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The *Drosophila* Polycomb Group proteins ESC and E(Z) bind directly to each other and co-localize at multiple chromosomal sites

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SUMMARY

The Polycomb Group gene *esc* encodes an evolutionarily conserved protein required for transcriptional silencing of the homeotic genes. Unlike other Polycomb Group genes, *esc* is expressed and apparently required only during early embryogenesis, suggesting it is required for the initial establishment of silencing but not for its subsequent maintenance. We present evidence that the ESC protein interacts directly with E(Z), another Polycomb Group protein required for silencing of the homeotic genes. We show that the most highly conserved region of ESC, containing seven WD motifs that are predicted to fold into a β-propeller structure, mediate its binding to a conserved N-terminal region of E(Z). Mutations in the WD region that perturb ESC silencing function in vivo also perturb binding to E(Z) in vitro. The entire WD region forms a trypsin-resistant structure, like known β-propeller domains, and mutations that would affect the predicted ESC β-propeller perturb its trypsin-resistance, while a putative structure-conserving mutation does not. We show by co-immunoprecipitation that ESC and E(Z) are directly associated in vivo and that they also co-localize at many chromosomal binding sites. Since E(Z) is required for binding of other Polycomb Group proteins to chromosomes, these results suggest that formation of an E(Z):ESC complex at Polycomb Response Elements may be an essential prerequisite for the establishment of silencing.

Key words: Polycomb group, ESC, E(Z), Transcriptional repression, Protein complex, *Drosophila*

INTRODUCTION

extra sex combs (*esc*) is one of the Polycomb Group (PcG) genes, whose products are required for transcriptional silencing of the homeotic genes in cells outside their normal expression domains. Initial expression of the homeotic genes in their characteristic spatially restricted patterns is established during the first few hours of embryogenesis by the activities of the transiently expressed activators and repressors encoded by the segmentation genes. After the first 4 hours of embryogenesis, these factors begin to disappear, but the spatially restricted domains of homeotic gene expression are stably maintained throughout the rest of development by activities of the PcG proteins. Like other PcG mutants, *esc* mutants exhibit normal spatially restricted patterns of homeotic gene expression during the first 4 hours of embryogenesis, but thereafter display derepression of the homeotic genes along the entire A-P axis of the embryo (Struhl and Akam, 1985; Wedeen et al., 1986; McKeon and Brock, 1991; Simon et al., 1992), leading to homeotic transformation of all segments to eighth abdominal segment identity (Struhl, 1981). This indicates that the PcG gene products are not required for the initial repression by the products of the gap genes, but are required for subsequent long-term maintenance of transcriptional repression.

More than a dozen PcG genes have been identified and more than thirty are predicted from genetic studies (Jürgens, 1985; Landecker et al., 1994). Ten have been isolated to date and all encode novel proteins, some of which share conserved motifs found in other chromosomal proteins involved in transcriptional regulation (Brunk et al., 1991; Paro and Hogness, 1991; DeCamillis et al., 1992; Jones and Gelbart, 1993; Lonie et al., 1994; Bornemann et al., 1996; Sinclair et al., 1998a).

The mechanisms underlying PcG-mediated silencing are unknown, but it has been suggested that it could involve the creation of highly condensed chromatin domains, perhaps similar to heterochromatin (Paro and Hogness, 1991), that could render transcription-factor-binding sites inaccessible (Bunker and Kingston, 1994; Zink and Paro, 1995; McCall and Bender, 1996) or prevent enhancer-promoter communication (Pirrotta and Rastelli, 1994; Pirrotta, 1997). Most PcG proteins have been shown to co-localize at many specific chromosomal sites, including the sites of known target genes (DeCamillis et al., 1992; Rastelli et al., 1993; Lonie et al., 1994; Carrington and Jones, 1996), suggesting that they act in concert and interact directly with their target genes. Some have been further shown to be present in the same high molecular weight complexes in vivo (Franke et al., 1992; Gunster et al., 1997; Satijn et al., 1997; Strutt and Paro, 1997) and to bind to one another in vitro (Alkema et al., 1997; Peterson et al., 1997;

Kyba and Brock, 1998), suggesting that silencing requires the assembly of large multiprotein complexes at target genes. None of the previously identified PcG proteins has demonstrable sequence-specific DNA-binding activity, suggesting that their targeting to specific genes likely depends on other protein(s) with such activities. Recently however, a *Drosophila* homolog of the multifunctional DNA-binding transcription factor YY1 has now been shown to be encoded by the PcG gene *pleiohomeotic* (*pho*), making it a candidate for a protein that directly tethers other PcG proteins to DNA (Brown et al., 1998).

Special Polycomb response elements (PREs) that possess autonomous silencing activity and can recruit PcG proteins in vivo have been identified in the Bithorax Complex (Simon et al., 1990; Chan et al., 1994; Busturia et al., 1997; Hagstrom et al., 1997) and the *engrailed* gene (Kassis, 1994). These elements are distinct from enhancers (Chan et al., 1994; Pirrotta et al., 1995) and appear to be critical nucleating elements for both the initial assembly and subsequent maintenance (Busturia et al., 1997) of mature silencing complexes, which ultimately contact discrete extended regions of chromatin surrounding PREs (Orlando and Paro, 1993; Strutt and Paro 1997).

The signals initiating PcG silencing are not known, but are strongly correlated with the initial transcriptional status of a target gene at the time PcG silencing is implemented during germ band extension (Poux et al., 1996; Pirrotta, 1997). This suggests that the initial establishment of PcG silencing may be inhibited by factors associated with transcriptionally active promoters, while assembly of mature PcG silencing complexes may be initiated by default whenever an inactive promoter is encountered.

The ESC protein is likely to have a distinct role in the initiation of PcG silencing. *esc* is unique among the PcG genes in being expressed only during oogenesis and the first several hours of embryogenesis (Frei et al., 1985; Sathe and Harte, 1995a; Simon et al., 1995). Other PcG genes are expressed and required continuously for PcG silencing (Jones and Gelbart, 1993; Martin and Adler, 1993; Paro and Zink, 1993; Lonie et al., 1994). The *esc* protein (ESC) is present in nuclei of early preblastoderm embryos, but is not detectable by the end of embryogenesis by immunohistochemical staining of whole embryos (Gutjahr et al., 1995) (T. F. and P. J. H., unpublished data). This suggests that ESC is only required for the initial establishment of silencing and not for its subsequent maintenance. The temperature-sensitive period of a t-s *esc* allele (Struhl and Brower, 1982) and experiments with a heat-inducible *esc* transgene (Simon et al., 1995) indicate that ESC is required for only a brief 3-4 hour period of embryogenesis, beginning after the first few hours of embryogenesis, i.e., after initial repression by gap gene products has already been established and shortly before derepression is first detected in *esc* mutants. Thus ESC is likely to provide a key to understanding how PcG-mediated silencing is initiated.

The ESC protein (425 residues, ~50 kDa) is composed of seven WD motifs, spanning the C-terminal 355 residues (Gutjahr et al., 1995; Sathe and Harte, 1995a; Simon et al., 1995; Ng et al., 1997), preceded by a 70 residue N-terminal region that is highly enriched in charged residues and serine and threonine residues, some of which are likely phosphorylation sites (Sathe and Harte, 1995a). WD motifs, originally identified in the beta subunit of the G protein transducin (G_β), are found in a large number of proteins with diverse functions, including a small but growing family of transcriptional co-repressors (Komachi et al., 1994; Paroush et al., 1994; Jiménez et al., 1997). A common theme emerging from the better characterized WD proteins is their participation in multiprotein complexes (Neer et al., 1994). In several cases, WD motifs have been directly implicated in mediating contacts with other proteins (Komachi et al., 1994; Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996; Jiménez et al., 1997; Komachi and Johnson, 1997). ESC has been highly conserved through evolution (Sathe and Harte, 1995b; Schumacher et al., 1996; Denisenko and Bomstyk, 1997; Ng et al., 1997), particularly its WD region, suggesting that the fundamental biochemical functions of ESC have been conserved and that its WD motifs are likely to be critical to these functions.

We report here that ESC interacts directly with another PcG protein, E(Z) (Enhancer of zeste) in vitro, co-immunoprecipitates with E(Z) from embryo extracts and co-localizes with E(Z) at many specific sites on chromosomes. We show that the highly conserved WD region of ESC mediates binding to a conserved N-terminal sequence of E(Z). In addition, mutations in the WD region that perturb ESC function in vivo also perturb binding to E(Z) in vitro. E(Z) has been shown to be required for the binding of other PcG proteins to chromosomes (Rastelli et al., 1993) and co-localizes with other PcG proteins at many sites (Carrington and Jones, 1996). Our results suggest that ESC may be directly associated with nascent PcG silencing complexes in the early embryo or, alternatively, together with E(Z), may act independently to promote changes in local chromatin structure that are essential for binding and/or assembly of complexes of other PcG proteins. The formation of E(Z):ESC complexes at PREs may thus be one of the early steps in the establishment of PcG silencing.

MATERIALS AND METHODS

Construction of ESC and E(Z) expression plasmids

An *Nde*I site was introduced at the 5′ end and an *Nsi*I site at 3′ end of the full-length ESC-coding sequence by PCR using the forward primer F1 (5′-GCTAGCCATATGAGCAGTGATAAAGTG-3′) and reverse primer R1 (5′-ATGCATTCAGATGGAAGTCGTTTGTCT-3′). The PCR product was ligated into the pGEM-T vector (Promega). The *Nde*I-*Nsi*I fragment containing ESC was isolated from this clone and inserted into the pET-11a vector, which places a 6xHis tag at the carboxyl terminus to yield the pET-ESC plasmid. The pET-$ESC_{61\text{-}425}$ construct containing just the WD region was constructed in same method using primer F2 (5′-CATATGGCCTACAAATACGACAC-3′) and primer R1 above. The pET-$ESC_{1\text{-}121}$ construct was derived from the full-length pET-ESC by *Bam*HI digestion to delete the carboxyl terminus of ESC followed by recircularization. The pET-$ESC_{\Delta(48\text{-}149)}$ was generated by removing the internal *Sac*II fragment of ESC and recircularizing.

The pET-E(Z) construct was generated similarly by PCR using the forward primer F3 (5′-GCTAGCCATATGAATAGCACTAAAGTG-3′) and reverse primer R2 (5′-ATGCATAACAATTTCCATTTCACG-3′). The pET-$E(Z)_{1\text{-}154}$ construct was generated by deleting the internal *Bam*HI fragment encoding the carboxyl terminus of E(Z) from pET-E(Z).

The $E(Z)_{1\text{-}73}$ construct was generated by PCR with forward primer

F4 (5′-CGGGATCCATGAATAGCACTAAAGTG-3′) and reverse primer R3 (5′-CGGAATTCCCCGCTTAACCGAGTC-3′) and the PCR product was cloned into the *Bam*HI-*Eco*RI sites of the pGEX-2TK vector (Pharmacia) for expression of GST-E(Z)$_{1-73}$ fusion protein. The expression plasmid was transformed into DH5α cells. After induction of the transformed cells by IPTG (isopropyl-β-D-thio-galactopyranoside), GST-E(Z)$_{1-73}$ fusion protein was purified with Glutathione Sepharose 4B (Pharmacia).

The E(Z)$_{105-531}$ construct was generated by inserting a *Pml*I cDNA fragment encoding these residues into the *Sma*I site of pGEX-2T vector (Pharmacia) by blunt end ligation. The E(Z)$_{601-760}$ construct was generated by PCR and inserted into *Eco*RI and *Xho*I cut pGEX-4T-1 vector.

ESC mutant constructs were generated by PCR site-directed mutagenesis. Each primer was designed to contain single base change to create a single amino acid substitution. The reverse primer 5′-ATGGAACTTCGGCTCGTTAAT-3′ was used for the L176P mutation, and forward primer 5′-TCGAGCGGCAAGGATCACTCG-3′ for the M236K mutation, 5′-AGGAACTATATGGACTGTGTG-3′ for the V289M mutation, 5′-TTGTGCTCTTCAAGTCCTGCG-3′ for the S301F mutation. The first PCR products, which contained the mutation, were added to the second PCR reaction with primers F1 and R1. The second PCR products, the cDNAs of ESC mutants, were constructed as the pET-ESC to yield the pET-ESC (L176P), pET-ESC(M236K), pET-ESC(V289M) and pET-ESC(S301F). The mutations were identified by DNA sequencing.

