this is a hypomorphic allele.

Database searches show that the Su(z)12 protein is highly conserved in vertebrates and, strikingly, that Su(z)12-related proteins also exist in plants (Fig. 5). In contrast, the worm and yeast genomes do not seem to encode Su(z)12-related proteins. The function of the highly conserved human homologue of Su(z)12, HsSU(Z)12 (Fig. 5), is not known but EMF2, FIS2 and VRN2, the three Su(z)12-related proteins in *Arabidopsis*, have been identified as regulators in plant development (Yang et al., 1995; Luo et al., 1999; N. Yoshida, personal communication; A. R. Gendall, personal communication). One characteristic feature of all these proteins is a single classical C_2H_2 zinc finger similar to the fingers found in sequence-specific DNA-binding proteins (Fig. 5). Attempts to show any DNA-binding activity of a polypeptide containing the Su(z)12 zinc finger have failed so far (A. K. S. and J. M., unpublished). A second stretch of amino acids that is conserved between Su(z)12, HsSU(Z)12, EMF2, VRN2 and FIS2 is located C-terminal to the zinc finger (Fig. 5). We term this part of the protein VEFS box (VRN2-EMF2-FIS2-Su(z)12 box). We note that the predicted protein products encoded by $Su(z)12^3$ and $Su(z)12^4$ lack both the zinc finger and the VEFS box, whereas the protein encoded by $Su(z)12^1$ would contain the zinc finger but lack the VEFS box.

DISCUSSION

We report the mutant phenotypes and cloning of *Su(z)12*, a novel *Drosophila* gene. The most notable phenotypes of *Su(z)12* mutants are strong homeotic transformations caused by the widespread misexpression of several HOX genes in embryos and in larvae. These phenotypes clearly classify *Su(z)12* as a PcG gene and our clonal analyses show that *Su(z)12* plays an essential role in HOX gene silencing throughout development. However, a number of properties distinguish *Su(z)12* from most other *Drosophila* PcG genes and we shall discuss these in turn.

Our genetic and molecular analyses suggest that $Su(z)12^3$ and $Su(z)12^4$ are most likely null or at least strong loss-of-function alleles, whereas $Su(z)12^2$ and $Su(z)12^5$ appear to be hypomorphic alleles. $Su(z)12^1$ is also a strong loss-of-function allele but in addition, it also shows properties of an antimorphic allele. Since $Su(z)12^3$ may contain a second, cell-lethal mutation, we will omit this allele for discussion of the *Su(z)12* mutant phenotype and presume that the phenotype of $Su(z)12^4$ represents the *Su(z)12* null phenotype. The analysis of $Su(z)12^1$ and $Su(z)12^4$ germline clones suggests that *Su(z)12* function is essential for germ-cell development and only germ cells carrying hypomorphic *Su(z)12* mutations develop into embryos. By contrast, germ cells mutant for most other PcG members complete oogenesis (Struhl, 1981; Lawrence et al., 1983; Breen and Duncan, 1986; Soto et al., 1995) and only *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). Although we do not know which processes in germ-cell development require *Su(z)12* function, the requirement for *Su(z)12* in the germline clearly distinguishes *Su(z)12* from most other PcG genes.

A second distinction between *Su(z)12* and most other PcG mutants is suggested by the suppression of PEV in *Su(z)12* mutants. Heterochromatin-mediated silencing has often been

compared to HOX gene silencing (e.g. Paro, 1990; Pirrotta and Rastelli, 1994). Although the two processes may use similar molecular mechanisms, they require two distinct sets of proteins; *Su(var)* mutants show no PcG phenotypes and most PcG mutations do not suppress w^{m4} variegation (Kennison, 1995; Sinclair et al., 1998). Among the exceptions (besides *Su(z)12*), mutations in the PcG gene *crm* suppress w^{m4} variegation (Yamamoto et al., 1997). *E(Pc)* mutations also suppress w^{m4} variegation (Kennison, 1995; Sinclair et al., 1998), but it is not clear whether *E(Pc)* is a PcG gene (Soto et al., 1995; Sinclair et al., 1998). Finally, *E(z)* mutations have been reported to weakly suppress w^{m4} variegation (Laible et al., 1997) or to enhance it (Sinclair et al., 1998). Although we favour the interpretation that Su(z)12 protein functions directly in heterochromatin-mediated gene silencing, (e.g. as a component of heterochromatin), we cannot exclude the possibility that the effect on PEV is indirect.

