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The *Drosophila* Polycomb Group proteins ESC and E(Z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3

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SUMMARY

The *Drosophila* Polycomb Group (PcG) proteins are required for stable long term transcriptional silencing of the homeotic genes. Among PcG genes, *esc* is unique in being critically required for establishment of PcG-mediated silencing during early embryogenesis, but not for its subsequent maintenance throughout development. We previously showed that ESC is physically associated in vivo with the PcG protein E(Z). We report here that ESC, together with E(Z), is present in a 600 kDa complex that is distinct from complexes containing other PcG proteins. We have purified this ESC complex and show that it also contains the histone deacetylase RPD3 and the histone-binding protein p55, which is also a component of the chromatin remodeling complex NURF and the chromatin assembly complex CAF-1. The association of ESC and E(Z) with p55 and RPD3 is conserved in mammals. We show that RPD3 is required for silencing mediated by a Polycomb response element (PRE) in vivo and that E(Z) and RPD3 are bound to the *Ubx* PRE in vivo, suggesting that they act directly at the PRE. We propose that histone deacetylation by this complex is a prerequisite for establishment of stable long-term silencing by other continuously required PcG complexes.

Key words: ESC, E(Z), Polycomb, histone deacetylase, p55, *Drosophila*

INTRODUCTION

The PcG proteins are required to maintain stable heritable repression of the homeotic genes and others. In *Drosophila*, the characteristic spatially restricted patterns of homeotic gene expression are established during the first few hours of embryogenesis through the action of the transiently expressed activators and repressors encoded by the segmentation genes. This process does not depend on PcG proteins. After the first 4 hours of embryogenesis, these activators and repressors disappear, but repression of the homeotic genes in cells outside their expression domains is stably maintained throughout the rest of development. This maintenance of repression requires the PcG proteins. More than a dozen PcG genes have been characterized (Bornemann et al., 1996; Brown et al., 1998; Brunk et al., 1991; DeCamillis et al., 1992; Jones and Gelbart, 1993; Jürgens, 1985; Landecker et al., 1994; Lonie et al., 1994; Paro and Hogness, 1991; Sinclair et al., 1998) and homologs of many have been identified in other species, including mammals (Alkema et al., 1997b; Denisenko and Bomstyk, 1998; Gunster et al., 1997; Hashimoto et al., 1998; Sewalt et al., 1998; van Lohuizen et al., 1991; van Lohuizen et al., 1998), suggesting that PcG-mediated silencing has been widely conserved in evolution.

PcG proteins function in concert. Some have been shown to bind to each other in vitro (Jones et al., 1998; Kyba and Brock, 1998a; Kyba and Brock, 1998b; Peterson et al., 1997; Tie et al., 1998) and these interactions are conserved in mammals (Alkema et al., 1997a; Gunster et al., 1997; Kyba and Brock, 1998b; Sewalt et al., 1998; Tomotsune et al., 1999). Individual PcG proteins that are tethered to DNA as lexA- or GAL4-fusion proteins can recruit other PcG proteins to form functional silencing complexes (Bunker and Kingston, 1994; Muller, 1995). Some have been shown to be present in the same high molecular weight complexes in vivo (Franke et al., 1992; Gunster et al., 1997; Hashimoto et al., 1998; Satijn et al., 1997a; Strutt and Paro, 1997) and a 2 MDa complex containing the PcG proteins PC, PH, PSC and SCM, was recently purified (Shao et al., 1999). Consistent with the existence of complexes containing multiple PcG proteins, most PcG proteins have been shown to bind to approximately 100 specific chromosomal sites (Carrington and Jones, 1996; DeCamillis et al., 1992; Franke et al., 1992; Lonie et al., 1994; Martin and Adler, 1993; Rastelli et al., 1993; Sinclair et al., 1998; Stankunas et al., 1998). However, despite their broad patterns of co-localization, the chromosomal distributions of individual PcG proteins often differ in detail. This suggests that there is a multiplicity of different PcG complexes that share some components but differ in others. Indeed, chromatin immunoprecipitation experiments have revealed PC-containing complexes of different

compositions bound to different target genes (Strutt and Paro, 1997). The phenotypes of individual PcG mutants, while generally similar, also differ in detail (McKeon and Brock, 1991; Simon et al., 1992; Soto et al., 1995), further indicating differential requirements for individual PcG proteins.

The mechanisms underlying PcG-mediated silencing are unknown. It has been suggested to involve the creation of altered chromatin domains (Paro and Hogness, 1991) that could render transcription factor-binding sites inaccessible (Bunker and Kingston, 1994; McCall and Bender, 1996) and/or prevent enhancer-promoter communication (Pirrotta, 1997; Pirrotta and Rastelli, 1994). The PC protein has recently been shown to bind to nucleosomes in vitro (Breiling et al., 1999), consistent with the possibility that it has a chromatin modifying function. Disruption of PRE-mediated silencing of a reporter can be induced by a potent activator and is accompanied by local accumulation of hyperacetylated histone H4, a signature of transcriptionally active genes (Cavalli and Paro, 1999). This indirectly suggests that local histone deacetylation may accompany PcG silencing. Recently, a purified complex, dubbed PRC1, containing the PcG proteins PC, PH, PSC and SCM, was shown to inhibit the in vitro ATP-dependent nucleosome remodeling activity of a purified human SWI/SNF complex (Shao et al., 1999). This raises the possibility that PRC1 alters chromatin in some way that renders it refractory to the action of nucleosome remodeling complexes, although the basis of its inhibition of remodeling is not yet understood.

PcG-mediated silencing is initiated during early embryogenesis. ESC plays a crucial early role in the initiation of PcG silencing, as well as some role in its subsequent maintenance. Genetic evidence indicates that ESC expression and function is required only transiently, between 2 and 6 hours of embryogenesis to establish PcG silencing and promote development of viable adults that display only weak partial transformations of second and third thoracic (T2 and T3) legs to T1 legs (Simon et al., 1995; Struhl and Brower, 1982). Consistent with this, *esc* mRNA is expressed during oogenesis and early embryogenesis, becoming undetectable after approx. 8 hours (Frei et al., 1985; Sathe and Harte, 1995; Simon et al., 1995). ESC protein is readily detectable during early embryogenesis, but is barely detectable by the end of embryogenesis (Gutjahr et al., 1995; Ng et al., 2000; T. F. and P. J. H., unpublished observations). In addition to its early critical requirement, genetic mosaic studies indicate that ESC is also required after embryogenesis to fully maintain silencing, at least in leg imaginal discs (Tokunaga and Stern, 1965). Consistent with this, ESC is detectable on third instar polytene chromosomes (Tie et al., 1998) and low levels are detectable in late larval extracts (Ng et al., 2000).

We have previously reported that ESC binds directly in vitro to the PcG protein E(Z), co-immunoprecipitates with E(Z) from embryo extracts and co-localizes with E(Z) on chromosomes (Jones et al., 1998; Tie et al., 1998). In addition, *esc* mutations that perturb ESC function in vivo also perturb ESC binding to E(Z), suggesting that the association of ESC and E(Z) in the early embryo is essential for PcG-mediated silencing (Tie et al., 1998). Similar results have been reported for mammalian ESC and E(Z) homologs (Denisenko and Bomstyk, 1998; Sewalt et al., 1998; van Lohuizen et al., 1998). Although the function of E(Z) is not well understood, it appears to be continuously required to maintain the binding of other PcG proteins to chromosomes (Rastelli et al., 1993) and to localize to chromosomal sites where other PcG proteins bind (Carrington and Jones, 1996).

In this report we show that ESC and E(Z) are present in a stable approx. 600 kDa complex in embryos that is distinct from other PcG complexes. We show that the ESC complex also contains the histone deacetylase RPD3 and the histone-binding protein p55, which is also a subunit of the CAF1 chromatin assembly factor (Tyler et al., 1996) and the nucleosome remodeling factor NURF (Martinez-Balbas et al., 1998). We show that RPD3 is required for PRE-mediated silencing in vivo and that E(Z) and RPD3 are bound to the *Ubx* PRE in vivo, suggesting they act directly at the PRE. Our findings provide a direct molecular link between the ESC complex and chromatin through p55, and implicate histone deacetylation in the mechanism of ESC-mediated silencing.

