Alternative ESC and ESC-Like Subunits of a Polycomb Group Histone Methyltransferase Complex Are Differentially Deployed during *Drosophila* Development

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The Extra sex combs (ESC) protein is a Polycomb group (PcG) repressor that is a key noncatalytic subunit in the ESC-Enhancer of zeste [E(Z)] histone methyltransferase complex. Survival of *esc* homozygotes to adulthood based solely on maternal product and peak ESC expression during embryonic stages indicate that ESC is most critical during early development. In contrast, two other PcG repressors in the same complex, E(Z) and Suppressor of zeste-12 [SU(Z)12], are required throughout development for viability and *Hox* gene repression. Here we describe a novel fly PcG repressor, called ESC-Like (ESCL), whose biochemical, molecular, and genetic properties can explain the long-standing paradox of ESC dispensability during postembryonic times. Developmental Western blots show that ESCL, which is 60% identical to ESC, is expressed with peak abundance during postembryonic stages. Recombinant complexes containing ESCL in place of ESC can methylate histone H3 with activity levels, and lysine specificity for K27, similar to that of the ESC-containing complex. Coimmunoprecipitations show that ESCL associates with E(Z) in postembryonic cells and chromatin immunoprecipitations show that ESCL tracks closely with E(Z) on *Ubx* regulatory DNA in wing discs. Furthermore, reduced *escl*⁺ dosage enhances *esc* loss-of-function phenotypes and double RNA interference knockdown of ESC/ESCL in wing disc-derived cells causes *Ubx* derepression. These results suggest that ESCL and ESC have similar functions in E(Z) methyltransferase complexes but are differentially deployed as development proceeds.

The Polycomb group (PcG) proteins of *Drosophila melanogaster* are chromatin components that maintain transcriptional off states during development (11, 38, 48). PcG repressors are implicated in silencing many target genes in the fly genome (13, 30, 39). The best-characterized targets are the fly *Hox* genes, such as *Ultrabithorax* (*Ubx*) (2, 28, 46, 52). Genetic studies reveal that most PcG repressors are needed continuously to repress *Hox* genes throughout development. The PcG requirement begins at about 3 to 4 h of embryonic development, when the initial *Hox* repressors such as Hunchback and Kruppel decay. PcG repressors then maintain *Hox* repression during the remainder of embryogenesis and through subsequent larval and pupal stages. Polycomb response elements (PREs), located in *Hox* regulatory regions, are also needed during postembryonic stages to ensure maintenance of repression (4). Thus, the PcG repression system provides stable gene silencing for long periods of developmental time and through many rounds of cell division in proliferating tissues.

There are approximately 15 fly PcG proteins with roles in *Hox* gene repression established by genetic studies. Biochemical studies have defined the compositions of complexes built from PcG proteins and have revealed some of their molecular functions in chromatin. These studies have generally employed

purification of epitope-tagged PcG complexes from fly embryo extracts, subunit identification, and analyses of in vitro functions using either native or recombinant PcG complexes. The two best-characterized fly PcG complexes are the Extra sex combs (ESC)-Enhancer of zeste [E(Z)] complex and Polycomb repressive complex 1 (PRC1) (9, 12, 32, 34, 41, 43, 55). These two protein complexes are biochemically separable and contain nonoverlapping sets of PcG subunits. In addition, the DNA-binding PcG proteins PHO and PHOL are required to target PcG complexes to *Hox* genes in vivo (3, 60).

The fly ESC-E(Z) complex contains four core subunits: Extra sex combs, Enhancer of zeste, Suppressor of zeste-12 [SU(Z)12], and NURF-55 (9, 32). This complex possesses enzyme activity that methylates histone H3 with primary specificity for lysine-27 (K27) in the N-terminal tail (9, 32). Chromatin complexes containing conserved human homologs have very similar subunit compositions and share histone methyltransferase activity for H3-K27 (5, 27). Since the Polycomb (PC) subunit of PRC1 binds specifically to methylated H3-K27, this chromatin modification is thought to help recruit PRC1 to target genes (5, 9, 10, 29). Indeed, genetic loss of methylated H3-K27 correlates with PC dissociation from a *Ubx* PRE in vivo (5). Thus, an ordered series of events has been hypothesized whereby the ESC-E(Z) complex marks the local chromatin for PcG repression and this in turn attracts PRC1, which is most directly responsible for keeping target genes transcriptionally silent (5, 47, 60).