Further details of all constructions can be obtained upon request.

ESC-H6 protein purification and trypsin digestion

The pET-ESC-H6 was generated as the pET-ESC except the PCR reverse primer 5′-ATGCATGATGGAAGTCGTTTGTCT-3′ which contained no stop codon and allowed a 6 histidine tag to attach to the carboxyl terminus of ESC. pET-ESC-H6-transformed *E. coli.* BL-21 cells were grown in LB medium containing 100 μg/ml ampicillin and induced by 0.1 mM IPTG overnight at room temperature. Cells were collected by centrifugation and resuspended in pH 8.0, 50 mM PBS containing 0.3 M NaCl, 0.1% Triton X-100, 0.2 mM PMSF and 2% (w/v) lauroylsarcosine, and then sonicated twice for 1 minute in an ice bath. The supernatant from centrifugation was mixed with Ni^{2+}-NTA Agarose (QIAGEN) for 1 hour on ice. Then beads were extensively washed with PBS, 0.3 M NaCl, 50-60 mM imidazole. ESC-H6 protein was finally eluted with the same buffer also containing 200 mM imidazole and dialyzed in 20 mM Hepes (pH 7.8), 0.1 M NaCl, 10% glycerol. ESC-H6 was incubated with sequencing grade trypsin (Sigma) at 37˚C. The digestion was terminated by adding 2× SDS-PAGE sample buffer. Proteins were analyzed by western blot using Anti-Penta-His Antibody (QIAGEN).

Production of ^{35}S-labeled ESC and E(Z) proteins by in vitro translation

The TNT T7 Quick Coupled Transcription/Translation System from Promega was used to synthesize ESC and E(Z) proteins in vitro. Plasmid DNA (1 μg of each pET-11a with insert of interest) purified by QIAGEN plasmid purification column, was mixed with 40 μl of TNT T7 Quick Master Mix and 2 μl of [^{35}S]methionine in total 50 μl of volume. The mixture was incubated at 30˚C for 60-80 minutes, then 12 μl of 5× protein binding buffer was added (1×BB: 25 mM Hepes pH 7.8, 5 mM KCl, 1 mM EDTA, 0.25 mM DTT, 1 mg/ml BSA, 10% glycerol) and stored at –20˚C for future use.

ESC$_{1-413}$ was generated by linearizing the pET-ESC with restriction enzyme *Dra*III to create a termination of transcription and translation at amino acid residue 413. The 3′ overhang created by *Dra*III was removed by the Klenow DNA polymerase. The pET-ESC was linearized by restriction enzyme *Afl*III for translation of ESC$_{1-406}$. Similarly, E(Z)$_{1-314}$ was generated by linearizing pET-E(Z) with *Sma*I.

Immunoprecipitation and GST fusion protein-binding assays

In vitro translated proteins (10 μl each) were mixed with 4.5 μl of affinity-purified E(Z) antibodies (Carrington and Jones, 1996; raised against E(Z)$_{8-155}$, a generous gift from Richard Jones) in 40 μl of 1×BB total volume and incubated at 37˚C for 30 minutes. The reaction mixture was then added to 60 μl of a 50% slurry of Protein A Sepharose CL-4B (Pharmacia) preincubated in 1× BB that contained 1 mg/ml BSA to reduce non-specific binding. After incubation overnight on ice, beads were washed four times with 200 μl of 1× BB. Bound proteins were eluted with 30 μl of 2× SDS-PAGE sample buffer and 15 μl of each sample was analyzed on SDS-polyacrylamide gel. The gel was soaked for 1 hour in 1 M sodium salicylate, dried and autoradiographed.

Glutathione Sepharose 4B was used for GST fusion protein-binding assays. Beads were bound with GST or GST-E(Z) fusion proteins and suspended in protein-binding buffer (1× BB). ^{35}S-labeled ESC proteins (8 μl) were mixed with 50 μl of a 50% slurry of beads (bound to approximately 200 ng of GST fusion protein) in 100 μl total volume. The binding reaction was incubated on ice for 2 hours, during which it was mixed gently several times. Beads were then washed twice with 200 μl of 1× BB and twice with 200 μl of washing buffer (20 mM Hepes, pH 7.8, 0.15 M NaCl, 10% glycerol). Bound proteins were eluted and analyzed as above.

Secondary structure predictions

Multiple alignment of the N-terminal 73 residues of the *Drosophila* and mammalian E(Z) homologs shown in Fig. 3 was used to obtain secondary structure and solvent accessibility predictions for the ESC-binding region of E(Z) using the PHDsec and PHDacc programs (Rost and Sander, 1993a,b, 1994; Rost et al., 1994) on the PHD server (EMBL, Heidelberg). To compensate for the near identities of mouse/human pairs mENX1/hEzh2 and mEZH1/hEzh1, which disproportionately weight the contributions of these sequences to predictions relative to E(Z), predictions were obtained by submitting only combinations of two different mouse and/or human homologs along with E(Z). All combinations gave virtually identical predictions.

P-element transformants expressing FLAG epitope-tagged ESC

P-element transformants were generated that express full-length ESC with an N-terminal FLAG epitope tag (Met-DYKDDDDK-) (Kodak) from the *esc* promoter or from the α1-tubulin promoter, which is expressed constitutively at moderate levels in all tissues (Matthews et al., 1989; O'Donnell et al., 1994). These constructs and characterization of transformants containing them will be described and in detail elsewhere (T. F. and P. J. H., unpublished data). Multiple insertions were recovered with each construct and were tested for the ability to rescue the maternal effect lethality and visible phenotypes of various *esc* mutations. All lines carrying either construct were capable of completely rescuing all aspects of the phenotype of *esc* null mutants, rendering them indistinguishable from wild-type animals, indicating that the FLAG epitope does not interfere with ESC function.

Co-immunoprecipitation of ESC and E(Z) from whole embryo extracts

Whole embryo extracts were prepared from 0- to 6-hour-old embryos from a wild-type strain (Oregon-R) and a transgenic line expressing FLAG-tagged ESC from α1-tubulin promoter. After dechorionation in 50% bleach and thorough washes in a 0.9% NaCl, 0.03% Triton-X solution, embryos were homogenized in a buffer containing 40 mM Hepes (pH 7.0), 350 mM NaCl, 0.1% Tween-20, 10% glycerol, 1 mM Benzamidine, 100 mg/ml PMSF, 2 mg/ml leupeptin, 2 mg/ml protinin and 2 mg/ml pepstatin. After ultracentrifugation for 40 minutes at 40,000 revs/minute in a Beckman SW50.1 rotor, the cleared supernatant was used for co-immunoprecipitation assays.

For immunoprecipitation of FLAG-ESC, anti-FLAG M2 affinity gel (Kodak) was equilibrated in a binding buffer (40 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mg/ml BSA, 10% glycerol), then incubated with whole embryo extract for 4 hours at 4˚C. After washing four times in the binding buffer, the bound proteins were eluted by adding an equal volume of 2× SDS-sample loading buffer. Detection of E(Z) on western blots of FLAG-ESC immunoprecipitates was performed using a mouse polyclonal E(Z) antibody at 1:1000 dilution, anti-mouse-HRP (Pierce) at 1:20,000 dilution, and Super Signal (Pierce). This mouse polyclonal E(Z) antibody was produced by injecting mice with a Ni-NTA-purified H6-E(Z)$_{1\text{-}154}$. The M5 anti-FLAG monoclonal Ab (Kodak) was also used, together with Protein G Sepharose (Pharmacia), for immunoprecipitation of FLAG-ESC. E(Z) was subsequently detected on western blots of these immunoprecipitates, but not after similar mock immunoprecipitations with Protein G without M5.

For immunoprecipitation of E(Z), Protein G Sepharose (Pharmacia) was equilibrated in the binding buffer, then incubated with mouse polyclonal E(Z) antibody (α-E(Z)) for 1 hour at room temperature. After washing four times in the binding buffer, α-E(Z) coupled Protein G beads were incubated with the whole embryo extract. Subsequent steps were done as described above. Detection of FLAG-ESC on western blots of anti-E(Z) immunoprecipitates was performed using affinity-purified polyclonal FLAG-probe D8 (Santa Cruz) at 1:1000 dilution, anti-rabbit-HRP (Pierce) at 1:20,000 and Super Signal (Pierce).

Immunostaining of polytene chromosomes

Polytene chromosomes were prepared as previously described (Chinwalla et al., 1994). Fixation was done for 20 seconds. Chromosomes were incubated simultaneously with affinity-purified rabbit polyclonal anti-E(Z) (1:50 dilution) and mouse M5 anti-FLAG monoclonal (Kodak) (1:50 dilution) primary antibodies overnight at 4˚C in a humidified chamber. Slides were rinsed briefly in PBS, washed for 15 minutes in 300 mM NaCl, 0.2% Tween-20, 0.2% NP-40, and 1× PBS, washed for 15 minutes in 400 mM NaCl, 0.2% Tween-20, 0.2% NP-40 and 1× PBS, and rinsed in PBS to remove excess NaCl. Primary antibodies were differentially detected with rhodamine-labeled anti-rabbit (1:200 dilution; for E(Z)) and FITC-labeled anti-mouse (1:200 dilution; for FLAG-ESC) secondary antibodies diluted in the blocking buffer containing 2% normal goat serum as previously described (Chinwalla et al., 1994). Slides were washed as described above, then mounted with anti-fade (Oncor). Images were obtained using the Bio-Rad MRC-600 confocal imaging system. Images of E(Z) and ESC were collected separately with red and green filters (for rhodamine and FITC, respectively) and then merged to reveal sites of co-localization (yellow).

RESULTS

ESC interacts directly with the N terminus of E(Z) in vitro

To determine whether the ESC protein interacts directly with any other PcG proteins, we began testing other identified PcG proteins for their ability to bind ESC using two in vitro binding assays: co-immunoprecipitation (co-IP) and 'GST pulldown' as described in the Materials and methods. As shown in Fig. 1A, using co-IP of in vitro translated proteins (lanes 1 and 2), we detected specific binding of ESC to E(Z) (lane 4).

Using the co-IP assay, we initially delimited the region of E(Z) that mediates ESC binding to the N-terminal 154 residues (Fig. 1B, lane 6). To further define the ESC-binding domain of E(Z), we used affinity-purified recombinant GST-E(Z) fusion proteins produced in *E. coli*. As shown in Fig. 2A (lane 3), GST-E(Z) fusion proteins that contain only E(Z)$_{1\text{-}73}$ retain full ESC-binding activity. No ESC binding was detected using GST-E(Z)$_{105\text{-}531}$ or GST-E(Z)$_{601\text{-}760}$ (data not shown). As shown in Fig. 3, E(Z)$_{1\text{-}73}$ contains multiple residues that are conserved in the four mouse and human E(Z) homologs (Chen et al., 1996; Hobert et al., 1996a,b; Laible et al., 1997). Secondary structure and solvent accessibility predictions for E(Z)$_{1\text{-}73}$ (Fig. 3, see Materials and Methods) show that the region spanning E(Z)$_{14\text{-}59}$ is predicted to form an extensively α-helical region that contains almost all of the invariant and conserved residues in E(Z)$_{1\text{-}73}$, many of which are also predicted to be solvent exposed, making them strong candidates for residues that are in direct contact with the ESC protein.