MATERIALS AND METHODS

Preparation of embryo extracts

A transgenic *Drosophila* line carrying a null mutation in the endogenous *esc* gene and whose only source of ESC protein is a fully functional FLAG epitope-tagged ESC (FLAG-ESC) expressed under the control of the constitutive α1-tubulin promoter (Tie et al., 1998) was used for overnight (0-18 hour) embryo collection. Embryo nuclear extract (NE) was prepared as previously described (Kamakaka et al., 1991) with the following modifications. Embryos were homogenized in buffer 1 (15 mM Hepes pH 7.4, 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 350 mM sucrose) containing protease inhibitors (1 mM PMSF and 1 μg/ml leupeptin, aprotinin and pepstatin) in ratio of 2 ml/g embryo. Nuclei were collected by centrifugation at 3000 ***g*** for 10 minutes and washed twice with the same buffer and then resuspended (2 ml/g nuclei) in nuclear extraction buffer (50 mM Hepes, pH 7.6, 110 mM KCl, 150 mM NaCl, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 0.1 mM EDTA) containing protease inhibitors and phosphatase inhibitors (5 mM KF, 1 mM sodium pyrophosphate, 1 mM glycerophosphate, 0.2 mM sodium molybdate, 0.2 mM sodium orthovanadate). *Drosophila* embryo total protein extract was prepared as described (Dingwall et al., 1995). Extract was centrifuged at 30,000 ***g*** for 1 hour at 4°C. Supernatant was carefully removed from tubes and filtered through a 0.2 μm filter (Nalgene), then stored at –80°C. Total protein concentration in NE was 2-3 mg/ml determined by Coomassie Protein Assay Reagent (Pierce). HeLa cell nuclear extract (5 mg/ml total protein) was kindly provided by Dr Donal Luse (Cleveland Clinic Foundation).

Chromatographic identification and purification of the ESC complex

For determination of the native molecular mass of ESC complexes, total protein extract (200 μl) from embryos (0-22 hour) was loaded onto a Pharmacia Superose 6 HR 10/30 column and eluted with 50 mM PBS, pH 7.4 containing 0.3 M NaCl at the flow rate of 0.2 ml/min by using a Pharmacia FPLC System. Fractions (0.5 ml/tube) were collected beginning after 5 ml elution and analyzed by western blot for the presence of the E(Z), FLAG-ESC and PC proteins.

To purify the ESC complex, 4-8 ml of nuclear extract were loaded onto a 2.6×80 cm Superdex 200 gel filtration column and eluted with 20 mM Hepes, pH 7.4, 150 mM NaCl, 5% glycerol at a flow rate of 0.8 ml/min. Eluate was collected in 4 ml fractions and the distribution of ESC and E(Z) were determined by western blot. Fractions containing both ESC and E(Z) were pooled and diluted to 100 mM NaCl with 40 mM Tris-HCl, pH 8.2. The mixture was loaded onto a DEAE-Sepharose Fast Flow column (2.6×12 cm) that was pre-equilibrated with 40 mM Tris-HCl, pH 8.2, 100 mM NaCl. 100-200

ml of the same buffer was used for washing the column at the flow rate of 1 ml/min and a gradient of 100-400 mM NaCl in 40 mM Tris-HCl buffer, pH 8.2 over a 600 ml volume was used for protein elution. 5 ml fractions were collected. Fractions containing ESC and E(Z), which started to elute at 220 mM NaCl, were pooled, adjusted to 150-200 mM NaCl and pH 7.4-7.6 and loaded onto an anti-FLAG M2-Agrose Affinity gel (Sigma) column (1.5 ml). The column was washed extensively with 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40. None of the proteins subsequently identified in the bound fraction was detectable in final wash fractions. Bound protein was eluted with FLAG peptide (150 μg/ml) (Sigma). Gel Filtration Standards from BioRad and Pharmacia were used for gel filtration column calibration. Chromatography was carried out at 4°C.

Protein sequencing

To identify polypeptide components of the purified ESC complex, after final affinity purification on M2 agarose gel, the purified sample was concentrated by TCA precipitation and separated on preparative 10% SDS-PAGE gel. A control sample of identically processed wild-type extract was also run to identify bands that were specifically bound by the anti-FLAG affinity gel in the final step. After Coloidal Blue (Sigma) staining and destaining, gel slices containing each polypeptide band of interest were excised, rinsed twice with distilled water and then 50% acetonitrile, frozen and shipped to the Harvard Microchemistry Facility (Cambridge, MA) for protein identification by Mass Spectrometry analysis. After in-gel reduction, S-carboxyamidomethylation and digestion with trypsin, individual MS/MS sequence spectra were obtained using microcapillary reverse-phase HPLC chromatography directly coupled to the electrospray ionization source of a quadrupole ion trap mass spectrometer (Finnegan LCQ, San Jose, CA). These MS/MS spectra were then correlated with known sequences in the NCBI Genpept and EST databases using the algorithm Sequest (Eng et al., 1994) and other algorithms (Chittum et al., 1998).

The following sequences derived from ESC were obtained from this analysis: DEPQVFATAGSNR, VTVYECPR, TSSPLLAAAGYR, LQLLLSGSK, IVSSGMDHSLK, IELSNTFSQEK, STLPFPTVTK, HFPDFSTR, FGFNPWQK and VIALGNQQGK. The following sequences derived from E(Z) were obtained: AIQPGEELFFDYR and FIEELIK. The following sequences derived from p55 were obtained: TPSSDVLVFDYTK, HPSKPEPSGECQPDLR and TVALWDLR.

Plasmid constructs and antibodies

Rabbit polyclonal anti-PC antibody was generously provided by Renato Paro. Plasmid constructs pET-E(Z) and pET-ESC were previously described (Tie et al., 1998). Affinity purified His6-E(Z)1-154 was used for generating anti-E(Z) rabbit antibodies. The GST-E(Z) construct was generated by excising full length E(Z) coding sequence from pET-E(Z) with *Nde*I-*Nsi*I and inserting it into a modified pGEX-2T vector. The GST-ESC1-60 and GST-EED1-81 constructs were generated by inserting PCR fragments containing ESC1-60 and EED1-81 into pGEX-2T at the *Bam*HI and *Eco*RI sites.

The pET-p55 plasmid and rabbit polyclonal antibodies against p55, ISWI and the CAF-1 p105 and p75 subunits were kindly provided by Drs Jessica K. Tyler and Jim Kadonaga (UC San Diego). The GST-p55 construct was generated by inserting PCR product encoding the full length p55 coding sequence into pGEX-2T vector at *Bam*HI and *Eco*RI sites.

Plasmid pOT2-RPD3 (accession no. AI456826) was produced and characterized by the Berkeley *Drosophila* Genome Project/HHMI EST Project (Rubin et al., 2000) and was obtained from Research Genetics. The GST-RPD3 construct was made by blunt-end ligation of an *Ecl*136I-*Nco*I fragment (blunted) containing the full-length RPD3 coding sequence into the *Eco*RI site (blunted) of pGEX-3X.

Rabbit anti-RPD3 polyclonal antibodies were a generous gift from Alan Wolffe (NICHD). They were raised against a region of the large catalytic domain of *Xenopus* RPD3 (residues 135-277) that is highly conserved in *Drosophila* RPD3 (88% identical) and they crossreact with *Drosophila* RPD3 (P. Wade and A. Wolffe, personal communication). We verified that they recognize *Drosophila* RPD3, which has an apparent molecular weight of ~70 kDa on SDS-PAGE, the same size as in vitro translated *Drosophila* RPD3. The M5 anti-FLAG monoclonal antibody (Sigma) was used for detection of FLAG-ESC on western blots. Monoclonal antibodies against RbAp48 and RbAp46 were from GeneTex. Polyclonal rabbit antibodies against human HDAC1 and HDAC2 were from Santa Cruz Biotech.