The SET domain of E(Z) provides the histone methyltransferase (HMTase) catalytic function (32). In agreement with

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A

	1		50
ESC	-----	-----	---MSSDKVK NGNEPEESEE
ESCLIKE	MTETNPSEIS	PPSEEP AED	ESTDHSPAEA VLSNNSSSVC NVVEPDDEHE
EED	-----	-----M	SEREVSTAPA GTDMPAAKKQ KLSSDENSNP
	51		100
ESC	SCGDES	ASYTTNSTTS	RSKSPSSSTR SKRR...GRR STKSKPKSRA
ESCLIKE	VNSVDREDTA	SLFSTTTTT	RSKSP..NTR KLN...LCR RIKAPKMQP
EED	DLSGDENDDA	VSIESGTNTE	RPDTPNTPN APGRKSWGKG KWKS.KKCKY
	101		150
ESC	AYKYDTHVKE	NHGANIFGVA	FNTLLGKDEP QVFATAGSNR VTVYECPRQ.
ESCLIKE	LYKYSSHVRE	DHNHQIFGVQ	FNPFLDRGQP QVFATVGKDR VSIYECERST
EED	SFKCVNSLIKE	DHNQPLFGVQ	FNWHSKEGDP LVFATVGSNR VTLYEC....
	WD1		
	151		200
ESC	GGMQL	LQCYADDPDP	EVFYTCAWSY DLKTSSPLLA AAGYRGVIRV
ESCLIKE	GQESCEGIRL	LQVYADPDTD	ESFYTCAWSY DSVTGDPVLA AAGYRGVIRI
EED	..HSQGEIRL	LQSYVDADAD	ENFYTCAWTY DSNTSHPLLA VAGSRGIIRI
	WD2		
	201		250
ESC	IDVEQNEAVG	NYIGHGQAIN	ELKFHPHKLQ LLLSGSKDHA IRLWNIQSHV
ESCLIKE	FNPVKHQCSK	NYIGHGHAIN	ELKFHPTRPQ LLLSGSKDHS LRLWNIQSDV
EED	INPITMQCIK	HYVGHGNAIN	ELKFHPRDPN LLLSVSKDHA LRLWNIQTDT
	WD3		
	251		300
ESC	CIAILGGVEG	HRDEVLSIDF	NMRGDRIVSS GMDHSLKLWC LNTPEFHHKI
ESCLIKE	CVAVFGGVEG	HRDEVLSVDF	DLRGDRIMSS GMDHSLKLWR LDKPDIKEAI
EED	LVAIFGGVEG	HRDEVLSADY	DLLGEKIMSC GMDHSLKLWR INSKRMMNAI
	WD4		
	301		350
ESC	ELSNFTSQEK	STLPFPTVTK	HFPDFSTRDI HRNYVDCVQW FGNFVLSKSS
ESCLIKE	ELSSGFSPNK	NTGPFPTIKE	HFPDFSTRDI HRNYVDCVQW FGDFVFSKSC
EED	KESYDYNPNK	TNRPFISQKI	HFPDFSTRDI HRNYVDCVRW LGDLILSKSC
	WD5		
	351		400
ESC	ENAIVCWKPG	QLHQSFQVK	PSDSSCTIIA EFFEYDECEIW FVRFGFNPWQ
ESCLIKE	ENSIVCWKPG	KLSESWHEIK	PQESATTVLH HFDYKMCEIW FVRFAFNAWQ
EED	ENAIVCWKPG	KMEDDIDKIK	PSESNTVILG RFDYSQCDIW YMRFSMDFWQ
	WD6		
	401		450
ESC	KVIALGNQQG	KVYVWELDPS	DPEGAHMTTL HNSRSVATVR QIAFSRDASV
ESCLIKE	KILALGNQLG	TTFVWELDCN	DPNLTKCSQL VHPKSNSTIR QTSFSKDGSI
EED	KMLALGNQVG	KLYVWDLEVE	DPHKAKCTTL THHKCGAAIR QTSFSRDSSI
	WD7		
	451		471
ESC	LVYVCDDATV	WRWNRRQTTS	I
ESCLIKE	LVCVCDDSTV	WRWDRVN---	-
EED	LIACVDDASI	WRWRLR---	-