The WD region of ESC is required for binding to E(Z)

The ESC protein is composed of seven WD motifs, spanning the C-terminal 355 residues (Gutjahr et al., 1995; Sathe and Harte, 1995a; Simon et al., 1995), preceded by a 70-residue N-terminal region that is highly enriched in charged residues and serine and threonine residues, some of which are likely phosphorylation sites (Sathe and Harte, 1995a). As shown in Fig. 2A (lane 12), only the WD region of ESC (residues 61-425) is required for strong interaction with E(Z) in vitro. An N-terminal polypeptide, ESC$_{1\text{-}121}$, which includes WD1 and the first few residues of WD2, shows no detectable binding to E(Z)

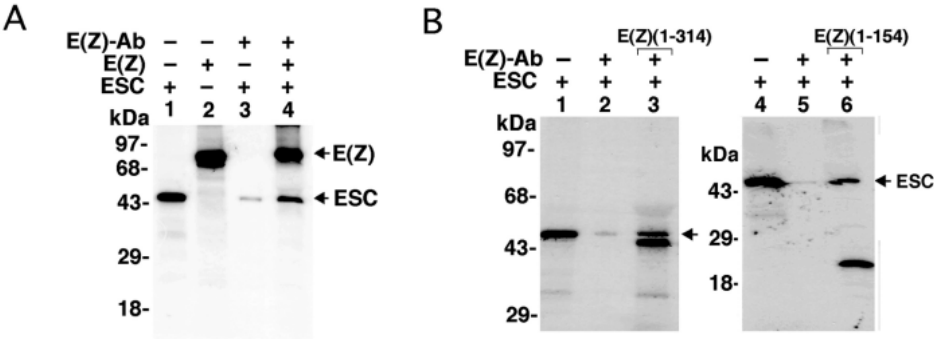

Fig. 1. ESC binds to E(Z) in vitro. (A) Co-immunoprecipitation (co-IP) of full-length ESC and E(Z). In vitro translated ^{35}S-labeled ESC and E(Z) proteins (lanes 1 and 2) were used for co-IP. Co-IP of ESC with E(Z) using Protein A Sepharose CL-4B and anti-E(Z) antibodies (E(Z)-Ab) in the absence of E(Z) (lane 3) and the presence of E(Z) (lane 4). Samples were analyzed by SDS-PAGE and visualized by autoradiography. A PhosphorImager (Molecular Dynamics) was used to quantify ESC and E(Z) signals. Lane 1, containing the total ESC input is set at 100%. The background ESC binding to protein A beads (lane 3) is 4.8% of the total ESC input (lane 1), while ESC binding to E(Z) (lane 4) is 42.1% of input. (B) Co-IP of ESC and truncated E(Z). Lanes 1 and 4 are translated ESC. Immunoprecipitation of ESC by E(Z)$_{1\text{-}314}$ and E(Z)$_{1\text{-}154}$ are indicated by arrows in lane 3 and 6 respectively. Lanes 2 and 5 show the non-specific background binding of ESC to protein A Sepharose beads. PhosphoImager measurements indicate that the specific ESC-binding signals (lanes 3 and 6) are 5-fold greater than background.

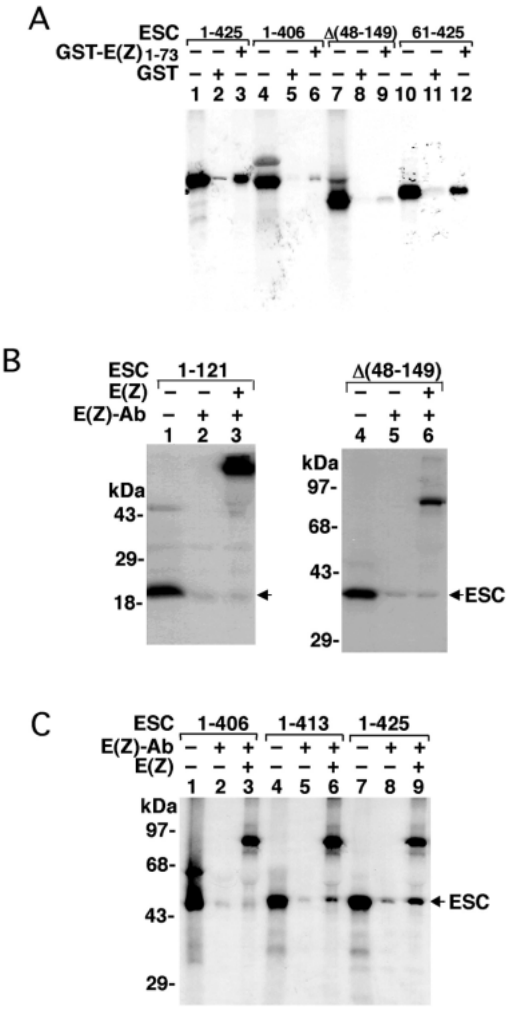

Fig. 2. In vitro binding data. (A) GST pulldown of ESC and deleted ESC proteins as indicated at top of respective lanes. Lanes 1, 4, 7 and 10 show the amounts of the various translated ESC proteins used in each binding reaction (100%). Lanes 2, 5, 8 and 11 serve as negative controls and show background binding of each ESC protein to GST alone. Lanes 3, 6, 9 and 12 show binding to the GST-E(Z)$_{1-73}$ fusion protein. All samples were analyzed as in Fig. 1A. (B,C) Co-IP of deleted ESC and E(Z). ESC fragments used are indicated at the top of respective lanes. Arrows indicate the immunoprecipated ESC (lanes 3 and 6 in B, lanes 3, 6 and 9 in C).

(lane 3 in Fig. 2B). A similar polypeptide product (ESC$_{1-112}$) is predicted to be produced in vivo by the null *esc*^{4} nonsense mutation (Q113X) (Sathe and Harte, 1995a), suggesting that the loss of E(Z) binding may be the basis for its null phenotype in vivo.

Attempts to subdivide the WD region revealed that even small deletions within it drastically affect binding to E(Z). Deletion of WD1, ESC$_{\Delta 48-149}$, almost abolishes binding to E(Z) (see lane 9 in Fig. 2A and arrow in lane 6 in Fig. 2B). Deletion of the C-terminal 19 residues, which includes the last 12 residues of WD7, greatly reduces binding (Fig. 2C, lane 3 and also Fig. 2A, lane 6). The in vivo phenotype of the similar *esc*^{2} mutation, which replaces the C-terminal 22 residues of ESC with 44 unrelated residues (Sathe and Harte, 1995a), is correspondingly strong, similar to that of a null mutation (Struhl, 1981, 1983). Even a deletion of the 12 C-terminal residues of ESC can perturb binding to E(Z) (Fig. 2C, lane 6). Other deletions within the WD region produce similar drastic effects on binding to E(Z) (data not shown). The data in Fig. 2 thus suggest that the entire WD region of ESC must be intact for strong interaction with E(Z) to occur. Results of all the in vitro binding experiments are summarized in Fig. 4.

Like ESC, the prototypical WD protein, the transducin beta subunit (Gβ), contains seven tandem WD motifs. Its crystal structure reveals that all seven WD motifs participate in forming a single large structural motif known as a β-propeller (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996). The 'propeller' (see Fig. 5) is composed of seven 'blades', i.e., seven twisted 4-stranded β-sheets organized into a toroid with approximate sevenfold symmetry about the central axis. Each WD motif contributes the inner three β-strands of one β-sheet; the outer (fourth) strand is formed by more variable sequences following each WD motif. The outer strand of the last (seventh) β-sheet is uniquely contributed by sequences immediately preceding the first WD motif, thereby linking the two ends of the WD region in the structure to achieve closure of the toroid. Loops connecting the individual β-strands within and between β-sheets contain residues that lie on the outer surface of the propeller, many of which in Gβ make direct contacts with the Gα and Gγ subunits.

The structural principles underlying the β-propeller motif and the stereotypical substructures of the individual WD motifs within it provide very strong predictions that the WD regions of ESC and all other WD proteins adopt a similar β-propeller structure (Wall et al., 1995; Lambright et al., 1996; Neer and

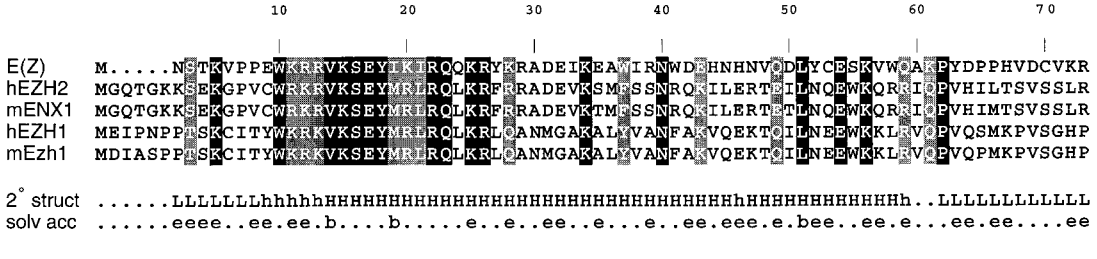

Fig. 3. Conservation and predicted secondary structure of the ESC-binding region of E(Z) in the four mammalian E(Z) homologs. E(Z)$_{1-73}$ is shown aligned with the two mouse (mENX1 and mEzh1) and two human (hEZH1 and hEZH2) homologs. Highlighting shows residues that are identical (black) or highly conserved (grey) in all five proteins. Note that mouse ENX1 and its human homolog EZH2 are more closely related to E(Z) in this region. Below the alignment are secondary structure and solvent accessibility predictions obtained using the PHDsec and PHDacc programs. H or h, helix (with capital H indicating an estimated accuracy >82% for the prediction); L, loop (with an estimated accuracy >69% for the prediction). '.', no prediction possible; e, exposed; b, buried. Note the extensive α-helical structure predicted to span this region and the numerous invariant and highly conserved residues predicted to be accessible.

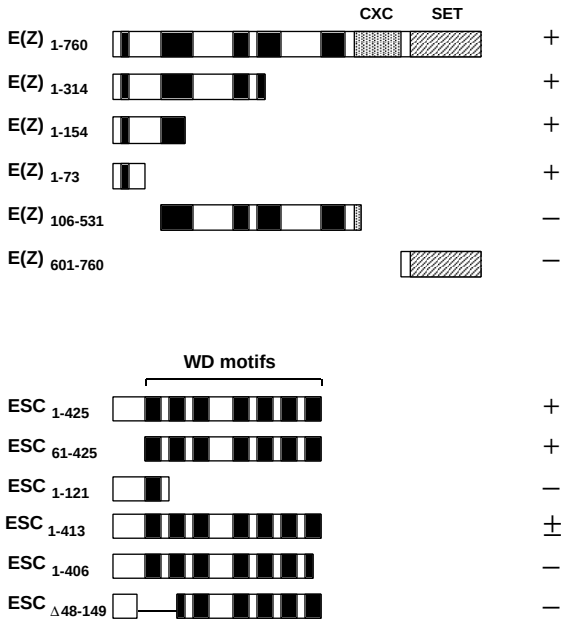

Fig. 4. Schematic summary of in vitro binding experiments and the E(Z) and ESC constructs tested. Subscripts indicate the residues present in each construct. For E(Z), the highly conserved SET domain and CXC motif containing 17 conserved cysteines are labeled. Other evolutionarily highly conserved regions of E(Z) are indicated by black boxes. For ESC, the positions of the seven highly conserved WD motifs, which contribute to the predicted β-propeller structural motif, are also indicated by black boxes. The top three E(Z) constructs were tested using the co-IP assay. The bottom three E(Z) constructs were tested as GST-E(Z) fusion proteins. The performance of each construct in the in vitro binding assays is indicated to the right (+/–). Note that the WD region (ESC_{61-425}) contains all the E(Z)-binding activity and that deletions throughout the WD region affect binding to E(Z) quite drastically. See text for details.

Smith, 1996; Sondek et al., 1996). Homology modeling of ESC suggests that its WD region adopts a β-propeller structure (Ng et al., 1997; P. J. H., unpublished data), providing a compelling rationale for the importance of the structural integrity of its entire WD region for effective binding to E(Z). Further, G_β binds to G_α through multiple contacts involving residues in all seven WD motifs; by analogy, perturbations of predicted β-propeller structure of ESC may cause simultaneous disruption of multiple E(Z) contacts, producing the observed drastic effects on E(Z) binding.

The WD region of ESC folds into a trypsin-resistant structure

The β-propeller portion of G_β forms a trypsin-resistant structure, despite the presence of 32 trypsin cleavage sites (Fong et al., 1986). The WD regions of at least five other WD proteins have also been shown to be trypsin resistant and, like G_β, to exhibit hydrodynamic properties of compact globular proteins (Garcia-Higuera et al., 1996). These data support structure-based predictions that all WD proteins are likely to form similar β-propeller structures (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996) and suggest that trypsin-resistance may be a characteristic feature of all β-propeller structures.