GST pull-down assays

GST fusion proteins were purified on Glutathione Sepharose 4B beads (Pharmacia). The Promega TNT T7 Quick Coupled Transcription/Translation System was used for production of ^{35}S-labeled proteins by in vitro translation. GST pull-downs of ^{35}S-labeled proteins were performed as previously described (Tie et al., 1998). For GST pull-downs from *Drosophila* nuclear extracts, 100 μl of embryo nuclear extract was incubated with 30 μl of Glutathione Sepharose beads containing bound GST fusion protein (300-400 ng) in a total volume of 200 μl for 1.5 hour at 4ºC. For GST pull-downs from human nuclear extracts, 40 μl of HeLa cell nuclear extract was incubated with 25 μl of Glutathione Sepharose beads containing bound GST fusion protein (300-400 ng) in a total volume of 100 μl for one hour at 4ºC. After extensive washing (five times with 400 μl of 50 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% NP-40), bound proteins were eluted with 40 μl of SDS sample buffer.

Immunoprecipitation

For co-immunoprecipitation assays 120 μl of nuclear extract was incubated with 6-8 μl of affinity-purified rabbit p55 antibody, E(Z) antibody or RPD3 antibody, and 40 μl of Protein G Sepharose (Pharmacia) in total volume of 240 μl for 3 hours. The beads were extensively washed. Proteins were eluted by adding 50 μl of 2× SDS sample buffer. 10 μl of sample was loaded on 10% SDS-PAGE for western blot analysis.

For co-immunoprecipitation of FLAG-ESC-associated proteins from extracts, 100 μl of embryo nuclear extract from the FLAG-ESC-expressing transgenic line was incubated with 30 μl of anti-FLAG M2-agrose affinity gel in a total volume of 200 μl for 1 hour at 4ºC. After extensive washing with 40 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.1% NP-40, proteins were eluted using 100 μl of 150 μg/ml FLAG peptide.

Immunofluorescent staining of chromosomes

Polytene chromosomes from late third instar salivary glands were prepared and stained as described (Chinwalla et al., 1995; Orlando et al., 1998). Antibodies were used at 1: 200 dilution and visualized with FITC-labeled secondary antibodies. Chromosomes were counterstained with propidium iodide to allow visualization of chromosome morphology and cytological mapping. Images were obtained with a Zeiss/BioRad confocal microscope as described (Orlando et al., 1998). Chromosomes from wild-type larvae were compared with those of transgenic line that contained a minimal *Ubx* PRE construct (a 670 bp *Pst*I/*Nde*I fragment) inserted at 65B, which was a previously characterized in V. Pirrotta's lab (Chan et al., 1994; Orlando et al., 1998). The appearance of new RPD3- and E(Z)-binding sites at the 65B site of insertion of the PRE construct was confirmed by analysis of more than 100 chromosomes.

Genetic analysis

To test whether RPD3 is required for PRE-mediated silencing, we crossed *Rpd3* mutant alleles, $Rpd3^{15\text{-}1}$ and $Rpd3^{def8}$, to transgenic flies harboring a single copy of a previously characterized *Ubx* PRE-*white* minigene construct that causes moderately strong variegated silencing of the mini-white reporter gene. Sibling progeny containing the PRE construct, either with or without the *Rpd3* mutation (heterozygous), were compared. The effect of the *Rpd3* mutations was similar in

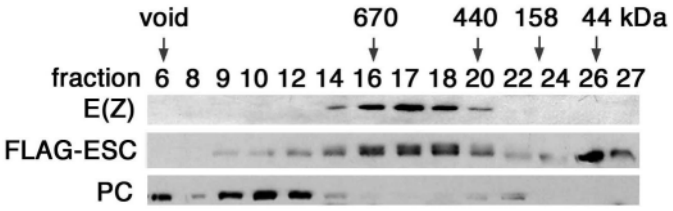

Fig. 1. The 600 kDa ESC complex is distinct from PC-containing complexes. Total protein extracts (approx. 20 mg/ml) from 0-22 hour old embryos were fractionated on a Superose 6 column. Fractions were analyzed by western blot for the proteins indicated at the left of each panel. Fraction numbers and sizes are indicated above lanes.

reciprocal crosses. The *Ubx* PRE construct is the 2.2 kb *Kpn*I-*Eco*RI fragment previously characterized in V. Pirrotta's lab (Chan et al., 1994). The *Rpd3*$^{15\text{-}1}$ mutation is a P-element insertion 1.8 kb 5′ of the *Rpd3* transcription unit. It has no detectable *Rpd3* RNA in eye-antennal discs (DeRubertis et al., 1996), where its affect was assayed here. We also tested several other recently characterized alleles, point mutations altering highly conserved residues in the *Rpd3*-coding sequence (Mottus et al., 2000).

RESULTS

ESC and E(Z) are components of a 600 kDa complex that is distinct from complexes containing other Polycomb Group proteins

We previously showed that ESC and E(Z) bind to each other in vitro, co-immunoprecipitate from embryo extracts and co-localize at approx. 100 specific sites on polytene chromosomes (Tie et al., 1998). To determine whether this reflects their presence in a stable complex, we fractionated total protein extracts from transgenic embryos whose only source of ESC protein is a fully functional FLAG epitope-tagged ESC (FLAG-ESC) expressed under the control of the constitutive α1-tubulin promoter (Tie et al., 1998). Fig. 1 shows that FLAG-ESC and E(Z) co-fractionate on a Superose 6 gel filtration column with identical peaks at ~600 kDa (fraction 17). They can be co-immunoprecipitated from peak fractions, indicating that their co-fractionation results from their physical association in a stable complex. Fig. 1 also shows that this complex is distinct from complex(es) containing the PC protein, which elute in the 2 MDa range (fraction 10), as also observed by Ng (Ng et al., 2000). A ~2 MDa PC-containing complex, PRC1, was recently purified and reported to also contain the PcG proteins PC, PH, PSC and SCM, but not E(Z) (Shao et al., 1999). Conversely, we also did not detect PC or PSC in immunoprecipitates of the ESC complex (data not shown). These results suggest that the function of the ESC complex is also likely to be distinct from that of PRC1. Mammalian homologs of ESC and E(Z) have also been shown to co-fractionate and co-immunoprecipitate with each other, but not with other PcG proteins (Sewalt et al., 1998; van Lohuizen et al., 1998), indicating that these distinct PcG complexes have been conserved in evolution.

The histone-binding protein p55 is a component of the ESC complex

To identify other polypeptide components of the ESC complex, the complex was purified from the FLAG-ESC-expressing

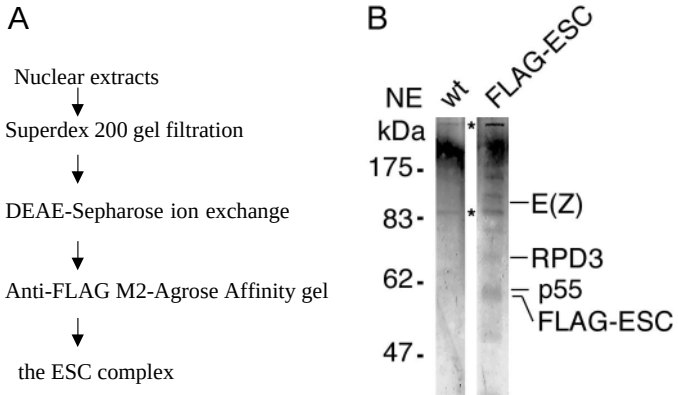

Fig. 2. (A) Scheme used to purify ESC complex from the FLAG-ESC expressing transgenic line. (B) Silver stained gel of purified ESC complex. Protein bands whose identities were confirmed by western blot and discussed in this paper are indicated to the right of the gel. As a stringent control, FLAG-ESC-associated proteins eluted from the final M2 column were compared with those obtained from identically processed wild type nuclear extracts (i.e. containing no FLAG-ESC). Proteins that were also recovered from wild-type nuclear extracts after identical purification steps (and therefore assumed to be nonspecifically binding to the M2 affinity gel) are indicated by asterisks.

transgenic line in three chromatographic steps, including gel filtration, ion exchange and antibody affinity columns as described in Materials and Methods, and outlined in Fig. 2A. Following the final immuno-affinity purification step, the purified complex was separated by SDS-PAGE. Fig. 2B shows a silver-stained gel of the purified complex. To distinguish proteins specifically associated with ESC from any proteins that might have bound nonspecifically to the final immuno-affinity gel, proteins purified from FLAG-ESC-containing extracts were compared with proteins recovered from identically processed wild-type nuclear extracts, which contain no FLAG-ESC (Fig. 2B).