this central role, E(Z) is required throughout fly development to maintain *Hox* gene repression. The tight correlation between E(Z) function, K27 methylation, and *Hox* silencing in vivo, as demonstrated with conditional *E(z)* alleles and site-directed E(Z) SET domain mutants (5, 7, 32), implies that the HMTase activity is itself needed continuously as development proceeds. In addition to E(Z), the noncatalytic ESC and SU(Z)12 subunits are required for HMTase activity in vitro and ESC is required for global H3-K27 methylation in embryos (6, 24, 33). Since E(Z) complexes have been purified so far only from embryos, much remains to be determined about the nature and composition of E(Z) complexes that maintain K27 methylation during postembryonic stages.

Genetic and expression studies on fly ESC suggest that subunit contributions to the HMTase complex may change during development. E(Z) and SU(Z)12 are critical for *Hox* repression during both embryonic and postembryonic stages. In contrast, ESC is critically required during embryogenesis, but its functional role is greatly diminished during postembryonic stages (18, 45, 53). Indeed, ESC is the only PcG member whose maternal supply to the embryo is sufficient for survival to adult stages in flies lacking zygotic product (50). In agreement with the genetic studies, ESC mRNA and protein levels are most abundant during embryonic stages but then are dramatically reduced during larval and pupal development (17, 20, 34, 40, 45).

These studies have defined a long-standing paradox: how can PcG repression be maintained stably throughout development despite changes in the requirement for ESC? In molecular terms, how can an E(Z) HMTase complex continue to function despite changes in the availability of a vital core subunit? A potential solution to this ESC paradox is provided by the discovery, through the fly genome project, of a second ESC-related gene product in flies. This gene, called *esc-like*, produces a protein which is strikingly similar to ESC (Fig. 1). We show here that ESC-Like (ESCL) is expressed at peak levels during postembryonic stages, which correspond to times of diminished ESC. We find overlapping requirements for ESC and ESCL in PcG repression via genetic studies in vivo and as assessed by RNA interference (RNAi) studies in postembryonic cultured fly cells. Chromatin immunoprecipitations show that ESCL associates and tracks with E(Z) in wing discs, and in vitro tests show that ESCL can replace ESC in catalytically active HMTase complexes. Our results suggest that the composition of E(Z) HMTase complexes changes during fly development, with the ESC subunit predominating in embryos and ESCL playing a major role during postembryonic stages.

MATERIALS AND METHODS

Drosophila strains. All standard fly stocks were obtained from the Bloomington stock center. *esc* is located at polytene cytological position 33A2, which corresponds to coordinates 2839 to 5061 of genomic scaffold sequence AE003634. *escl* is located at cytological position 33B5 and coordinates 166586 to

168478 of AE003634. *Df(2L)esc¹⁰* [Df(2L)33A1; 33B2] deletes *esc* but not *escl*. The proximal breakpoint of *Df(2L)esc⁴⁰* is ~140 kb proximal to *esc* and ~20 kb distal to *escl* (14). *Df(2L)Phl* [Df(2L)32F1-3; 33F1-2] deletes both *esc* and *escl* (15). *esc⁶* is an apparent null allele resulting from a splice site mutation that truncates the protein at residue G20, and *esc²* is an apparent null allele resulting from a frameshift at residue V404 (20, 40, 50). *esc⁵* is a nonsense allele, Q171stop (40).

Generation of *escl* transgenes and germ line transformants. A 2.3-kb genomic *escl* fragment was obtained by PCR using wild-type adult fly DNA as the template and tailed primers positioned 0.3 kb upstream of the start codon and 0.5 kb downstream of the stop codon. These primers lie within the coding regions of the closely flanking genes CG16969 and CG31866, respectively. The 2.3-kb fragment was inserted into pCasper4 to generate the transformation construct pCas-ESCL2.3. A Flag-tagged version was also constructed in pCasper4, which is identical except for insertion of a Flag-encoding oligonucleotide just upstream of the ESCL start codon. Germ line transformants were generated in a *y Df(1)w^{67c23}* genetic background.