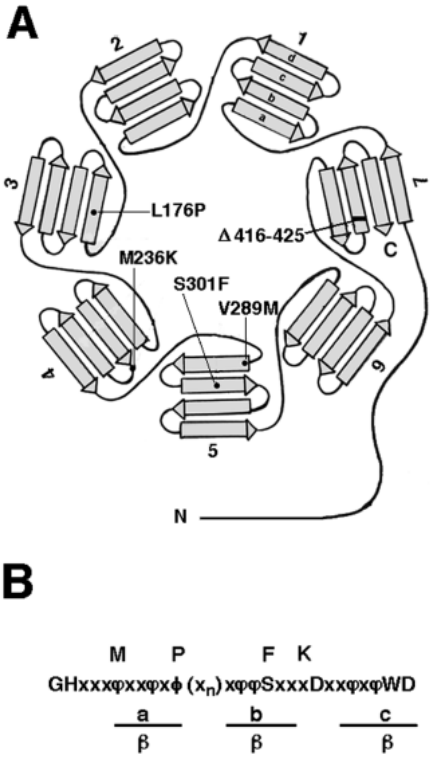

Fig. 5. (A) Schematic diagram of predicted ESC β-propeller structure (adapted from Sondek et al., 1996) showing the position of the ESC mutations analyzed. The seven four-strand β-sheets (blades) are numbered 1 through 7 and individual β-strands labeled a through d. Intrastrand loops connecting β-strands within and between β-sheets are not to scale. Note how N-terminal residues preceding β-blade 1 contribute the d strand of β-sheet 7 to close of the toroidal structure. The N-terminal region preceding the β-propeller is depicted as unstructured. The positions of ESC mutations analyzed are also indicated. (B) The positions of the ESC mutations are depicted above a consensus WD motif (Neer et al., 1994; Neer and Smith, 1996) positions indicated. x, nonconserved; x_n, variable number; φ, hydrophobic; Φ, aromatic; p, polar. The residues involved in β-strands a, b and c are indicated below.

To further explore whether the WD region of ESC forms a single compact structure like other β-propellers, we tested the trypsin-sensitivity of purified recombinant ESC protein containing a C-terminal 6xHis tag (ESC-H6) produced in *E. coli* (Fig. 6A). Much of the recombinant protein remained insoluble (Fig. 6A, lane 4). The soluble fraction (Fig. 6A, lane 3), obtained in 2% sarcosyl, was purified on Ni^{2+}-NTA Agarose. As shown in Fig. 6B, trypsin digestion of purified full-length ESC-H6 yields a single C-terminal trypsin-resistant fragment (detected with anti-6xHis antibody) with an apparent molecular weight of ~41 kDa on SDS-PAGE (Fig. 6B, lane 3). The N-terminal region preceding the WD motifs appears to be highly sensitive to trypsin (Fig. 6B, lane 2), consistent with our previous suggestion that this highly charged Ser- and Thr-rich region constitutes a flexible, unstructured, solvent-exposed region of ESC (Sathe and Harte, 1995a). The C-terminal trypsin-resistant fragment is just large enough to include the entire WD region and is similar in size to in vitro translated ESC_{61-425} (Fig. 6C, lane 5). We also subjected in vitro translated ESC_{61-425} to trypsin cleavage and find that it is

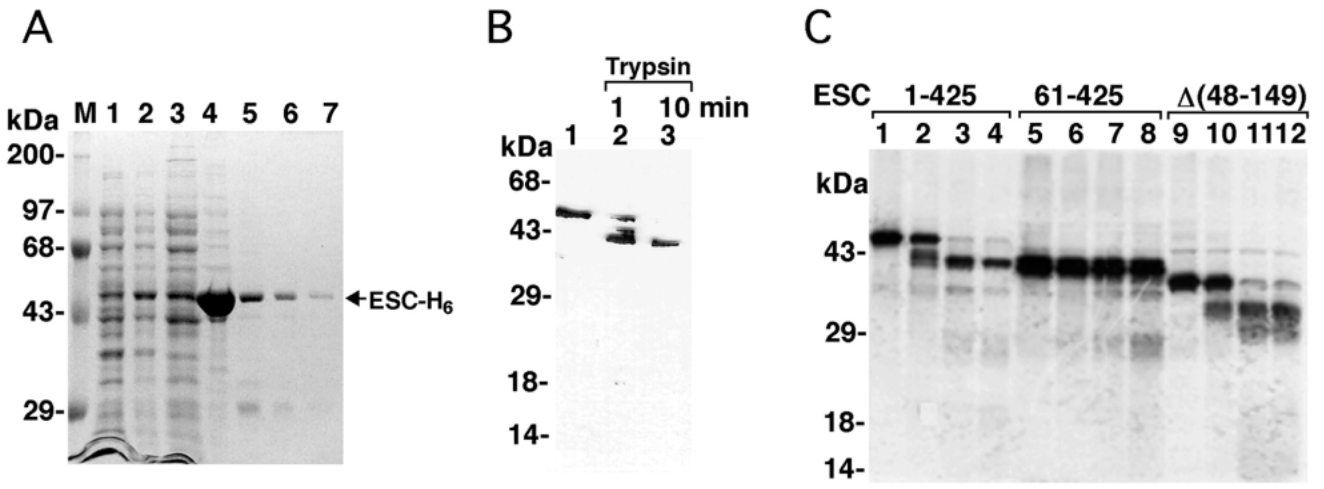

Fig. 6. The entire WD region of ESC adopts a trypsin-resistant structure. (A) Purification of ESC-H6 on Ni^{2+}-NTA Agarose. SDS-PAGE was analyzed by Coomassie blue staining. The samples are whole cell proteins from uninduced (lane 1) and IPTG-induced (lane 2) *E. coli*, supernatant (lane 3) and insoluble inclusions (lane 4), and fractions eluted from Ni^{2+}-NTA column with 0.2 M imidazole (lane 5-7). (B) Trypsin cleavage of ESC-H6. Purified ESC-H6 proteins were incubated with 0.8 μM trypsin at 37˚C. Samples were analyzed by western blot using anti-penta-His antibodies. (C) Trypsin cleavage of full-length ESC and various ESC deletion mutants. Translated ESC proteins (lanes 1, 5 and 9) as indicated at the top of their respective lanes were incubated with 0.1 μM trypsin (lanes 2, 6 and 10) or 0.8 μM trypsin (lanes 3, 4, 7, 8, 11 and 12) for 10 minutes (lanes 2, 4, 6, 8, 10 and 12) or 2 minutes (lanes 3, 7 and 11) at 37˚C. Note that ESC contains 47 potential trypsin cleavage sites, of which 32 lie within the predicted β-propeller region.

trypsin resistant (Fig. 6C, lanes 6-8), indicating that all seven WD motifs (containing 32 trypsin cleavage sites) lie within the trypsin-resistant structure. $ESC_{\Delta(48\text{-}149)}$, which deletes WD1, is much more trypsin sensitive (Fig. 6C, lanes 10-12). These results strongly suggest that the entire WD region of ESC forms a single compact globular domain, consistent with its predicted β-propeller structure.

Point mutations in the WD motifs of ESC perturb its binding to E(Z)

To further test the involvement of the WD region in E(Z) binding, we tested four different ESC point mutations that cause single amino acid substitutions in the WD region for their effects on ESC binding to E(Z) in vitro. All were isolated in classical genetic screens and abolish transcriptional silencing by ESC in vivo. One we previously reported (Sathe and Harte, 1995a) and two are reported for the first time here. In addition, we tested one other that mimics a null mutation reported in the mouse ESC homolog, EED (Schumacher et al., 1996; Denisenko and Bomstyk, 1997). All these mutations alter residues that are invariant in the eight ESC homologs identified to date, suggesting that they are critical for the structure and/or function of ESC. The position of each mutant residue within the predicted structure and WD consensus is indicated in Fig. 5. As shown in Fig. 7A, full-length ESC proteins containing these mutations bind poorly or not at all to E(Z).

esc^{21} (S301F in WD5) has a drastic effect on E(Z) binding. The Ser residue affected in *esc*^{21} in particular is one of three highly conserved residues in WD motifs (Neer et al., 1994), which form the 'structural triad' that preserves the common network of interactions that stabilize the infrastructure of each WD motif in Gβ. Its side chain hydroxyl forms two structurally critical hydrogen bonds with the highly conserved His_2 (G**H**...) and signature Trp (...**W**D) at opposite ends of the same WD motif, both of which are also conserved in WD5 of ESC. Thus, this nonconservative substitution is predicted to perturb at least the structure of β-sheet 5 (WD5) and perhaps the global structure of the whole protein. Consistent with this, the WD region of this mutant protein is much more trypsin sensitive than wild-type ESC (Fig. 7B, lane 6).

L176P, which mimics a mouse EED mutation, also strongly affects E(Z) binding (Fig. 7A, lane 6). It affects a position that is predicted to be structurally important for formation of the 'a' β-strand of β-sheet 3 (WD3). This position is occupied by an invariant Leu in all eight ESC homologs and tends to be occupied by a hydrophobic residue with an aliphatic side chain in most WD motifs (Neer et al., 1994). Paralleling its strong effect on binding in vitro, it behaves as a null allele in vivo in the mouse (Schumacher et al., 1996). The WD region of this mutant protein is also much more sensitive to trypsin digestion than wild-type ESC (Fig. 7B, lane 10), suggesting that this mutation not only perturbs local β-sheet structure but global structure as well, consistent with the importance of every WD motif to the structural integrity of the entire WD region.

esc^{17} (V289M in WD5) affects a residue predicted to initiate the 'a' β-strand of β-sheet 5 (WD5), corresponding to a position in Gβ adjacent to a residue that makes a direct contact with Gα. It makes a conservative substitution at a position that is occupied by an invariant Val in all seven ESC homologs and that tends to be occupied by a hydrophobic residue with an aliphatic side chain in almost all known WD motifs (Neer et al., 1994). This mutation is also temperature sensitive in vivo (Struhl and Brower, 1982), indicating that it does not entirely abolish ESC function. Consistent with this and the predicted less drastic structural effect of this conservative substitution, it exhibits some residual binding to E(Z) (Fig. 7A, lane 15) and is not as trypsin sensitive as L176P (Fig. 7B, lane 8).

esc^{9} (M236K in WD4) makes a nonconservative Lys

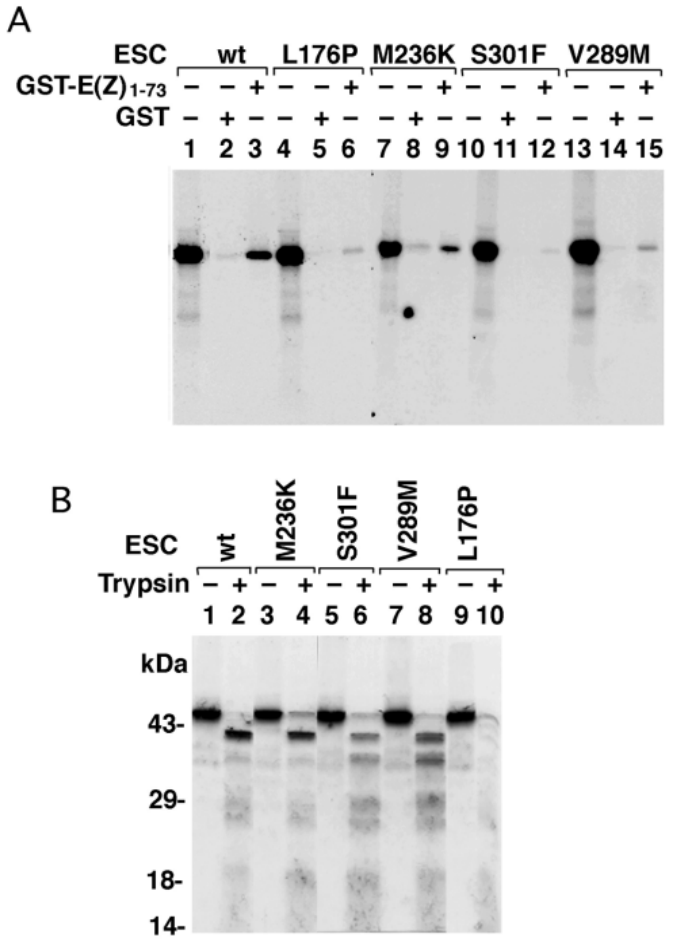

Fig. 7. (A) Effects of ESC mutations on binding to E(Z). GST pulldown of wild-type (wt) and mutant ESC proteins as indicated at top of respective lanes. Lanes 1, 4, 7, 10 and 13 show the amounts of the various wild-type and mutant ESC proteins used in each binding reaction. Lanes 2, 5, 8, 11 and 14 serve as negative controls and show background binding of each ESC protein to GST alone. Lanes 3, 6, 9, 12 and 15 show binding to the GST-E(Z)$_{1-73}$ fusion protein. All samples were analyzed as in Fig. 1A. (B) Trypsin cleavage of wild-type and mutant ESC proteins. In vitro translated wild-type and mutant ESC proteins (lanes 1, 3, 5, 7 and 9) were incubated with 0.8 μM trypsin for 10 minutes at 37˚C (lanes 2, 4, 6, 8 and 10). Samples were analyzed by SDS-PAGE and autoradiography.