The identity of individual polypeptides was determined by mass spectrometry of tryptic peptides as described in Materials and Methods. We confirmed the presence of ESC and E(Z) in the complex both by western blot analysis of the purified fraction and by peptide sequencing. One of the new proteins identified was the histone-binding protein p55 (also known as CAF-1 p55 and Nurf-55). Three different peptide sequences derived from p55 were identified (see Materials and Methods). p55 is an abundant nuclear protein that is present throughout *Drosophila* development (Tyler et al., 1996). It was previously identified as a histone-binding subunit of the *Drosophila* chromatin assembly factor CAF-1 (Tyler et al., 1996) and as a subunit of the *Drosophila* nucleosome remodeling factor NURF (Martinez-Balbas et al., 1998). p55 is the *Drosophila* homolog of mammalian RbAp48, originally identified as an Rb-binding protein (Huang et al., 1991; Qian and Lee, 1995; Qian et al., 1993) and subsequently as a histone deacetylase-bound protein (Taunton et al., 1996) and a histone-binding subunit of the human CAF-1 complex (Verreault et al., 1996). A closely related protein, RbAp46, with a similar histone-binding activity, is a component of the cytoplasmic histone acetyltransferase complex HAT1 in humans (Verreault et al.,

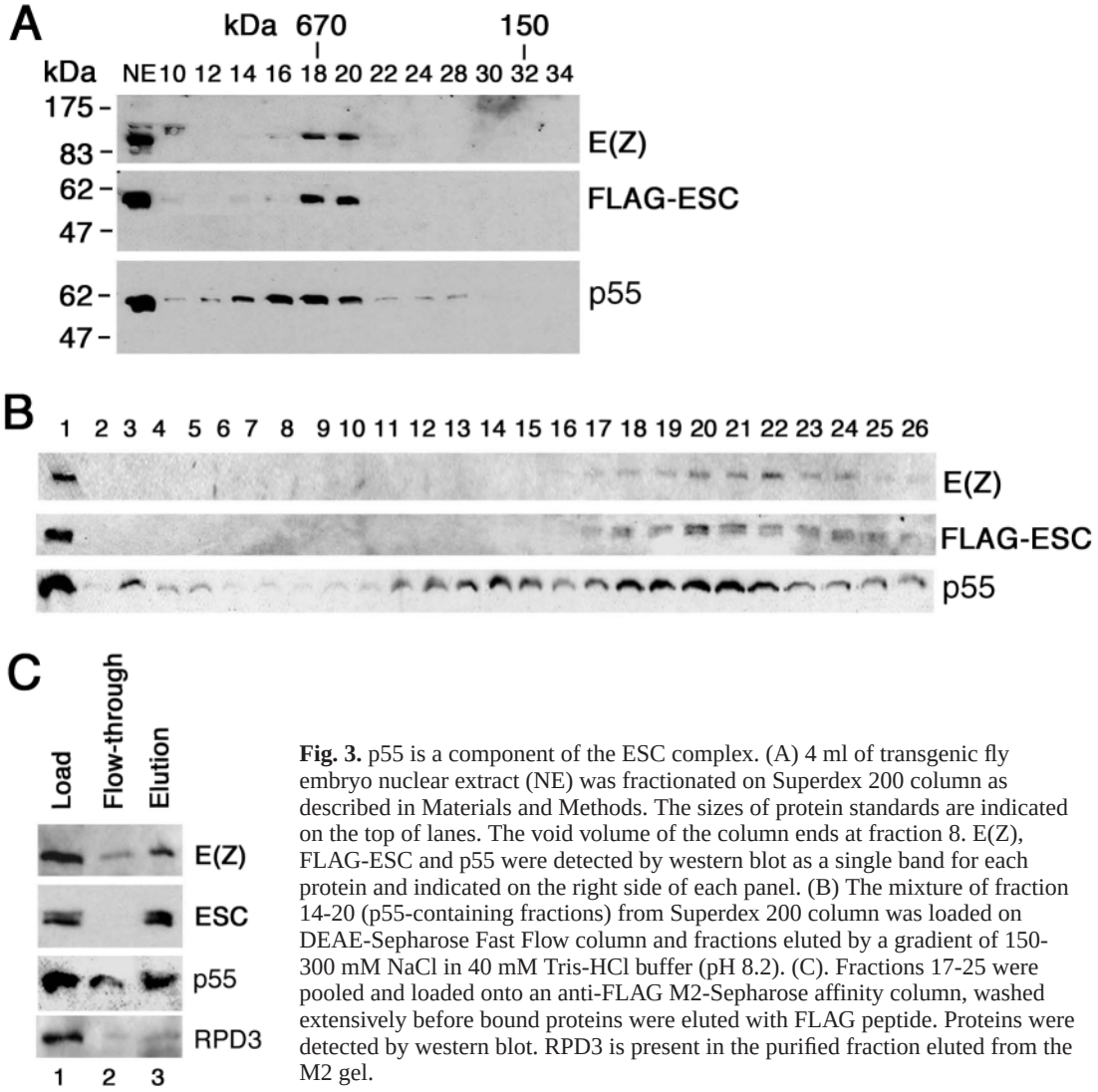

Fig. 3. p55 is a component of the ESC complex. (A) 4 ml of transgenic fly embryo nuclear extract (NE) was fractionated on Superdex 200 column as described in Materials and Methods. The sizes of protein standards are indicated on the top of lanes. The void volume of the column ends at fraction 8. E(Z), FLAG-ESC and p55 were detected by western blot as a single band for each protein and indicated on the right side of each panel. (B) The mixture of fraction 14-20 (p55-containing fractions) from Superdex 200 column was loaded on DEAE-Sepharose Fast Flow column and fractions eluted by a gradient of 150-300 mM NaCl in 40 mM Tris-HCl buffer (pH 8.2). (C). Fractions 17-25 were pooled and loaded onto an anti-FLAG M2-Sepharose affinity column, washed extensively before bound proteins were eluted with FLAG peptide. Proteins were detected by western blot. RPD3 is present in the purified fraction eluted from the M2 gel.

1998) and yeast (Parthun et al., 1996). Highly conserved homologs of p55 are found throughout the animal, plant and fungal kingdoms (Ach et al., 1997; Lu and Horvitz, 1998; Vermaak et al., 1999). Like ESC, p55 contains seven WD motifs, predicted to fold into a beta-propeller structure, similar to that of the Gβ subunit (Tyler et al., 1996).

The profile of p55 on a preparative gel filtration column is shown in Fig. 3A. A small proportion of p55 co-fractionates with FLAG-ESC and E(Z) in fraction 18-20. On a DEAE-Sepharose column, p55 elutes in multiple peaks and co-fractionates with FLAG-ESC and E(Z) in fractions 18-25 (Fig. 3B). On an anti-FLAG M2 agarose affinity gel column, p55 is present in the bound fraction and is co-eluted by FLAG peptide along with FLAG-ESC and E(Z) (Fig. 3C, lane 3). When control wild-type extracts (i.e. not containing FLAG-ESC) are subjected to identical prior fractionation steps, no p55 is recovered from the final M2 agarose column, indicating that p55 is specifically associated with FLAG-ESC.