Antibodies, Western blots, and coimmunoprecipitations. Rabbit anti-ESCL and anti-ESC antisera were raised against His₆ fusion proteins containing ESCL residues 2 to 96 and ESC residues 2 to 64, respectively. Anti-ESCL and anti-ESC antibodies were affinity purified, using the same respective fusion proteins, as previously described (36) except that antisera were first preadsorbed against a His₆-dMes4 fusion containing residues 420 to 633. Protein extracts from embryos, larvae, and pupae for use in Western blots were prepared as described previously (34). Relative protein concentrations were determined by Coomassie blue staining of proteins after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Western blots were performed using affinity-purified anti-ESCL at 1:1,000 and goat anti-rabbit immunoglobulin-horseradish peroxidase at 1:10,000 (Bio-Rad) and signals were developed using an ECL chemiluminescence detection kit (Amersham). Protein extracts for use in coimmunoprecipitations were prepared from MCW12 cells as described for SL2 cells (60). Immunoprecipitations were performed as previously described (22) using 10 μ l of affinity-purified anti-ESCL or anti-ESC antibodies.

Cell culture and RNA interference. MCW12 cells, which were derived from wing imaginal discs, were a generous gift from Deborah Cottam and Martin Milner (University of St. Andrew). Aliquots were thawed and $\sim 1.5 \times 10^6$ cells were cultured in six-well plates in SS3 medium (Sigma) supplemented with 2% heat-inactivated fetal calf serum, 2.5% fly extract, and 12.5 IU/100 ml insulin. Fly extract was prepared as described previously (8). Because the MCW12 cells grew for only approximately 2 weeks before they ceased dividing, fresh aliquots of cells were thawed for each experiment.

Reverse transcription (RT)-PCR analysis of transcript levels was performed as previously described for SL2 cells (60) using the following gene-specific primers: *Anip*, 5'-AGCAACAGCCCTCGCAGAAC-3' and 5'-AACTCCCGACTGCTGCTGGT-3'; *Ubx*, 5'-CGAGGAAATCCGTCAGCAGAC-3' and 5'-CAGAGTAACCAATTTGTTTTCAC-3'; *Abd-B*, 5'-CTCCCTCGCAATTACCAAGG-3' and 5'-TGCCGTGTGCCGCTTGACCG-3'; and *Rp1140*, 5'-CCTGCTGGATCGTGATTAACGC-3' and 5'-GTTGATGATGAAGTAGCCACCG-3'.

Double-stranded RNA was prepared as previously described (60) and included the following sequences: *Pc*, 935 bp, extending from bp 120 to 955 downstream of the ATG; *esc*, 598 bp, extending from 50 bp upstream of the ATG to 545 bp downstream; *escl*, 915 bp, extending from bp 700 to 1615 downstream of the ATG; and green fluorescent protein (GFP), 662 bp, extending from bp 25 to 687 downstream of the ATG. Freshly thawed aliquots of MCW12 cells were cultured for 1 week and then transfected with double-stranded RNA as previously described (5, 60) except that the second transfection was performed 4 days after the first transfection due to the lower growth rate of MCW12 cells than that of SL2 cells.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays of hand-dissected wing imaginal discs were performed essentially as previously described (5, 60). Wing disc ChIP input control PCRs contained total DNA extracted from fixed and sonicated chromatin equivalent to DNA from 10, 1, or 0.1% of an imaginal disc. Each PCR performed on DNA from immunoprecipitated chro-

FIG. 1. Amino acid sequence and conservation of the *Drosophila* ESC-Like protein. (A) Sequence alignment of fly ESC, fly ESC-LIKE, and mouse EED proteins. The seven WD repeats are enclosed in boxes. Residues in red correspond to predicted loops shown in red in B and are absolutely conserved in all three proteins. Other residues that are identical in all three proteins are shown in bold. In pairwise comparisons, ESC is 55% identical to EED and ESCL is 60% identical to EED. (B) Side and top views showing predicted structure (35) of the ESC β -propeller formed from the WD repeats. Loops in red are identical among ESC, ESCL, and EED. *, positions of loss-of-function *esc* missense alleles (20, 40); X, positions of site-directed *esc* mutations that cause loss of function in vivo (34, 35).

matin contained the equivalent of one wing imaginal disc. Wing discs were isolated from either normal [*E(z)*⁶¹ larvae reared at 18°C] or *esc* mutant larvae. In order to obtain *esc* mutant wing discs, *esc*⁵/*In*(2*LR*), *Gla Bc Elp* adults were crossed to *esc*⁶/*In*(2*LR*), *Gla Bc Elp* adults and wing discs were dissected from *Bc*⁺ late-third-instar larval progeny. ChIP assays of MCW12 cells were performed essentially as previously described for SL2 cells (60). Input control PCRs contained total DNA from fixed and sonicated chromatin equivalent to 10% of the immunoprecipitated chromatin used in each ChIP PCR.