substitution for an invariant Met in a position that is predicted to lie in the exposed b-c loop between the b and c β-strands of β-sheet 4 (WD4), a position not predicted to be important for β-propeller structure. A Lys occurs at this position in other WD motifs (Neer et al., 1994), including WD3 of ESC, further suggesting that it is not likely to be structurally detrimental. Consistent with this, the trypsin-resistance of the mutant protein is indistinguishable from that of wild-type ESC (Fig. 7B, lane 4), despite the fact that the mutation itself introduces an additional trypsin cleavage site. In Gβ the residue at this same position is involved in a direct contact with Gα. In vivo, *esc*^{9} is a partial loss-of-function mutant and exhibits dominant negative properties (T. F. and P. J. H., unpublished data), further indicating that the mutant protein is likely to be structurally intact and retains some wild-type function(s). This mutation also compromises binding to E(Z) in vitro, but it does not abolish it (Fig. 7A, lane 9). It has a less severe effect on binding than *esc*^{17}, which likely causes at least some structural perturbation. Since Met$_{236}$ is not predicted to be important for β-propeller structure, it might contribute to E(Z) binding through a direct contact, or the Lys substitution might indirectly perturb a contact made by a nearby residue. Given the wild-type trypsin-resistance of the *esc*^{9} protein, its higher residual E(Z)-binding activity is also likely due to contacts made by residues in other parts of the structurally intact β-propeller.

The strong correlation between the in vitro binding activities of these mutant proteins, their predicted structural effects and trypsin-sensitivity, as well as their in vivo phenotypic characteristics, provides a strong argument that ESC and E(Z) are associated in vivo through contacts made within predicted β-propeller region of ESC and that the basis for the ESC mutant phenotypes in vivo, whether entirely or in part, is the inability of the mutant proteins to bind E(Z) in vivo.

Co-immunoprecipitation of ESC and E(Z) from embryo extracts

To determine whether ESC and E(Z) are physically associated in vivo, we attempted to co-immunoprecipitate them from embryo extracts. For this purpose, we used transgenic lines expressing a FLAG epitope-tagged ESC (FLAG-ESC) from the constitutive α1-tubulin promoter (see Materials and Methods). These constructs, like similar untagged constructs, rescue all aspects of the *esc* mutant phenotype indicating that the epitope tag does not interfere with the function of ESC and constitutive expression of ESC from this promoter has no detectable detrimental effects (T. F. and P. J. H., unpublished data). Fig. 8A (right lane) shows that when the M2 anti-FLAG monoclonal antibody was used for immunoprecipitation from FLAG-ESC-containing embryo extracts, E(Z) was detected in the immunoprecipitate by western analysis. In contrast, E(Z) was not detected in immunoprecipitates when the identical immunoprecipitation procedure was performed with wild-type embryo extracts (Fig. 8A, left lane), which contain endogenous ESC but no FLAG-ESC. E(Z) was also not detected after mock immunoprecipitations from the FLAG-ESC-containing extracts when anti-FLAG antibody was omitted (data not shown, see Materials and Methods).

Reciprocal immunoprecipitation experiments with anti-E(Z) antibody yielded similar results. When anti-E(Z) antibody was used for immunoprecipitation from embryo extracts containing FLAG-ESC, the FLAG-ESC protein was detected in the immunoprecipitate by western analysis (Fig. 8B, right lane). FLAG-ESC was not detected in similar mock immunoprecipitations from which anti-E(Z) antibody was omitted (Fig. 8B, middle lane). Also, no signal was detected in immunoprecipitates from wild-type embryo extracts, which contain no FLAG-ESC, demonstrating the specificity of the anti-FLAG antibody (Fig. 8B, left lane). These results demonstrate that the ESC and E(Z) proteins are physically associated in vivo in the early embryo, consistent with the in vitro binding results above.

ESC and E(Z) co-localize at many specific sites on chromosomes

The E(Z) protein has been shown to bind to at least 44 specific sites on the polytene chromosomes of the larval salivary

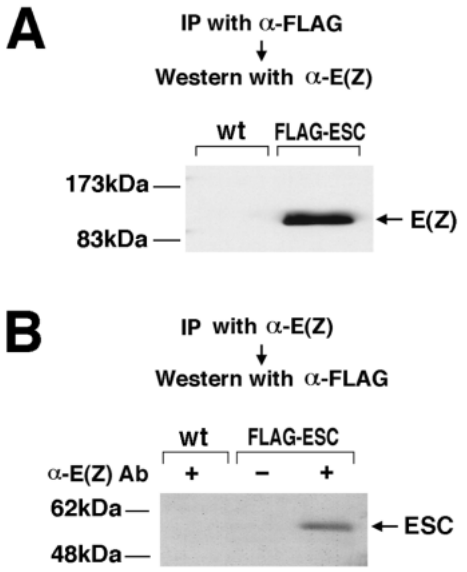

Fig. 8. Co-immunoprecipitation of ESC and E(Z) from whole embryo extracts. (A) Detection of E(Z) in ESC immunoprecipitates. An extract of either Oregon-R wild-type (wt) or transgenic embryos expressing FLAG-tagged ESC (FLAG-ESC) was incubated with agarose beads directly coupled to M2 anti-FLAG monoclonal Ab. A western blot of the immunoprecipitates probed with anti-E(Z) Ab is shown. E(Z) is co-immunoprecipitated with FLAG-ESC from the extract containing FLAG-ESC, but E(Z) is absent in the wild-type control which does not contain FLAG-ESC, demonstrating the specificity of this co-immunoprecipitation assay. (B) Detection of FLAG-ESC in E(Z) immunoprecipitates. An extract of either wild-type or transgenic embryos expressing FLAG-tagged ESC was incubated with anti-E(Z) Ab bound to Protein G-sepharose. A western blot with anti-FLAG Ab (See Material and Methods) is shown. FLAG-ESC is co-immunoprecipitated with E(Z) only in the presence of anti-E(Z) antibody, but not in the absence of the antibody (middle lane, Protein G alone). The wild-type control shows that no nonspecific signal is detected with the α-FLAG Ab in immunoprecipitates from embryos that do not contain FLAG-ESC.

gland, most of which appear to correspond to previously reported binding sites of other PcG proteins (Carrington and Jones, 1996). To determine whether ESC binds to chromosomes and whether ESC and E(Z) are associated with the same sites, we visualized both proteins simultaneously on the polytene chromosomes by immunofluorescence. Initially, we used transgenic lines expressing a FLAG-ESC from the constitutive α1-tubulin promoter. Fig. 9 shows that FLAG-ESC binds to many specific sites on chromosomes and that ESC and E(Z) are virtually completely co-localized. In addition to many euchromatic sites, we also detect both FLAG-ESC and E(Z) associated with the heterochromatic chromocenter (see Discussion). Surprisingly, the identical pattern of ESC binding is seen when FLAG-ESC is expressed from the *esc* promoter (data not shown). Since the latter case reflects the normal temporally restricted expression of the endogenous *esc* gene, it suggests that ESC protein is sufficiently stable to persist on salivary gland chromosomes until the third instar. We therefore conclude that the co-localization of ESC and E(Z) seen on third instar polytene chromosomes reflects the persistence of an authentic

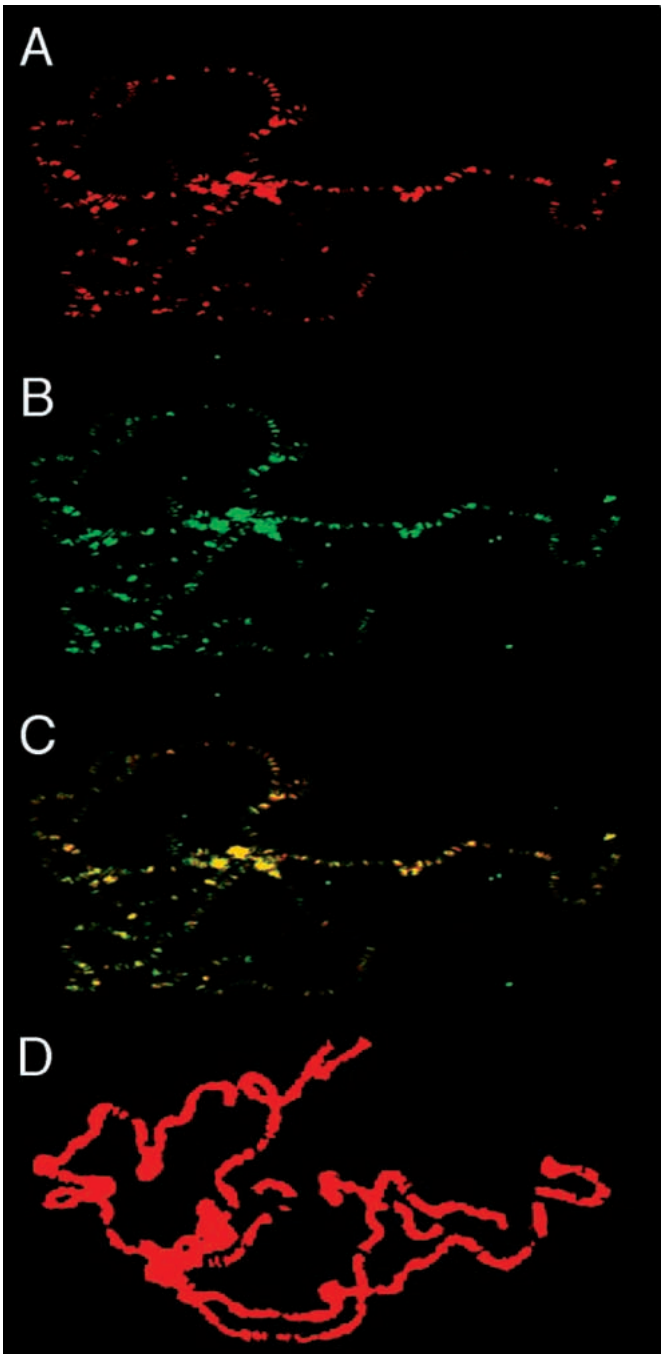

Fig. 9. Co-localization of ESC and E(Z) on salivary gland polytene chromosomes. Simultaneous immunofluorescence localization of E(Z) (A) and FLAG epitope-tagged ESC (B) showing that virtually all of their binding sites coincide (C). Note that in addition to their many specific euchromatic sites, both proteins are also present in the chromocenter. The E(Z) antibodies were detected with rhodamine-labelled secondaries (red). The FLAG monoclonal was detected with FITC-labelled secondaries (green). (D) A control showing that the anti-FLAG antibody (green) produces no signal on chromosomes from wild-type animals (counterstained in red with propidium-iodide), indicating it is specifically detecting the FLAG-ESC protein.

association established in the early embryo, when PcG silencing is first implemented. However, we suspect that this

persistence of ESC in salivary gland beyond embryogenesis may not be functionally significant (see Discussion). Taken together, the co-localization of ESC and E(Z) and the co-immunoprecipitation data indicate that the E(Z) and ESC proteins are physically associated in vivo.