To further verify that p55 is specifically associated with the ESC complex, we tested whether p55 co-immunoprecipitates with FLAG-ESC from embryos extracts. Nuclear extracts from transgenic embryos expressing FLAG-ESC or from wild-type embryos (control) were mixed with anti-FLAG M2 agarose gel. As expected, because there is no FLAG-ESC present in wild-type extract, all detectable E(Z) and p55 protein was present in the unbound fraction (Fig. 4, lane 2), and neither protein was detected in the bound fraction eluted with FLAG peptide (Fig. 4, lane 3). In contrast, the FLAG-ESC and E(Z) present in the transgenic extract (lane 4) were recovered by anti-FLAG affinity gel (lane 6). Although most of the p55 was present in the unbound fraction from the anti-FLAG affinity gel, a substantial amount of p55 was detected in the bound fraction eluted with FLAG peptide (compare Fig. 4, lane 6 with lane 3).

Since p55 is present in at least two other complexes (CAF-1 and NURF) that are abundant in embryos and similar in size to the ESC complex, we determined whether the association of p55 with the ESC complex might be due to adventitious association of the ESC complex with CAF-1 or NURF. We tested whether other subunits of the CAF-1 and NURF complexes co-immunoprecipitate with FLAG-ESC from embryo nuclear extracts using antibodies that specifically recognize the ISWI subunit of NURF or other CAF-1-specific subunits (p105 and p75) (Tyler et al., 1996) (J. Tyler and J.

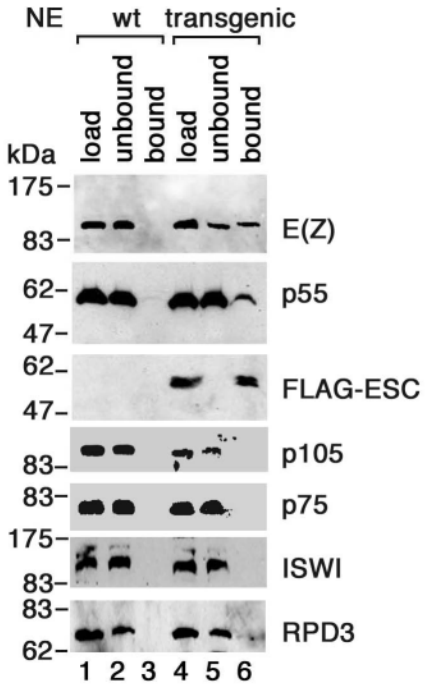

Fig. 4. p55 is specifically associated with ESC and E(Z) in vivo. Anti-FLAG M2 affinity gel was used for immunoprecipitation from FLAG-ESC embryo nuclear extracts (transgenic) and wild-type control extracts (wt). Proteins present in immunoprecipitates were analyzed by western blot as indicated in Materials and Methods. Each lane was loaded with an equal amount of sample (Lanes 1 and 4 show 100% of NE used). Lanes 2 and 5 show unbound protein present in the flow-through prior to washing. Lanes 3 and 6 show bound proteins eluted with FLAG peptide. The top three panels show that p55 is present in the bound fraction from transgenic extracts (lane 6), along with FLAG-ESC and E(Z). The next three lower panels show that the p105 and p75 subunits of CAF-1 as well as the ISWI subunit of NURF (other p55-containing complexes) are not present in the same bound fraction from transgenic extracts. The bottom panel shows that RPD3 is also present in the same bound fraction from transgenic but not wild-type extracts.

Kadonaga, unpublished observations). All of the p105, p75 and ISWI detected in the input nuclear extract was found in the unbound fraction of the M2 agarose gel; none was detected in the bound fraction with FLAG-ESC (Fig. 4, lane 6). This strongly suggests that the presence of p55 in the ESC complex is not due to adventitious association of the CAF-1 or NURF complexes with ESC. When affinity purified p55 antibody was used for the complementary immunoprecipitation from embryo nuclear extract, ESC was also detected in the immunoprecipitate (see next section and Fig. 5). We conclude that ESC, E(Z) and p55 are specifically and stably associated with each other in vivo. Our data indicate that less than 5% of the p55 in embryo extracts is present in the ESC complex.

The histone deacetylase RPD3 is a component of the ESC complex

In addition to its presence in CAF-1 and NURF, p55 and its orthologs in other organisms have been found associated with class I histone deacetylases related to yeast RPD3 (Hassig et al., 1997; Lu and Horvitz, 1998; Taunton et al., 1996; Tyler et al., 1996; Vermaak et al., 1999; Wade et al., 1998; Zhang et al., 1997; Zhang et al., 1999). Furthermore, the mammalian RPD3 homologs HDAC1 and HDAC2 have been reported to be associated with EED, a mammalian homolog of ESC (van der Vlag and Otte, 1999). We therefore tested whether *Drosophila* RPD3 was associated with the ESC complex. We observed that the RPD3 protein elutes from a Superdex 200 gel filtration column in a broad range in fraction 10-20. RPD3 was detected in the purified ESC complex after subsequent affinity purification on anti-FLAG M2 agarose (lane 3 in Fig. 3C).

To further verify that RPD3 is specifically associated with the ESC complex in vivo, we tested whether RPD3 co-immunoprecipitates with FLAG-ESC from embryo nuclear extracts. As shown in the bottom panel of Fig. 4, lane 6, RPD3 was detected along with FLAG-ESC in the bound fraction of an M2 agarose gel. In addition, when anti-E(Z), anti-RPD3 and anti-p55 antibodies were used for immunoprecipitation, FLAG-ESC was detected in the immunoprecipitates (Fig. 5A).

We observed that endogenous RPD3 is readily degraded during preparation of extracts and purification and often yields relatively weak signals on westerns. Therefore we also performed GST pull-downs from nuclear extract to further verify the physical association of p55 and RPD3 with the ESC complex in vivo. Our previous results indicated that a GST fusion protein containing the N-terminal 73 residues of E(Z), GST-E(Z)1-73, binds directly to the WD region of ESC (Tie et al., 1998). As shown in Fig. 5B, GST-E(Z)1-73 pulls down RPD3, p55 and ESC (lane 3) from embryo nuclear extract, but

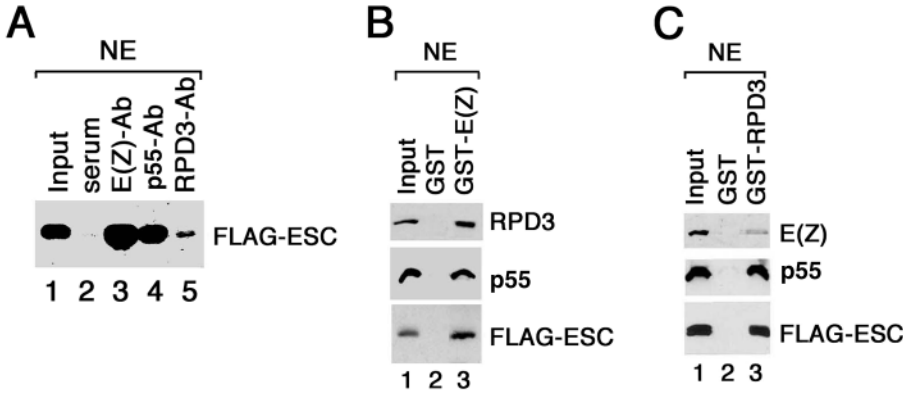

Fig. 5. p55 and RPD3 are physically associated with ESC and E(Z) in vivo. (A) Co-immunoprecipitation was performed as indicated in Methods. Lane 1 shows 20% of NE used in lane 2-5. Lanes 2-5 show the Protein G Sepharose immunoprecipitation samples. Lane 2, normal rabbit serum, serves as a negative control. FLAG-ESC detected by western blot using anti-FLAG M5 monoclonal antibody is indicated at the right side of panel. (B) GST-E(Z)1-73 pulls down RPD3, p55 and ESC from nuclear extract (NE). Lane 1 shows input NE (10%). RPD3, p55 and FLAG-ESC were detected by western blot as indicated at the right side of each panel. (C) GST-RPD3 pulls down E(Z), p55 and FLAG-ESC from NE. Lane 1 shows 25% of NE used in lanes 2 and 3. Lane 2 shows GST alone pull-down sample as a negative control. Lane 3 shows GST-RPD3 pull-down sample. Proteins detected by western are indicated at the right side of each panel.