Generation and analysis of recombinant ESC-E(Z) and ESCL-E(Z) complexes. Baculovirus expression of recombinant complexes was performed using the Bac-to-Bac system (Invitrogen). Full-length cDNAs encoding Flag-ESC, E(Z), SU(Z)12, and NURF-55 inserted into pFastBac1 were described previously (32). A Flag-ESCL construct was produced by inserting a 1.8-kb *escl* cDNA fragment into pFastBac1, followed by insertion of a Flag oligonucleotide at the N terminus. Anti-Flag immunoaffinity purification of complexes was done essentially as described previously (32), except washes in BC buffer were performed up to 1.2 M KCl and elutions were performed in batches for 1 h with 0.8 mg/ml Flag peptide.

The ESCL-E(Z) complex was purified twice independently and in parallel with an ESC-E(Z) complex used for comparative HMTase tests. HMTase assays were performed as described previously (32), with duplicate assays performed on independently prepared complexes. Polynucleosome substrate, consisting of 8- to 12-mers purified from HeLa cells, was used for the experiment in Fig. 6B and was prepared as described previously (24). The histone substrates used in Fig. 6C were histone H3/H4 tetramers containing wild-type or a K9A or K27A mutant form of histone H3 and were kindly provided by Nicole Francis (Harvard University).

RESULTS

The *Drosophila esc-like* gene encodes an ESC-related protein. One of the unexpected findings from the *Drosophila* genome project was the discovery of a second *esc*-related gene, designated CG5202. This gene, *esc-like* (*escl*), encodes a protein that shares about 60% overall identity with fly ESC and with the mammalian homolog, EED (Fig. 1). The bulk of ESC and ESCL is comprised of a domain with seven WD repeats, which is 63% identical between the two proteins. Each protein also possesses a smaller and less conserved (37% identical) N-terminal tail. The ESCL tail is 35 amino acids longer than the ESC tail, which contributes to a slightly larger ESCL protein of 462 residues and an expected mass of 52 kDa.

A predicted three-dimensional structure for ESC was derived previously (35) based upon the folding of multiple WD repeats into a β -propeller (58). This circular structure serves as a scaffold for protein interactions and features variable surface loops that emanate above and below the plane of the propeller. The surface loops highlighted in red (Fig. 1B) correspond to regions that are absolutely identical in ESC, ESCL, and EED (Fig. 1A) and are thus likely to be functionally important. Indeed, analysis of *esc* alleles and site-directed *esc* mutations in these loops (indicated in Fig. 1B) have implicated these ESC regions in repressor function in vivo (20, 34, 35, 40) and in E(Z) binding and HMTase function of the complex in vitro (22, 24, 54). This pattern of conservation suggests that the molecular function of ESCL is similar to the function of ESC. Thus, we pursued genetic and molecular tests to address the roles of ESCL in vivo and in vitro.

Evidence for overlap of ESC and ESC-Like functions in vivo. There are no preexisting mutant alleles of the *escl* gene. However, since *escl* is closely linked to *esc*, about 160 kb proximal within cytogenetic region 33B, deficiencies are available that remove just *esc* or both *esc* and *escl*. We used such deficiencies in genetic interaction tests to address if heterozygosity for *escl* enhances the severity of the phenotypes observed with loss

FIG. 2. Genetic enhancement of *esc* loss by reduced *escl* dosage. Extra sex combs phenotypes (A) and antenna-to-leg transformations (B) are shown for the indicated genotypes. T1, T2, and T3 indicate the first, second, and third thoracic legs, respectively. The arrow under T2 indicates an example of a partial extra sex comb. Arrows in panel B indicate partial transformations of antennae towards legs. Genetic enhancement is evident by comparing the phenotypes of *esc*²/*Df(esc)*¹⁰ (second row) and *esc*²/*Df(Prl)* (third row) flies. The enhanced *esc*²/*Df(Prl)* phenotype is rescued by a single copy of an *escl* transgene (fourth row).

of *esc* function. Our tests employed *Df(2L)esc*¹⁰, which removes *esc* but leaves *escl* intact; *Df(2L)Prl*, which deletes both *esc* and *escl*; and two apparent *esc* null alleles, *esc*⁶ and *esc*². *esc*⁶ disrupts the intron 1 splice donor, leading to a prematurely truncated protein of only 24 amino acids (20). *esc*² creates a frameshift within the seventh WD repeat that replaces the normal 22 C-terminal amino acids with 44 novel residues (20, 40).