DISCUSSION

The WD region of ESC binds to the N terminus of E(Z)

We have demonstrated binding of the ESC protein to the E(Z) protein in vitro and shown that this interaction is mediated by the N terminus of E(Z) and the entire WD region of ESC. The region of E(Z) that mediates the binding is conserved in the four mammalian E(Z) homologs and is predicted to have an extended α-helical structure and many of the most highly conserved residues are predicted to be exposed for potential interactions with ESC. In contrast, we were unable to align $E(Z)_{1-73}$ with any portion of the three *Arabidopsis* E(Z) homologs, CLF (Goodrich et al., 1997), MEA (Grossniklaus et al., 1998) and T10M13.3 (GenBank accession no. 2104526), all of which are otherwise extensively homologous to E(Z). Since no ESC homolog has been reported yet in plants, this could indicate either (1) that the interaction of E(Z) with ESC may have evolved only in the animal kingdom after its divergence from plants or (2) if an ESC:E(Z) complex exists in plants, that the two proteins co-evolved to preserve their interaction, but underwent sequence divergence between animals and plants in that process.

We have also shown that mutations in invariant residues in all ESC homologs that were isolated in classical genetic screens and abolish ESC function in vivo also affect the binding of ESC to E(Z) in vitro. Three of the mutations that we analyzed affect residues at positions that would play structurally important roles in the predicted β-propeller motif of ESC and so are likely to perturb either global β-propeller structure or at least the local structure in the vicinity of residues directly involved in E(Z) binding. We showed that, as for other WD proteins, the entire WD region of ESC folds into a trypsin-resistant structure, consistent with a compact β-propeller structure. Mutations that would affect the predicted ESC structure also result in reduced trypsin resistance, consistent with a predicted β-propeller structure. Since all the individual WD motifs in G_β contribute to the stability of the structure, by analogy, it is likely that many mutations within the WD region of ESC abolish binding to E(Z) by perturbing the overall structure of the WD region. The esc^9 mutation (M236K) affects a residue that would not affect the predicted β-propeller structure; it is predicted to lie in a surface-exposed loop between β-strands b and c of blade 4 (WD4). Consistent with this prediction, the mutant protein has the same trypsin-resistance as the wild-type ESC protein. The residual in vitro E(Z)-binding activity of the esc^9 mutant protein further suggests that the M236K mutation perturbs a functionally important E(Z)-contact in this region of ESC, but leaves other contacts in the β-propeller unperturbed, as might be expected for a protein that makes multiple contacts with its partner. This is further supported by the dominant negative nature of the esc^9 mutation (T. F. and P. J. H., unpublished data), which clearly indicates that it retains some activity of the wild-type protein in vivo. Interestingly, the residue at the corresponding position in G_β makes a direct contact with G_α (one of 17 G_α contacts in its β-propeller region).

Stability and persistence of the ESC protein

The persistence of the ESC protein in the salivary gland is surprising, since *esc* RNA is no longer detectable by mid-embryogenesis and ESC protein has been reported to be no longer detectable by the end of embryogenesis. However, the chromosome binding assay appears to be exquisitely sensitive and perhaps chromosomally bound ESC is also protected from degradation. Furthermore, since the embryonic precursors of the salivary gland undergo no divisions after stage 10-11 (5.3-7.3 hours) (Campos-Ortega and Hartenstein, 1985), when *esc* RNA is still detectable, dilution of ESC protein by cell division is minimal in this tissue.

Could the presence of ESC in the third instar salivary gland be due to previously undetected zygotic *esc* expression after embryogenesis? If so, it is unlikely to be absolutely essential, since the normal maternal expression of *esc* alone is sufficient to rescue the embryonic lethality of homozygous *esc* mutants and produce viable adults that exhibit only a weak extra sex combs phenotype with low penetrance (Struhl, 1981). A single pulse of early embryonic *esc* expression from the hsp70 promoter can also promote substantial rescue to adults of *esc* embryos lacking maternal *esc* RNA [Simon, 1995 #1403]. Furthermore, we have found that higher levels of exclusively maternal expression completely rescue *esc* embryos to phenotypically normal fertile adults and that even maternally derived ESC protein can still be detected on polytene chromosomes (T. F. and P. J. H., unpublished data). This indicates that there is no intrinsic requirement for any zygotic *esc* expression and that ESC protein synthesized during early embryogenesis is sufficiently stable to persist on third instar salivary gland chromosomes. We conclude that the persistence of ESC in the salivary gland is likely to be simply a reflection of its stability and lack of mitotic dilution in that tissue. Nevertheless, the possibility that there is some postembryonic *esc* expression has not been definitively excluded. Indeed, Tokunaga and Stern (1965) reported the cell autonomy of *esc* phenotypes in genetic mosaics arising from X-ray-induced somatic recombination after embryogenesis. Their results imply that there is some *esc* transcription after embryogenesis, at least in leg imaginal discs and that its elimination can have phenotypic consequences. In contrast, Struhl and Brower (1982) reported that wing imaginal discs from 'maternally rescued' *esc* larvae (whose only potential source of ESC protein is maternally synthesized mRNA) remain stably determined when cultured in vivo to allow them to go through extra divisions. Unlike most larval cells, which undergo no postembryonic divisions, the imaginal discs grow from ~25 founder cells to ~50,000 during the larval period. Even an unusually stable protein synthesized during embryogenesis would experience ~2000-fold dilution, likely reducing its concentration below any functionally significant or even detectable level. Thus their results would argue against a continuous requirement for ESC. Interestingly, the mammalian ESC homolog *eed* appears to be expressed continuously and *eed* RNA is present in adult tissues (Schumacher et al., 1996). This issue deserves further investigation.

Implications of ESC:E(Z) association for PcG-mediated silencing

The binding of ESC and E(Z) in vitro, their physical association in a complex in embryos and their co-localization at many discrete chromosomal sites suggest a number of possible interpretations. It suggests that ESC is likely to be directly involved in the establishment of PcG silencing at its target genes. The virtually complete overlap of ESC and E(Z) chromosomal binding sites also suggests that formation of an ESC:E(Z) complex may be obligatory, possibly for entry or retention of both proteins in the nucleus, for binding to chromosomes, or for other aspects of their function (but see below). In contrast, the chromosomal binding sites of many other PcG proteins do not completely overlap with one another (Rastelli et al., 1993; Lonie et al., 1994; Sinclair et al., 1998a), suggesting that there are a variety of different complexes composed of different combinations of PcG proteins (Pirrotta, 1997; Strutt and Paro, 1997). However, if the formation of an ESC:E(Z) complex is obligatory, it can only be so when ESC is normally present. Since E(Z) itself is required continuously to maintain the binding of other PcG proteins to specific chromosomal sites (Rastelli et al., 1993; Platero et al., 1996), it might also be required to recruit or tether ESC to these same sites, where ESC might participate in recruitment of other PcG proteins and/or the assembly of the first mature silencing complexes. Alternatively, ESC might be required to initially recruit E(Z) to sites destined for PcG silencing. We are currently investigating this question by analyzing E(Z) and ESC chromosome binding in *esc* and *E(z)* mutants to determine whether binding of either or both proteins to chromosomes is dependent on the other.

Some transcriptional silencing functions of E(Z) appear to be ESC-independent (Moazed and O'Farrell, 1992; Pelegri and Lehmann, 1994) and E(Z) has other functions besides PcG-mediated silencing (Gatti and Baker, 1989; Phillips and Shearn, 1990; Laible et al., 1997). E(Z) is required for the growth and development of imaginal discs and the germ line (Shearn et al., 1971, 1978a,b; Phillips and Shearn, 1990) suggesting there may be a general cell cycle requirement for E(Z) in all cells. The abundant maternally synthesized *E(z)* mRNA 'rescues' homozygous *E(z)* mutant progeny of heterozygous females until the late larval period (Shearn et al., 1978b). Metaphase spreads of imaginal neuroblasts from late larval brains of *E(z)* mutants reveal an extremely low mitotic index in these normally rapidly dividing cells. The few metaphase cells present reveal defects in chromosome condensation and integrity (Gatti and Baker, 1989; Phillips and Shearn, 1990). Similarly, the interphase polytene chromosomes from larvae carrying a temperature-sensitive *E(z)* mutation appear generally decondensed at the restrictive temperature. They have much fainter bands and are missing their characteristic constrictions, i.e., specific regions of highly condensed chromatin, including the landmark constriction associated with the Bithorax Complex (Rastelli et al., 1993; Carrington and Jones, 1996), which presumably reflects its transcriptionally silent state in the salivary gland. This chromosomal phenotype suggests that E(Z) may have a general role in regulating chromatin condensation states globally throughout the chromosome. Consistent with this, Carrington and Jones (1996) reported that, when high concentrations of E(Z) antibody are used, they detected a low level of E(Z) binding that is uniformly distributed along the entire length of the chromosomes, in addition to the prominent discrete sites of E(Z) binding. This could reflect general functions of E(Z) in regulating aspects of chromosome structure that are not ESC-dependent. Perhaps the formation of the prominent sites of E(Z):ESC colocalization represent a specialized ESC-dependent organization of generally distributed E(Z) at sites of PcG silencing. Other PcG proteins are proposed to regulate chromatin packaging locally, but unlike E(Z), they are not required for germline or imaginal disc proliferation (Breen and Duncan, 1986; Soto et al., 1995), suggesting they do not play a more general global role in chromosome architecture or condensation during mitosis. Indeed, the PcG proteins PC, PH and PSC dissociate from chromosomes during mitosis (Buchenau et al., 1998).

Recent evidence that E(Z) is required for the heterochomatin-mediated silencing associated with Position Effect Variegation (PEV) (Laible et al., 1997) further suggests that E(Z) has a more general function than other PcG proteins, most of which do not play a role in PEV (Sinclair et al., 1998b). Interestingly, we detect both E(Z) and ESC binding to the largely heterochromatic chromocenter of polytene chromosomes. E(Z) was previously reported to be absent from the chromocenter (Carrington and Jones, 1996). We suspect that the differences in our results may be due to the generally greater sensitivity of the immunofluorescence detection method and confocal microscopy used here, although differences in fixation or processing of chromosomes might also contribute. (On the contrary, our failure to detect any low level of uniform chromosome binding seen previously (Carrington and Jones, 1996) is probably due to the lower E(Z) antibody concentration used as well as the signal cutoff threshold used when obtaining these images.) The reported role of E(Z) in PEV suggests that its binding to the chromocenter could reflect a functionally significant interaction with heterochromatin, rather than an adventitious association (Platero et al., 1998). However, E(Z) does not appear to be required for the heterochromatin-specific binding of HP1 (Platero et al., 1996), a protein required for PEV that is believed to be a structural component of heterochromatin (Eissenberg et al., 1990, 1992). ESC presently has no known connection with PEV, but its association with the chromocenter raises the possibility that ESC might also be involved in the packaging of heterochromatin.

The involvement of E(Z) in regulating global chromosome architecture and condensation during mitosis could represent a more ancient function than its role in PcG silencing. The presence of highly conserved E(Z) homologs in the plant kingdom implies that it is a very ancient protein. Recruitment of E(Z) by ESC at specific sites of PcG silencing, if it occurs, could represent a more recent evolutionarily acquisition for targeting chromatin condensation activities of E(Z) that originally evolved to serve more general functions to new contexts.

While E(Z) is continuously required for the binding of other PcG proteins, at present there is no evidence that the E(Z):ESC is physically associated with complexes containing other PcG proteins. Indeed, it was previously suggested that ESC acts independently from PC based on the ability of

mutations in *Polycomb* to further enhance the *esc* null phenotype (Struhl, 1983). Temporal restriction of ESC to embryogenesis would also imply that it is not an integral component of PcG complexes present throughout development. Consistent with this, we have identified a ~500 kDa complex containing ESC and E(Z) that is distinct from complexes containing other PcG proteins (F. T. and P. J. H., unpublished data). It remains quite possible that complexes containing ESC:E(Z) act independently of other PcG proteins, perhaps making some initial alteration in local chromatin structure that is essential for recognition and binding by other PcG proteins or their assembly into functional silencing complexes.