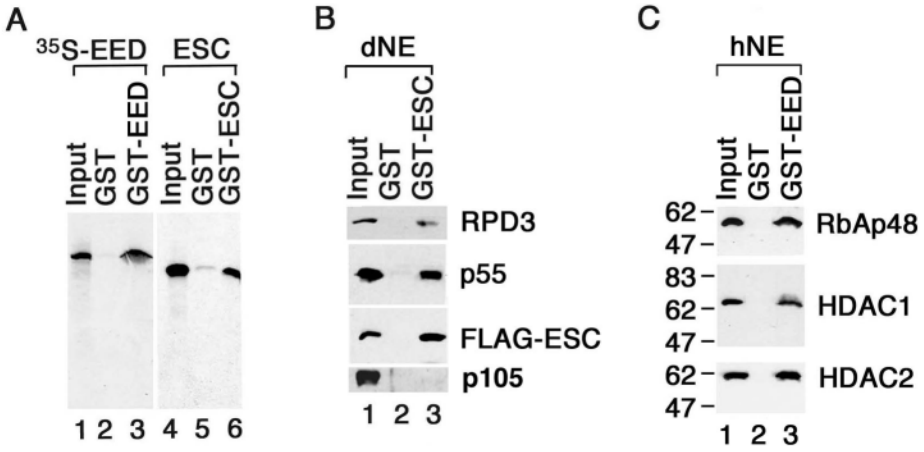

Fig. 6. Association of p55 with the ESC complex is conserved in mammals. (A) ESC1-60 and EED1-81 directly interact with ESC and EED respectively in vitro. In vitro translated full-length ESC and EED were used for GST pull-down assay. Lane 1 and lane 4 show 20% of total protein used. (B) The results of a GST-ESC1-60 pull-down from *Drosophila* nuclear extract (d-NE). Lane 1 shows 20% of input. Proteins detected by western blot are indicated at the right side of each panel. Note that while RPD3, p55 and FLAG-ESC are pulled down, the CAF-1 p105 subunit is not pulled down (lowest panel). (C) The results of a GST-EED1-81 pull-down from HeLa cell nuclear extract (hNE). Lane 1 is 20% of total hNE. RbAp48, HDAC1 and 2 were detected by western blot.

GST alone (lane 2) did not. We have determined that GST-E(Z)1-73 does not interact directly with RPD3 or p55, i.e., does not pull down in vitro translated RPD3 or p55 proteins (data not shown). This strongly suggests that GST-E(Z)1-73 pulls down a complex containing ESC, RPD3 and p55 via its interaction with ESC. A similar pull-down assay indicates that GST-RPD3 can pull down E(Z), p55 and ESC from nuclear extract (Fig. 5C, lane 3), further demonstrating that ESC, E(Z), p55 and RPD3 are associated with each other in vivo. Evidence for direct binding of these proteins to each other in vitro is presented below.

Association of p55 and RPD3 with ESC is conserved in mammals

To test whether the association of ESC and E(Z) with p55 and RPD3 has been conserved in mammals, we analyzed the human complex containing the ESC homolog EED for the presence of RPD3 and p55 homologs. Database searches reveal that *Drosophila* RPD3 is most closely related to two human histone deacetylases, HDAC1 and HDAC2 (77% and 75% identical to RPD3). Similarly, there are two closely related p55 homologs in mammals, RbAp48 and RbAp46 (91% and 86% identical to p55). RbAp48 and RbAp46 have also been found together in the SIN3 and Mi-2 deacetylase complexes, as have HDAC1 and HDAC2 (Hassig et al., 1997; Taunton et al., 1996; Zhang et al., 1997). We therefore tested whether all four proteins were associated with the human EED complex.

We previously observed that a GST-ESC fusion protein encoding full-length ESC can pull down full-length in vitro translated ESC (F. T. and P. J. H., unpublished observations) and a GST-ESC1-60 fusion protein encoding just the N-terminal 60 residues of ESC is sufficient to pull down full-length in vitro translated ESC (Fig. 6A, lane 6). Similarly, GST-EED1-81, which contains the corresponding N-terminal region of EED (Schumacher et al., 1996), binds directly to in vitro translated EED (Fig. 6A, lane 3). As shown in Fig. 6B (lane 3), in addition to FLAG-ESC, GST-ESC1-60 also pulls down p55 and RPD3 from *Drosophila* embryo nuclear extract. (Note the lower panel of Fig. 6B, which again shows that the CAF-1 p105 subunit is not associated with the ESC complex.) Combined with the co-IP data in Figs 4, 5A, this strongly suggests that GST-ESC1-60 specifically pulls down the ESC complex. Fig. 6C shows that GST-EED1-81 pulls down HDAC1, HDAC2 and RbAp48 from HeLa cell nuclear extract (lane 3). We have also detected RbAp46 (data not shown). Thus, the association of ESC with p55 and RPD3 is mirrored in the conserved association of mammalian EED with RbAp48, RbAp46 and HDAC1 and HDAC2. Our results confirm the previously reported association of EED with HDAC1 and HDAC2 (van der Vlag and Otte, 1999).

Direct interactions among p55, E(Z), RPD3 and ESC in vitro

We previously showed that ESC and E(Z) are associated in vivo and interact directly through the N-terminus of E(Z) and the WD region of ESC (Tie et al., 1998). To identify direct interactions among p55, E(Z), ESC and RPD3, we used GST pull-down assays. As shown in Fig. 7A, a GST-p55 fusion

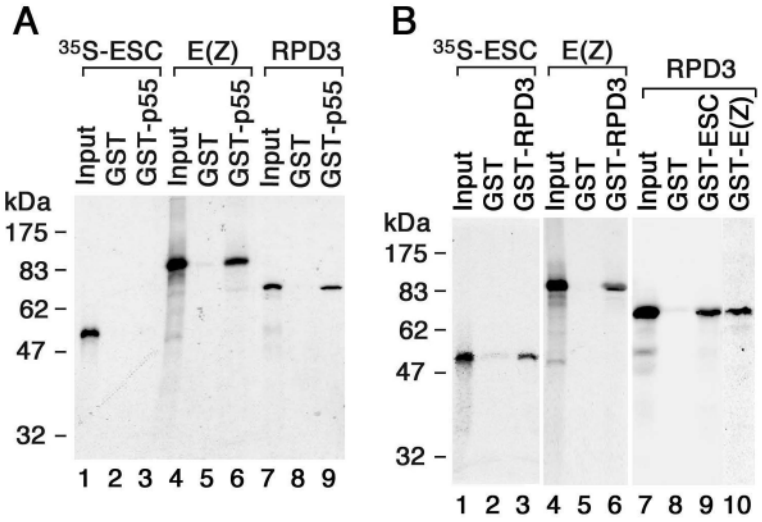

Fig. 7. Direct interactions of p55 and E(Z), ESC, RPD3 in vitro. (A) GST-p55 was used to pull down in vitro translated full-length ESC, E(Z) and RPD3. Lane 1, 4, 7 show 20% of input. p55 directly interacts with E(Z) (lane 6) and RPD3 (lane 9) but not with ESC (lane 3) in vitro. (B) GST-RPD3 directly interacts with ESC (lane 3) and E(Z) (lane 6). GST-ESC and GST-E(Z) directly interacts with RPD3 (lane 9 and 10) respectively.