A third, striking feature of *Su(z)12* is its conservation not only in vertebrates but also in plants. Most *Drosophila* PcG proteins have vertebrate homologues and studies on PcG mutant mice showed that these proteins are needed to repress HOX gene transcription outside of the normal HOX expression domains (reviewed by van Lohuizen, 1998). The Su(z)12 protein is highly conserved in humans and hence, it seems likely that vertebrate Su(z)12 homologues are also needed for silencing of HOX genes. Of the other *Drosophila* PcG genes, only *E(z)* and *esc* are also conserved in plants and previous studies showed that the E(z) homologue CURLY LEAF (CLF) is needed for repression of floral homeotic genes in leaves (Goodrich et al., 1997). *Su(z)12* shows sequence similarity with three *Arabidopsis* proteins; FIS2, VRN2 and EMF2. Each of these proteins functions as a regulator to suppress a particular developmental process during plant development. FIS2 is needed to repress seed development in the absence of fertilization, a process that also requires the E(z)- and esc-related proteins FIS1/MEA and FIS3/FIE (Grossniklaus et al., 1998; Luo et al., 1999). VRN2 is needed for the stable repression of FLC, a key regulator that controls flowering (Sheldon et al., 2000, A. R. Gendall, personal communication). Particularly intriguing is the similarity between Su(z)12 and EMF2 (N. Yoshida personal communication). EMF2 acts as a floral repressor by suppressing the onset of reproductive development; EMF2 mutants show misexpression of the floral homeotic genes *APETALA1* (*AP1*) and *AGAMOUS* (*AG*) in germinating seedlings (Chen et al., 1997). Thus, it appears that repression of HOX genes in *Drosophila* and repression of floral homeotic genes in *Arabidopsis* both depend on a conserved set of PcG proteins, Su(z)12 and E(z) in flies and EMF2 and CLF in plants.

The hallmarks of Su(z)12, EMF2, FIS2 and VRN2 are a single C_2H_2 zinc finger and a conserved stretch of amino acids that we named the VEFS-box. In all four genes, the VEFS-box is located C-terminal to the zinc finger. In DNA-binding assays, we have found no evidence that the Su(z)12 zinc finger by itself binds to DNA (A. K. S. and J. M., unpublished data). However, most other PcG proteins also do not bind to DNA directly but bind to chromatin as multiprotein complexes that contain different PcG members (Franke et al., 1992; Strutt and Paro, 1997; Shao et al., 1999; Ng et al., 2000; Tie et al., 2001). It is possible that the Su(z)12 protein also functions in a chromatin-binding protein complex and that in the context of such a complex, the zinc finger is needed for making DNA or protein contacts. As discussed in the following, the comparison of $Su(z)12^4$ and $Su(z)12^1$ mutant phenotypes suggests that the zinc finger and the VEFS box are probably two distinct functional domains. In embryos, $Su(z)12^1$ homozygotes show more extensive misexpression of HOX genes than $Su(z)12^4$ or *Df(3L)kto2* homozygotes, and in imaginal discs, $Su(z)12^1$ mutant clones show a more rapid loss of HOX gene silencing than $Su(z)12^4$ mutant clones. As already discussed, the stronger phenotype of $Su(z)12^1$ mutants may be attributed to the interference of an aberrant $Su(z)12^1$ product with persisting $Su(z)12^+$ protein molecules. The lesion in $Su(z)12^1$ may result in the expression of a truncated polypeptide that contains the zinc finger but lacks the VEFS box, whereas the short polypeptide encoded by the $Su(z)12^4$ allele lacks both the zinc finger and the VEFS box. One possible molecular explanation for the stronger phenotype of $Su(z)12^1$ mutants would therefore be that the truncated $Su(z)12^1$ protein, containing the C_2H_2 zinc finger, competes with wild-type Su(z)12 protein for binding to its natural target (i.e., a DNA sequence or another protein) but is not functional since it lacks the VEFS box and the C terminus. It is possible that the VEFS box is needed for interaction with other (PcG) proteins or, alternatively, that it is a catalytic domain providing an enzymatic activity needed for silencing.

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Note added in proof

A very recent study (Koontz et al., 2001) reports that endometrial stromal tumors in humans show chromosomal rearrangements in which the human homologue of *Su(z)12*, *HsSU(Z)12*, is fused to a zinc-finger protein.

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