Dual functions of E(Z) in transcriptional activation and silencing

In addition to its requirement for silencing of the homeotic genes, E(Z) is also required for maintaining their expression. *E(z)* loss-of-function mutations enhance the phenotypes of *trx* and *ash1* mutants in double heterozygotes and, in addition to ectopic expression, can also cause reductions in homeotic gene expression in imaginal discs, similar to trithorax group (trxG) mutants (LaJeunesse and Shearn, 1996). Similar dual effects of altered E(Z) gene dosage on *esc* phenotypes have also been observed. Reducing *E(z)* gene dosage strongly enhances *esc* mutant phenotypes in embryos (Campbell et al., 1995), as expected from the requirement of both in PcG silencing. But surprisingly, increasing *E(z)* gene dosage also enhances the weak *esc* mutant phenotypes seen in adults (Campbell et al., 1995), similar to the effect of increasing *trx* gene dosage (Capdevila and García-Bellido, 1981). At present we do not know whether these two opposing phenotypic effects of E(Z) reflect alternative, fundamentally different biochemical activities of the E(Z) protein or simply two different 'pathways' of E(Z) action that both impinge differently on the phenotype of *esc* mutants. It is possible that excess E(Z) sequesters sufficient ESC into nonfunctional complexes to effectively reduce the amount of chromosomally bound ESC. Mutations in some of the other PcG genes have been shown to have similar *trx*-like effects (Sinclair et al., 1992) (J. Gildea and A. Shearn, personal communication).

There is at least one precedent for such apparently opposing dual functions: GAGA factor. Mutations in GAGA exhibit dominant *trx*-like phenotypes (Farkas et al., 1994), but they also suppress PcG/PRE-mediated silencing of a mini-*white* reporter (Hagstrom et al., 1997), indicating that GAGA is required both for the maintenance of active and repressed transcriptional states. In this case, both effects are likely to be due to a single underlying function of GAGA: facilitating binding of both trxG and PcG proteins to chromosomes (E. P. Jane and P. J. H., unpublished). Whether there is also a single function/activity of E(Z) underlying its dual effects on transcriptional maintenance and silencing is not yet known, but the fact that *E(z)* mutants also exhibit reduced binding of TRX (Kuzin et al., 1994) as well as PcG proteins (Rastelli et al., 1993) to polytene chromosomes suggests this possibility. If E(Z) has a direct role in promoting both 'open' and 'closed' chromatin states, it might do so as a more general component of chromosome architecture to which other chromosomal proteins that more directly regulate chromatin structure are tethered. Its associations with different proteins in different transcriptional contexts might determine its different functions. In such a scenario, rather than ESC recruiting E(Z) to specific sites, E(Z) might participate in tethering ESC and other proteins to specific sites to promote silencing. Analysis of E(Z) and ESC chromosome binding in *esc* and *E(z)* mutants should establish whether binding of either protein to chromosomes is dependent on the other. Further identification of other proteins associated with ESC and E(Z) should further clarify the role of each in PcG silencing.

After this work was completed, Jones et al. (1998) reported similar in vitro binding and co-IP data showing that ESC and E(Z) interact directly. Our in vitro binding and co-IP results are consistent with theirs and further show that the WD region of ESC is sufficient for E(Z) binding and adopts a trypsin-resistant structure, consistent with its predicted β-propeller structure. Different ESC mutants were analyzed in each study and the results complement one another. Jones et al. (1998) further mapped the ESC-binding region of E(Z) to $E(Z)_{33\text{-}66}$ and by in vitro mutagenesis identified a single amino acid substitution within this region of E(Z) that perturbs its in vitro binding to ESC.

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REFERENCES

Alkema, M. J., Bronk, M., Verhoeven, E., Otte, A., van't Veer, L. J., Berns, A. and van Lohuizen, M. (1997). Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian Polycomb complex. *Genes Dev.* **11**, 226-240.

Bornemann, D., Miller, E. and Simon, J. (1996). The *Drosophila* Polycomb group gene *Sex comb on midleg* (*Scm*) encodes a zinc finger protein with similarity to polyhomeotic protein. *Development* **122**, 1621-1630.

Breen, T. R. and Duncan, I. M. (1986). Maternal expression of genes that regulate the bithorax complex of *Drosophila melanogaster*. *Dev. Biol.* **118**, 442-456.

Brown, L., Mucci, D., Whiteley, M., Dirksen, M.-L. and Kassis, J. (1998). The *Drosophila* Polycomb group gene *pleiohomeotic* encodes a sequence-specific DNA binding protein with homology to the multifunctional mammalian transcription factor YY1. *Molecular Cell* (in press).

Brunk, B. P., Martin, E. C. and Adler, P. N. (1991). *Drosophila* genes *Posterior Sex Combs* and *Suppressor two of zeste* encode proteins with homology to the murine *bmi-1* oncogene. *Nature* **353**, 351-353.

Buchenau, P., Hodgson, J., Strutt, H. and Arndt-Jovin, D. J. (1998). The distribution of Polycomb-Group proteins during cell division and development in *Drosophila* embryos: impact on models for silencing. *J. Cell Biol.* **141**, 469-481.

Bunker, C. A. and Kingston, R. E. (1994). Transcriptional repression by *Drosophila* and mammalian *Polycomb* group proteins in transfected mammalian cells. *Mol. Cell. Biol.* **14**, 1721-1732.

Busturia, A., Wightman, C. D. and Sakonju, S. (1997). A silencer is required for maintenance of transcriptional repression throughout *Drosophila* development. *Development* **124**, 4343-4350.

Campbell, R. B., Sinclair, D. A., Couling, M. and Brock, H. W. (1995). Genetic interactions and dosage effects of *Polycomb* group genes of *Drosophila*. *Mol. Gen. Genet.* **246**, 291-300.

Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster* Berlin: Springer-Verlag.

Capdevila, M. P. and García-Bellido, A. (1981). Genes involved in the

activation of the Bithorax Complex of *Drosophila*. *Roux's Arch. Dev. Biol.* **190**, 339-350.

Carrington, E. A. and Jones, R. S. (1996). The *Drosophila Enhancer of zeste* gene encodes a chromosomal protein: examination of wild-type and mutant protein distribution. *Development* **122**, 4073-4083.

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

Chen, H., Rossier, C. and Antonarakis, S. E. (1996). Cloning of a human homolog of the *Drosophila Enhancer of zeste* gene (EZH2) that maps to chromosome 21q22.2. *Genomics* **38**, 30-37.

Chinwalla, V., Jane, E. P. and Harte, P. J. (1994). The *Drosophila trithorax* protein binds to specific chromosomal sites and is co-localized with *Polycomb* at many sites. *EMBO J.* **14**, 2056-2065.

DeCamillis, M., Cheng, N. S., Pierre, D. and Brock, H. W. (1992). The *polyhomeotic* gene of *Drosophila* encodes a chromatin protein that shares polytene chromosome-binding sites with *Polycomb*. *Genes Dev.* **6**, 223-232.

Denisenko, O. N. and Bomstyk, K. (1997). The product of the murine homolog of the *Drosophila extra sex combs* gene displays transcriptional repressor activity. *Mol. Cell. Biol.* **17**, 4707-4717.

Eissenberg, J. C., James, T. C., Foster-Hartnett, D. M., Hartnett, T., Ngan, V. and Elgin, S. C. (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **87**, 9923-9927.

Eissenberg, J. C., Morris, G. D., Reuter, G. and Hartnett, T. (1992). The heterochromatin-associated protein HP-1 is an essential protein in *Drosophila* with dosage-dependent effects on position-effect variegation. *Genetics* **131**, 345-352.

Farkas, G., Galloni, M., Gausz, J., Reuter, G., Gyurkovics, H. and Karch, F. (1994). *Trithorax-like*, the gene encoding the *Drosophila* GAGA factor, regulates homeotic loci and suppresses heterochromatin-induced position effect variegation. *Nature* **371**, 806-808.

Fong, H. K. W., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F. and Simon, M. I. (1986). Repetitive segmental structure of the transducin β subunit: homology with the CDC4 gene and identification of related mRNAs. *Proc. Natl. Acad. Sci. USA* **83**, 2162-2166.

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

Frei, E., Baumgartner, S., Edström, J. E. and Noll, M. (1985). Cloning of the *extra sex combs* gene of *Drosophila* and its identification by P element-mediated gene transfer. *EMBO J.* **4**, 979-987.

Garcia-Higuera, I., Fenoglio, J., Li, Y., Lewis, C., Panchenko, M. P., Reiner, O., Smith, T. F. and Neer, E. J. (1996). Folding of proteins with WD repeats: comparison of six members of the WD-repeat superfamily to the G protein β subunit. *Biochemistry* **35**, 13985-13994.

Gatti, M. and Baker, B. S. (1989). Genes controlling essential cell-cycle functions in *Drosophila melanogaster*. *Genes Dev.* **3**, 438-453.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E. M. and Coupland, G. (1997). A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. *Nature* **386**, 44-51.

Grossniklaus, U., Vielle-Calzada, J.-P., Hoeppner, M. A. and Gagliano, W. B. (1998). Maternal control of embryogenesis by *MEDEA*, a Polycomb Group gene in *Arabadopsis*. *Science* **280**, 446-450.

Gunster, M. J., Satijn, D. P., Hamer, K. M., den Blaauwen, J. L., de Bruijn, D., Alkema, M. J., van Lohuizen, M., van Driel, R. and Otte, A. P. (1997). Identification and characterization of interactions between the vertebrate Polycomb-Group protein BMI1 and human homologs of polyhomeotic. *Mol. Cell. Biol.* **17**, 2326-2335.

Gutjahr, T., Frei, E., Spicer, C., Baumgartner, S., White, R. A. H. and Noll, M. (1995). The Polycomb-group gene, *extra sex combs*, encodes a nuclear member of the WD-40 repeat family. *EMBO J.* **14**, 4296-4306.

Hagstrom, K., Muller, M. and Schedl, P. (1997). A Polycomb and GAGA dependent silencer adjoins the Fab-7 boundary in the *Drosophila* bithorax complex. *Genetics* **146**, 1365-1380.

Hobert, O., Jallal, B. and Ullrich, A. (1996a). Interaction of Vav with ENX-1, a putative transcriptional regulator of homeobox gene expression. *Mol. Cell. Biol.* **16**, 3066-3073.

Hobert, O., Sures, I., Ciossek, T., Fuchs, M. and Ullrich, A. (1996b). Isolation and developmental expression analysis of *Enx-1*, a novel mouse Polycomb group gene. *Mech. Dev.* **55**, 171-184.

Jiménez, G., Paroush, Z. and Ish-Horowicz, D. (1997). Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes and Development* **11**, 3072-3417.

Jones, R. S. and Gelbart, W. M. (1993). The *Drosophila* Polycomb-Group gene *Enhancer of zeste* shares a domain of sequence similarity with *trithorax*. *Mol. Cell. Biol.* **13**, 6357-6366.

Jones, C. A., Ng, J., Peterson, A. J., Morgan, K., Simon, J., Jones, R. S. (1998). The *Drosophila* esc and E(z) proteins are direct partners in Polycomb Group-mediated repression. *Mol. Cell. Biol.* **18**, 2825-2834.

Jürgens, G. (1985). A group of genes controlling the spatial expression of the Bithorax Complex in *Drosophila*. *Nature* **316**, 153-155.

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

Komachi, K. and Johnson, A. D. (1997). Residues in the WD repeats of Tup1 required for interaction with α2. *Mol. Cell. Biol* **17**, 6023-6028.

Komachi, K., Redd, M. J. and Johnson, A. D. (1994). The WD repeats of Tup1 interact with the homeo domain protein α2. *Genes Dev.* **8**, 2857-2867.

Kuzin, B., Tillib, S., Sedkov, Y., Mizrokhi, L. and Mazo, A. (1994). The *Drosophila trithorax* gene encodes a chromosomal protein and directly regulates the region-specific homeotic gene *fork head*. *Genes Dev.* **8**, 2478-2490.

Kyba, M and Brock, H. W. (1998). The *Drosophila* Polycomb Group protein PSC contacts PH and PC through specific conserved domains. *Mol. Cell Biol.* **18**, 2712-2720.