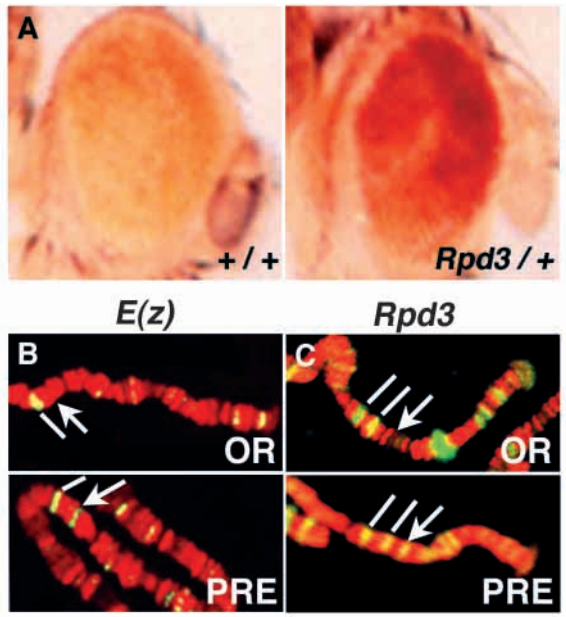

Fig. 8. (A) PRE-mediated silencing of a *white* reporter construct is disrupted in an *Rpd3* / + mutant background. Shown are representative sibling progeny that contain a *Ubx* PRE construct and are either wild type for *Rpd3* or heterozygous for the *Rpd3*$^{15\text{-}1}$ mutation inherited from the mother. (B,C) E(Z) and RPD3 bind to the *Ubx* PRE in vivo. Shown are the chromosomal localization of E(Z) (B) and RPD3 (C) on the distal portion of chromosome arm 3L in a wild-type larva (OR) and in a transformant (PRE) containing the 670 bp *Pst*I/*Nde*I fragment of the *Ubx* PRE inserted at 65B. Note the new E(Z) and RPD3 binding sites at the insertion site of the PRE construct in the transformant (arrows) and its absence in wild type (arrow). Landmark bands are indicated by white lines.

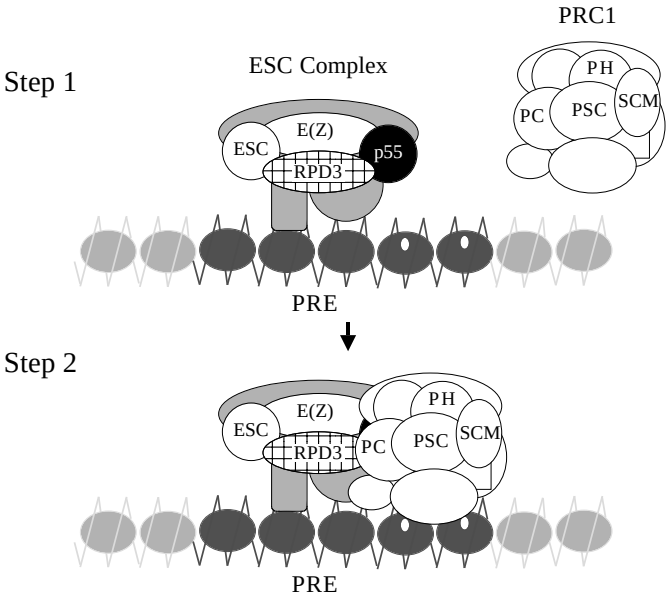

Fig. 9. Model for ESC complex action. The ESC complex is recruited to a PRE where it mediates local deacetylation of nucleosomal histones, which is a prerequisite for the efficient recruitment, formation or function of other PcG complexes, such as PRC1, that are required to maintain silencing throughout the rest of development. The requirement for ESC after embryogenesis in imaginal discs suggests that histone deacetylation by the ESC complex is also required for full silencing in these actively dividing cells throughout the larval period, perhaps to ensure efficient re-binding of other PcG complexes to PREs after each round of replication. Nuclosomes are depicted as light gray ovals, the PRE as dark gray ovals. The white dots indicate deacetylated sites on nucleosomes.

protein specifically pulls down E(Z) (lane 6) and RPD3 (lane 9) but not ESC (lane 3). Fig. 7B shows that GST-RPD3 pulls down ESC (lane 3) and E(Z) (lane 6). Conversely, GST-ESC and GST-E(Z) pull down RPD3 (lane 9 and 10). (A summary of the direct interactions among ESC, E(Z), p55 and RPD3 is illustrated in Fig. 9.)

RPD3 is required for PRE-mediated silencing in vivo

PcG silencing is mediated by PREs, complex DNA regulatory elements that recruit PcG proteins in vivo and exhibit autonomous silencing activity when placed next to a reporter gene (Pirrotta, 1999). E(Z) and a number of other PcG proteins have been shown to be required for PRE-mediated reporter silencing in vivo (Chan et al., 1994). Fig. 8A shows that RPD3 is also required for PRE-mediated silencing. Flies heterozygous for the *Rpd3*$^{15\text{-}1}$ mutation (DeRubertis et al., 1996) display reduced silencing of a previously characterized reporter construct containing the mini-*white* gene flanked by a *Ubx* PRE fragment that causes variegated silencing of *white*. We have observed a similar effect with several recently characterized *Rpd3* point mutations (Mottus et al., 2000) (data not shown). Although it is not possible to conclude from this result that the ESC complex per se is responsible for this silencing, rather than some other RPD3-containing complex, these results are consistent with this possibility. A similar test of whether p55 is required for PRE-mediated silencing in vivo is not yet possible, since no p55 mutations have been identified.

RPD3 and E(Z) are bound to the *Ubx* PRE in vivo

To address whether RPD3 and E(Z) are required directly at PREs, we determined whether they are recruited in vivo to constructs containing just a minimal *Ubx* PRE. RPD3, like PcG proteins, binds to ~100 specific euchromatic sites on polytene chromosomes (J. P.-S. and P. J. H., unpublished observations). Fig. 8B,C shows that new chromosomal binding sites for E(Z) and RPD3, which are absent in wild-type chromosomes, are associated with the insertion site of a construct containing an isolated *Ubx* PRE fragment. This suggests that E(Z) and RPD3 act directly at the PRE to mediate silencing. Since we previously showed that ESC and E(Z) co-localize completely on chromsomomes, we presume, but have not shown, that ESC is also likely to bind to the *Ubx* PRE (Tie et al., 1998). Again, while it is not possible to conclude from the presence of E(Z) and RPD3 at the PRE that the ESC complex per se binds to the PRE, these results are consistent with this possibility. We should point out that we have not yet determined whether ESC complex components bind only to the PRE and not other parts of the *Ubx* gene, such as the promoter region. We have also not yet been able to determine by this assay whether p55 is also specifically associated with the PRE, due to the high number of endogenous p55-binding sites on polytene chromosomes (Martinez-Balbas et al., 1998).

DISCUSSION

The association of p55 with the ESC Complex: a direct link to chromatin

The presence of p55 in the ESC complex provides a direct molecular link to chromatin. The highly conserved mammalian p55 homologs, RbAp48 and RbAp46, have been shown to bind directly to histone H4 and possibly H2A, but not H2B or H3 (Vermaak et al., 1999; Verreault et al., 1996; Verreault et al., 1998). The N- and C-terminal regions of RbAp48 that mediate binding to histone H4 are virtually identical to the corresponding regions of *Drosophila* p55 (Tyler et al., 1996), strongly suggesting that p55 has the same histone-binding specificity.

What, then, is the role of p55 in the ESC complex? It is unlikely that p55 is responsible for the selective recruitment or targeting of ESC and E(Z) to the ~100 specific chromosomal sites at which they co-localize (Tie et al., 1998). The histone-binding activity of p55 does not, by itself, suggest a mechanism for such specificity and p55 binds to many more sites on the polytene chromosomes than ESC and E(Z) (Martinez-Balbas et al., 1998), presumably reflecting its distribution in other complexes, such as CAF1 and NURF. It seems more likely that p55 acts after the ESC complex is recruited and serves to direct the deacetylase activity of RPD3 to local histone substrates. This is analogous to the role proposed for RbAp46 in the heterodimeric HAT1 complex. RbAp46 greatly stimulates the acetyltransferase activity of the non-histone-binding HAT1 catalytic subunit, presumably by tethering it to its substrate via its histone-binding activity (Parthun et al., 1996; Verreault et al., 1998). Similarly, although recombinant RPD3 can deacetylate histone H4 in vitro, free RPD3 does not bind to H4 when the two are co-expressed in vivo (Vermaak et al., 1999) and is unlikely to be able to deacetylate nucleosomal histones. This suggests that p55 may play a similar essential role in the ESC complex by targeting RPD3 to histone substrates for deacetylation.