As described previously (50), we observed that animals with the genotype *esc*⁶/*Df(2L)esc*¹⁰ survive to adulthood and display an extra sex combs phenotype, a hallmark of partial PcG loss of function. Although complete loss of *esc*⁺ product is embryonic lethal, these *esc*⁶/*Df(2L)esc*¹⁰ adults survive due to maternal *esc*⁺ product, which supplies sufficient function for viability. In contrast, we found that *esc*⁶/*Df(2L)Prl* is a lethal genotype. This result is consistent with heterozygos-

TABLE 1. Rescue of extra sex combs phenotype by ESCL transgenes

Genotype	Avg no. of legs with sex comb teeth ^b
<i>esc²/Df(esc)¹⁰</i>	3.7 (38)
<i>esc²/Df(Prl)</i>	5.9 (9)
<i>esc²/Df(Prl); P[ESCL]/+^a</i>	2.4 (18)
<i>esc²/Df(Prl)</i>	5.5 (8)
<i>esc²/Df(Prl); P[FL-ESCL]/+^a</i>	2.0 (8)

^a Two independent tests for rescue of the extra sex combs phenotype in *esc²/Df(Prl)* adults were performed using either an untagged ESCL transgene or a Flag-tagged transgene. The number of legs with sex comb teeth ranges from two (wild type) to six (extreme mutant phenotype).

^b Numbers in parentheses are the number of adult males of the indicated genotypes recovered and scored from among approximately 300 *esc²*-containing male progeny each in the *P[ESCL]* and *P[FL-ESCL]* rescue crosses.

ity for *escl* enhancing the zygotic loss of *esc* to create synthetic lethality. However, the lethality could also be due to other lethal mutations on the *esc⁶* chromosome located within the *Df(Prl)* interval. To directly address whether *escl⁺* dosage is responsible, a transgenic copy of *escl* was tested for its ability to restore viability to *esc⁶/Df(2L)Prl* animals. A 2.3-kb genomic fragment which extends into the predicted flanking genes on either side of *escl* was generated by PCR using wild-type adult DNA as the template. This fragment was inserted into the P element vector pCasper4 and transformant lines carrying a single copy of this *escl⁺* transgene were produced. Crosses were performed to produce animals with the genotype *esc⁶/Df(2L)Prl; P[escl⁺]/+*. These individuals survive to adulthood with an extra sex combs phenotype similar to that of *esc⁶/Df(2L)esc¹⁰* flies. Thus, the lethality is rescued by increasing *escl⁺* dosage from one to two copies. These results suggest that there is overlap between the functions of *esc* and *escl* in vivo.

A similar set of genetic tests using *esc²* instead of *esc⁶* were also performed. Although genetic enhancement of *esc* by *escl* was again observed, the severity of the phenotypes was less extreme, involving visible homeotic transformations rather than synthetic lethality. Specifically, *esc²/Df(2L)esc¹⁰* males display an intermediate extra sex combs phenotype (Fig. 2A; Table 1) that is less severe than that seen with *esc⁶/Df(2L)esc¹⁰*. The *esc²/Df(2L)esc¹⁰* animals typically showed sex combs on only three or four out of six legs and, when present, the ectopic sex combs typically contained only one to four teeth (Fig. 2A, second row). In addition, they appeared wild type with respect to antennal development. In contrast, *esc²/Df(2L)Prl* animals show nearly full sex combs on all six legs (Fig. 2A; Table 1) as well as partial antenna-to-leg transformations (Fig. 2B). Thus, the severity of homeotic transformations is enhanced here by heterozygosity for *escl*. Once again, the enhancement is specifically due to *escl* dosage because addition of an *escl⁺* transgene rescues these defects (Fig. 2, bottom; Table 1). These phenotypes further support *esc/escl* overlap and also indicate that *escl* function in vivo includes a role in *Hox* gene repression.