Laible, G., Wolf, A., Dorn, R., Reuter, G., Nislow, C., Lebersorger, A., Popkin, D., Pillus, L. and Jenuwein, T. (1997). Mammalian homologues of the Polycomb-group gene *Enhancer of zeste* mediate gene silencing in *Drosophila* heterochromatin and at *S. cerevisiae* telomeres. *EMBO J* **16**, 3219-3232.

LaJeunesse, D. and Shearn, A. (1996). *E(z)*: a Polycomb group gene or a trithorax group gene? *Development* **122**, 2189-2197.

Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E. and Sigler, P. B. (1996). The 2.0 Å crystal structure of a heterotrimeric G protein. *Science* **379**, 311-319.

Landecker, H. L., Sinclair, D. A. and Brock, H. W. (1994). Screen for enhancers of *Polycomb* and *Polycomblike* in *Drosophila melanogaster*. *Dev. Genet.* **15**, 425-434.

Lonie, A., D'Andrea, R., Paro, R. and Saint, R. (1994). Molecular characterization of the *Polycomblike* gene of *Drosophila melanogaster*, a transacting negative regulator of homeotic gene expression. *Development* **120**, 2629-2636.

Martin, E. C. and Adler, P. N. (1993). The Polycomb group gene *Posterior Sex Combs* encodes a chromosomal protein. *Development* **117**, 641-655.

Matthews, K. A., Miller, D. F. and Kaufman, T. C. (1989). Developmental distribution of RNA and protein products of the *Drosophila* alpha-tubulin gene family. *Dev. Biol.* **132**, 45-61.

McCall, K. and Bender, W. (1996). Probes for chromatin accessibility in the *Drosophila* Bithorax Complex respond differently to Polycomb-mediated repression. *EMBO J.* **15**, 569-580.

McKeon, J. and Brock, H. W. (1991). Interactions of the Polycomb group of genes with homeotic loci of *Drosophila*. *Roux Arch. Dev. Biol.* **199**, 387-396.

Moazed, D. and O'Farrell, P. H. (1992). Maintenance of the *engrailed* expression pattern by Polycomb group genes in *Drosophila*. *Development* **116**, 805-810.

Neer, E. J., Schmidt, C. J., Nambudripad, R. and Smith, T. F. (1994). The ancient regulatory-protein family of WD-repeat proteins. *Nature* **371**, 297-300.

Neer, E. J. and Smith, T. F. (1996). G protein heterodimers: new structures propel new questions. *Cell* **84**, 175-178.

Ng, J., Li, R., Morgan, K. and Simon, J. (1997). Evolutionary conservation and predicted structure of the *Drosophila* extra sex combs repressor protein. *Mol. Cell. Biol.* **17**, 6663-6672.

O'Donnell, K. H., Chen, C. T. and Wensink, P. C. (1994). Insulating DNA directs ubiquitous transcription of the *Drosophila melanogaster* alpha 1-tubulin gene. *Mol. Cell Biol.* **14**, 6398-6408.

Orlando, V. and Paro, R. (1993). Mapping *Polycomb*-repressed domains in the bithorax complex using *in vivo* formaldehyde cross-linked chromatin. *Cell* **75**, 1187-1198.

Paro, R. and Hogness, D. S. (1991). The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **88**, 263-267.

Paro, R. and Zink, B. (1993). The *Polycomb* gene is differentially regulated

during oogenesis and embryogenesis of *Drosophila melanogaster*. *Mech. Dev.* **40**, 37-46.

Paroush, Z., Finley, R. L., Kidd, T., Wainwrirht, S. M., Ingham, P. W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for *Drosophila* neurogenesis, segmentation and sex determination and interacts directly with Hairy-related bHLH proteins. *Cell* **79**, 805-815.

Pelegri, F. and Lehmann, R. (1994). A role of Polycomb Group genes in the regulation of gap gene expression in *Drosophila*. *Genetics* **136**, 1341-1353.

Peterson, A. J., Kyba, M., Bornemann, D., Morgan, K., Brock, H. W. and Simon, J. (1997). A domain shared by the Polycomb group proteins Scm and ph mediates heterotypic and homotypic interactions. *Mol. Cell. Biol.* **17**, 6683-6692.

Phillips, M. D. and Shearn, A. (1990). Mutations in *polycombeotic*, a *Drosophila* Polycomb-group gene, cause a wide range of maternal and zygotic phenotypes. *Genetics* **125**, 91-101.

Pirrotta, V. (1997). PcG complexes and chromatin silencing. *Curr. Op. Gen. Dev.* **7**, 249-258.

Pirrotta, V., Chan, C.-S., McCabe, D. and Qian, S. (1995). Distinct parasegmental and imaginal enhancers and the establishment of the expression pattern of the *Ubx* gene. *Genetics* **141**, 1439-1450.

Pirrotta, V. and Rastelli, L. (1994). *white* gene expression, repressive chromatin domains, and homeotic gene regulation in *Drosophila*. *BioEssays* **16**, 549-556.

Platero, J. S., Csink, A. K., Quintanilla, A. and Henikoff, S. (1998). Changes in chromosomal localization of heterochromatin-binding proteins during the cell cycle in *Drosophila*. *J. Cell Biol.* **140**, 1297-1306.

Platero, J. S., Sharp, E. J., Adler, P. N. and Eissenberg, J. C. (1996). In vivo assay for protein-protein interactions using *Drosophila* chromosomes. *Chromosoma* **104**, 393-404.

Poux, S., Kostic, C. and Pirrotta, V. (1996). Hunchback-independent silencing of late *Ubx* enhancers by a Polycomb group response element. *EMBO J.* **15**, 4713-4722.

Rastelli, L., Chan, C. S. and Pirrotta, V. (1993). Related chromosome binding sites for *zeste*, suppressors of *zeste* and *Polycomb* group proteins in *Drosophila* and their dependence on *Enhancer of zeste* function. *EMBO J.* **12**, 1513-1522.

Rost, B. and Sander, C. (1993a). Improved prediction of protein secondary structure by use of sequence profiles and neural networks. *Proc. Natl. Acad. Sci. USA* **90**, 7558-7562.

Rost, B. and Sander, C. (1993b). Prediction of protein structure at better than 70% accuracy. *J. Mol. Biol.* **232**, 584-599.

Rost, B. and Sander, C. (1994). Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19**, 55-72.

Rost, B., Sander, C. and Schneider, R. (1994). PHD – an automatic mail server for protein secondary structure prediction. *CABIOS* **10**, 53-60.

Sathe, S. S. and Harte, P. J. (1995a). The *Drosophila* Extra sex combs protein contains WD repeats essential for its function as a repressor of homeotic genes. *Mech. Dev.* **52**, 77-87.

Sathe, S. S. and Harte, P. J. (1995b). The Extra sex combs protein is highly conserved between *Drosophila virilis* and *Drosophila melanogaster*. *Mech. Dev.* **52**, 225-232.

Satijn, D. P., Gunster, M. J., van der Vlag, J., Hamer, K. M., Schul, W., Alkema, M. J., Saurin, A. J., Freemont, P. S., van Driel, R. and P, O. A. (1997). RING1 is associated with the Polycomb Group protein complex and acts as a transcriptional repressor. *Mol. Cell Biol.* **17**, 4105-4113.

Schumacher, A., Faust, C. and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. *Nature* **383**, 250-253.

Shearn, A., Hersperger, G. and Hersperger, E. (1978a). Genetic analysis of two allelic temperature-sensitive mutants of *Drosophila melanogaster*, both of which are zygotic and maternal-effect lethals. *Genetics* **89**, 341-353.

Shearn, A., Hersperger, G., Hersperger, E., Pentz, E. S. and Denker, P. (1978b). Multiple allele approach to the study of genes in *Drosophila melanogaster* that are involved in imaginal disc development. *Genetics* **89**, 355-370.

Shearn, A., Rice, T., Garen, A. and Gehring (1971). Imaginal disc abnormalities in lethal mutants of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **68**, 2695-2698.

Simon, J., Bornemann, D., Lunde, K. and Schwartz, C. (1995). The *extra sex combs* product contains WD40 repeats and its time of action implies a role distinct from other Polycomb group products. *Mech. Dev* **53**, 197-208.

Simon, J., Chiang, A. and Bender, W. (1992). Ten different *Polycomb* group genes are required for spatial control of the *abd-A* and *Abd-B* homeotic products. *Development* **114**, 493-505.

Simon, J., Peifer, M., Bender, W. and O'Connor, M. (1990). Regulatory elements of the bithorax complex that control expression along the anterior-posterior axis. *EMBO J.* **9**, 3945-3956.

Sinclair, D., Milne, T., Hodgson, J., Shellard, J., Salinas, C., Kyba, M., Randazzo, F. and Brock, H. (1998a). The *Additional sex combs* gene of *Drosophila* encodes a chromatin protein that binds to shared and unique Polycomb group sites on polytene chromosomes. *Development* **125**, 1207-1216.

Sinclair, D. A., Campbell, R. B., Nicholls, F., Slade, E. and Brock, H. W. (1992). Genetic analysis of the *Additional sex combs* locus of *Drosophila melanogaster*. *Genetics* **130**, 817-825.

Sinclair, D. A., Clegg, N. J., Antonchuk, J., Milne, T. A., Stankunas, K., Ruse, C., Grigliatti, T. A., Kassis, J. A. and Brock, H. W. (1998b). *Enhancer of Polycomb* is a suppressor of position-effect variegation in *Drosophila melanogaster*. *Genetics* **148**, 211-220.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E. and Sigler, P. B. (1996). Crystal structure of a G_A protein $\beta\gamma$ dimer at 2.1 Å resolution. *Science* **379**, 369-374.

Soto, M. C., Chou, T. B. and Bender, W. (1995). Comparison of germline mosaics of genes in the Polycomb group of *Drosophila melanogaster*. *Genetics* **140**, 231-243.

Struhl, G. (1981). A gene product required for correct initiation of segmental determination in *Drosophila*. *Nature* **293**, 36-41.

Struhl, G. (1983). Role of the *esc*$^+$ gene product in ensuring the selective expression of segment-specific homeotic genes in *Drosophila*. *J. Embryol. Exp. Morphol.* **76**, 297-331.

Struhl, G. and Akam, M. (1985). Altered distributions of *Ultrabithorax* transcripts in *extra sex combs* mutant embryos of *Drosophila*. *EMBO J.* **4**, 3259-3264.

Struhl, G. and Brower, D. (1982). Early role of the *esc*$^+$ gene product in the determination of segments in *Drosophila*. *Cell* **31**, 285-292.

Strutt, H., Cavalli, G. and Paro, R. (1997). Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. *EMBO J.* **16**, 3621-3632.

Strutt, H. and Paro, R. (1997). The Polycomb group protein complex of *Drosophila* melanogaster has different compositions at different target genes. *Mol. Cell. Biol.* **17**, 6773-6783.

Tokunaga, C. and Stern, C. (1965). The developmental autonomy of *extra sex combs* in *Drosophila melanogaster*. *Dev. Biol.* **11**, 50-81.

Wall, M. A., Coleman, D. E., Lee, E., Iñiguez-Lluhi, J. A., Posner, B. A., Gilman, A. G. and Sprang, S. R. (1995). The structure of the G protein heterotrimer Giα1β1γ2. *Cell* **83**, 1047-1058.

Wedeen, C., Harding, K. and Levine, M. (1986). Spatial regulation of Antennapedia and bithorax gene expression by the *Polycomb* locus in *Drosophila*. *Cell* **44**, 739-748.

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

Note added in proof

Interactions between EED and ENX1/EZH2, the mammalian homologs of ESC and E(Z) respectively, were also recently reported (Jones et al., 1998).

Sewalt, R. G. A. B., van der Vlag, J., Gunster, M. J., Hamer, K. M., den Blaauwen, J. L., Satijn, D. P. E., Hendrix, T., van Driel, R. and Otte, A. P. (1998). Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. *Mol. Cell. Biol.* **18**, 3586-3595.

van Lohuizen, M., Tijms, M., Voncken, J. W., Schumacher, A., Magnuson, T. and Wientjens, E. (1998). Interaction of mouse polycomb-group (Pc-G) proteins enx1 and enx2 with eed: indication for separate Pc-G complexes. *Mol. Cell. Biol.* **18**, 3572-3579.