The role of RPD3 histone deacetylation in PcG-mediated silencing

The presence of RPD3 in the ESC complex suggests that histone deacetylation is an intrinsic activity of the ESC complex and that RPD3 is required for PRE-mediated silencing. The related mammalian EED complex has been shown to contain the RPD3 homologs HDAC1 and HDAC2, and immunoprecipitates containing this complex can deacetylate a histone H4 tail-peptide in vitro (van der Vlag and Otte, 1999). In yeast, RPD3-dependent repression in vivo has been shown to be associated with deacetylation of histones H4 and H3 (Kadosh and Struhl, 1998; Rundlett et al., 1998). Which nucleosomes would be deacetylated by the ESC complex? Histone deacetylation by yeast RPD3 appears to be highly localized, extending only one or two nucleosomes from a site to which it is recruited (Kadosh and Struhl, 1998; Rundlett et al., 1998). Since components of the ESC complex are physically associated with the *Ubx* PRE in vivo, ESC-mediated deacetylation may be restricted to nucleosomes comprising and immediately adjacent to PREs. Nucleosomes outside the PRE might also be targeted if the PRE has long-range interactions with the promoter or if the ESC complex itself also binds to the promoter or other regions outside the PRE, a possibility that the data presented here do not rule out.

Although we observe an effect of several *Rpd3* mutations on silencing of a PRE-mini-white reporter, which is an extremely sensitive assay, PcG phenotypes have not been reported for *Rpd3* mutants. A hypomorphic *Rpd3* allele associated with the insertion of a P-element transposon in the noncoding 5′ untranslated region has been analyzed in the most detail. Homozygous mutant embryos derived from germline clones of this allele do not exhibit PcG phenotypes, but have a pair-rule phenotype similar to that of *ftz* mutants (Perrimon et al., 1996; Mannervik and Levine, 1999). Abundant ubiquitously distributed *Rpd3* RNA and protein of maternal origin are detectable in early (0-2 hour) wild-type embryos, but are reduced no more than fivefold in these *Rpd3* mutant embryos derived from germline clones. By stage 9-10, the level of maternally derived *Rpd3* RNA and protein is greatly diminished. Localized zygotic expression of *Rpd3* becomes detectable in the brain and ventral nervous system of wild-type embryos, but is not detectable in these mutant embryos (Chen et al., 1999; Mannervik and Levine, 1999), suggesting that this *Rpd3* allele may have a stronger effect on zygotic expression than maternal expression. If RPD3 protein derived from maternally synthesized RNA is sufficient to promote development of a normal cuticular phenotype, then it remains possible these mutant embryos may contain sufficient maternally derived protein to do so and that germline clones of a true null *Rpd3* allele would display PcG phenotypes.

Alternatively, it is possible that the function of RPD3 in the ESC complex is not absolutely essential for ESC-dependent silencing or is redundant, i.e. when eliminated, it can be compensated by another histone deacetylase, either one normally associated with the ESC complex or a related one that can associate with the complex in the absence of RPD3. A number of other histone deacetylases have been identified in *Drosophila* and at least two are reported to be ubiquitously distributed in the early embryo (Mannervik and Levine, 1999). However, unlike mammals, which have two very closely related RPD3 orthologs (HDAC1 and HDAC2), both of which are associated with mouse EED, the *Drosophila* genome contains no equally closely related homolog of RPD3. The next most closely related *Drosophila* HDAC is an unequivocal ortholog of mammalian HDAC3 (Johnson et al., 1998), which is a class I HDAC like RPD3. Interestingly, mouse HDAC3 has been reported to interact with the mouse ESC homolog EED in a yeast two-hybrid assay (van der Vlag and Otte, 1999), consistent with the possibility that RPD3 function in the ESC complex might be at least partially redundant. Further genetic analysis of RPD3 should help to clarify its role of in the ESC complex.

The function of the ESC complex in relation to other PcG complexes

The 600 kDa ESC complex is distinct from complexes containing PC and other PcG proteins. This suggests that the ESC complex and other PcG complexes are likely to have separate functions. Furthermore, in embryos lacking any functional ESC protein, some weak residual PC-dependent silencing activity is still detected (Struhl, 1983), also supporting separate, if interdependent, functions. Similar conclusions have been drawn for the homologous mammalian PcG complexes (Gunster et al., 1997; Satijn et al., 1997a; Satijn et al., 1997b; Sewalt et al., 1998), which have been reported to

be expressed in temporally distinct stages of B cell differentiation, further suggesting they have distinct functions (Raaphorst et al., 2000). In *Drosophila*, derepression of homeotic genes is detected slightly earlier in *esc* mutants than in other PcG mutants (Soto et al., 1995), raising the possibility that ESC complex function might be required earlier than other PcG complexes. However, unlike the apparent temporal separation of the homologous complexes during mammalian B cell development, both ESC- and PC-containing complexes are present together throughout most of embryogenesis, before ESC disappears, and E(Z), like other PcG proteins, is required continuously throughout development. The phenotypic similarities between *esc*, *E(z)* and other PcG mutants, the genetic interactions among them and their common association with PREs, suggests that their functions, however distinct at the biochemical level, are interdependent.

What role might ESC-mediated histone deacetylation play in PcG silencing? Given the critical early requirement for ESC, ESC-mediated deacetylation of PRE-associated nucleosomes might be an essential prerequisite for the initial binding of one or more components of PRC1 or other PcG complexes to PREs. Fig. 9 presents a schematic model for such a function of the ESC complex in which ESC complex-mediated deacetylation of PRE associated histones is a critical step in establishing stable long-term PcG silencing. Alternatively, the ESC complex may be required for events subsequent to the initial binding of other PcG proteins to a PRE, perhaps for their assembly into active silencing complexes or for interaction of PRE-bound PcG complexes with the promoter. Indeed, repression of a reporter gene by a tethered GAL4-PC fusion protein remains dependent on endogenous ESC and E(Z) as well as other PcG proteins (Muller, 1995). This indicates that, at least for PC, constitutive binding to DNA does not bypass the requirement for ESC and E(Z). This also suggests that while our biochemical data and that of others (Ng et al., 2000; Shao et al., 1999) reveals no stable direct association of the ESC complex with other PcG complexes, it remains possible that there is a transient or less stable association in vivo that is essential for establishing PcG silencing.

Conservation of the ESC complex in mammals

The association of mammalian EED with the two closely related HDACs and two histone-binding proteins could reflect the existence of two separate EED complexes or some different functionality of the EED complex compared with the ESC complex. Consistent with this latter possibility, EED has recently been shown to be required after embryogenesis for aspects of adult hematopoietic development (Lessard et al., 1999; Raaphorst et al., 2000). Interestingly, analysis of the complete *Drosophila* genome sequence (Adams et al., 2000) using the BLASTP and TBLASTN algorithms (Altschul et al., 1990; Altschul et al., 1997) reveals that p55 has no other closely related *Drosophila* homologs, strongly suggesting that it is the functional counterpart of both RbAp48 and RbAp46 in *Drosophila*. Likewise, RPD3 is the only *Drosophila* counterpart of mammalian HDAC1 and HDAC2. Given the remarkably high degree of similarity between RbAp48 and RbAp46 and HDAC1 and HDAC2, it is not yet clear whether each of these proteins has a distinct or redundant role in the EED complex. Perhaps this situation reflects a greater degree of functional specialization or versatility within the mammalian EED complexes. Since HDAC1 and HDAC2 have also been found together with RbAp48 and RbAp46 in other co-repressor complexes, it is also possible that the EED and ESC complexes represent specialized relatives of these complexes, perhaps more dedicated to a specific subset of genes. We are currently characterizing the other components of the ESC complex, which should provide a clearer picture of the mechanism underlying ESC complex-mediated repression in the early embryo.

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