ESC-like expression peaks during postembryonic development. In order to examine ESCL expression during develop-

ment and its associations with other PcG proteins, we generated polyclonal antibodies against the ESCL N-terminal tail. The ESCL immunogen spanned residues 2 to 96, a region which lacks significant sequence similarity to ESC. Figure 3A shows that this antibody detects a species of approximately the correct size, ~60 kDa, on a Western blot of total extract from wild-type pupae. This same species plus a slightly larger one are detected in pupal extract from a transformant line that contains a Flag-ESCL transgene (Fig. 3A lane 2). This transgene produces functional ESCL product since, like the untagged transgene, it rescues the synthetic lethality of *esc⁶/Df(2L)Prl* animals as well as the homeotic phenotypes of *esc²/Df(2L)Prl* animals (Table 1). The detection of this doublet identifies the lower species as endogenous ESCL and indicates that Flag-ESCL is expressed at a comparable level. Western blot analysis of purified recombinant Flag-ESCL versus recombinant Flag-ESC (Fig. 3A, lanes 3 and 4) confirms antibody specificity for ESCL and lack of cross-reactivity with ESC.

Figure 3B shows a developmental Western blot performed on extracts from wild-type embryos, larvae, and pupae of the indicated stages. In contrast to ESC, which peaks during mid-embryogenesis and then is dramatically reduced (17, 34), ESCL accumulation persists through late embryonic and larval stages and then peaks during early pupal development. These differential ESC and ESCL expression profiles are also apparent at the mRNA level (*Drosophila* Developmental Gene Expression Timecourse, <http://genome.med.yale.edu/Lifecycle/>). Thus, ESCL is most abundant at developmental times that correspond to times of diminished ESC.

ESC-Like associates with E(Z) on target gene chromatin. ESC functions in vivo as a noncatalytic subunit in E(Z) HMTase complexes (9, 24, 32, 33). The high degree of ESC/ESCL similarity (Fig. 1), including regions required for E(Z) binding, suggests that ESCL also functions together with E(Z). To test this, we performed chromatin immunoprecipitation assays to determine if ESCL colocalizes with E(Z) on *Hox* gene DNA in vivo. Previous ChIP analyses using wing imaginal discs have shown that E(Z) associates with the transcription start

FIG. 3. Western blot analysis of ESCL expression during development. (A) Western blot demonstrating reactivity of affinity-purified anti-ESCL antibody. Leftmost lanes contain mid-pupal extracts from the wild type (lane 1) and Flag-ESCL germ line transformants (lane 2). Rightmost lanes contain approximately 25 ng of purified recombinant Flag-ESCL-E(Z) complex (lane 3) and purified recombinant Flag-ESC-E(Z) complex (lane 4). (B) Developmental Western blot using anti-ESCL antibody and extracts from the indicated stages. Embryonic stages are in hours, and L3 indicates crawling third-instar larvae. Pupal stages: white, prepupae; tan, midstage pupae; and late pupae with eye and body pigmentation.

FIG. 4. Associations of E(Z), ESCL and ESC with *Ubx* regulatory DNA in wing imaginal discs. Chromatin immunoprecipitations were performed to compare distributions of E(Z), ESCL, and ESC on fragments (shown in panel A) from the *Ubx* promoter region and an upstream PRE. Fragment numbering is as described previously (60). (B) Distributions on the *Ubx* promoter in wild-type wing discs. (C) Distributions on the *Ubx* PRE in wild-type wing discs. (D) Distributions on the *Ubx* promoter and PRE in *esc* mutant wing discs. In each panel, the antibodies used in the immunoprecipitations are indicated at the top and PCR-amplified regions are indicated to the left. "Mock" indicates control immunoprecipitation with crude rabbit preimmune antiserum, and "Rp" indicates a control fragment from the *RpIII40* promoter.

region and with a Polycomb response element of the *Ubx* gene (5, 60).

Within these regulatory regions, E(Z) binding is detected primarily on fragment p2, which includes the start site, and on fragments b4 and b5 of the upstream PRE (Fig. 4A to C). We find that ESCL tracks precisely with E(Z) on these same chromatin fragments in wing discs (Fig. 4B and C). Based upon coimmunoprecipitations from soluble extracts (Fig. 5D) and analysis of a recombinant ESCL-E(Z) complex (see below), this close tracking likely reflects a complex containing both ESCL and E(Z).

In addition, we performed ChIP assays to determine if ESC is also present in *Hox* gene chromatin from wing discs. Somewhat surprisingly, despite its diminished expression level in larvae, ESC is readily detected on the same chromatin fragments as E(Z) and ESCL (Fig. 4B and C). When these ChIP experiments were repeated using wing discs from *esc* null mutant larvae, ESC is no longer detected but E(Z) and ESCL remain associated with the *Ubx* promoter and PRE (Fig. 4D). Thus, E(Z) and ESCL can bind to target sites in wing disc chromatin in the absence of ESC. These results are consistent with E(Z) complexes in this tissue containing either ESC or ESCL, or possibly a combination of both, reinforcing the possibility that these proteins have overlapping functions in larvae.

Requirement for ESCL and ESC in *Ubx* repression in postembryonic cells. The contributions of ESCL and ESC to *Ubx* repression in wing imaginal discs were tested using a cell

line, MCW12, that was derived from wing imaginal discs (D. Cottam and M. Milner, unpublished). These cells have retained wing imaginal disc-like patterns of *Hox* gene expression; RT-PCR analysis indicated expression of *Antp* but a lack of *Ubx*, *abdA*, or *AbdB* mRNA in control MCW12 cells (Fig. 5A and data not shown). However, RNAi-mediated knockdown of PC resulted in dramatic *Ubx* derepression (Fig. 5A), reminiscent of *Ubx* derepression observed in wing disc somatic clones of PcG mutant alleles in vivo (2). In addition, ChIP assays revealed the same distributions of PC and E(Z) at the *Ubx* PRE and promoter regions in MCW12 cells as in wing imaginal discs (Fig. 5B and C) (5, 60). Thus, we conclude that MCW12 cells have retained PcG-mediated silencing of *Ubx* as in wing imaginal discs in vivo.

Coimmunoprecipitations demonstrated that E(Z) is physically associated with both ESC and ESCL in MCW12 cell extracts (Fig. 5D). To determine whether ESC and/or ESCL are functionally required for PcG-mediated silencing of *Ubx*, we performed RNAi-mediated knockdown experiments. MCW12 cells were transfected with *esc* double-stranded RNA, *escl* double-stranded RNA, or an equal mixture of both and the effects on *Ubx* expression were monitored by RT-PCR (Fig. 5E). Knockdown of either ESC or ESCL alone had no effect on the level of *Ubx* RNA. However, simultaneous knockdown of both ESC and ESCL resulted in *Ubx* derepression (Fig. 5E, right panel) similar to that observed following PC knockdown (Fig. 5A). These results indicate that ESC and ESCL are both

FIG. 5. Analysis of PcG functions in wing disc-derived MCW12 cells. (A) RNA interference assay using MCW12 cells. RT-PCR analysis of *Ubx* and *Rpl1140* expression in MCW12 cells transfected with *Pc* or *GFP* double-stranded RNAs. Panels B and C show ChIP analysis of the distributions of E(Z) and PC on the *Ubx* promoter (B) and *Ubx* PRE (C) in MCW12 cells. Fragments are shown in Fig. 4A. “Rp” indicates the control fragment from the *Rpl1140* promoter, “mock” indicates a control immunoprecipitation with crude rabbit preimmune antiserum, and “Gen” indicates input control PCRs on genomic DNA. (D) Coimmunoprecipitations from MCW12 cell extracts showing association of E(Z) with ESC or ESCL. Proteins were immunoprecipitated with anti-ESC or anti-ESCL antibodies or protein A alone, as indicated above the panels, and Western blots were probed with anti-E(Z) antibodies. Input lanes contain crude extract equivalent to 10 or 20% of the extract, used in anti-ESC (left panel) and anti-ESCL (right panel) immunoprecipitations, respectively. (E) RNA interference assay using MCW12 cells. RT-PCR analysis of *Ubx* and *Rpl1140* expression in MCW12 cells transfected with *esc* double-stranded RNA (left panel), *escl* double-stranded RNA (middle panel) or a mixture of both (right panel). In panels A and E, “RT+” indicates reverse transcriptase added, and “RT-” indicates reverse transcriptase omitted from the reverse transcription reaction.

functionally required for *Ubx* repression in this wing disc-derived cell line. Taken together with the coincident distributions of E(Z), ESC, and ESCL in *Ubx* wing disc chromatin (Fig. 4B and C), these results provide further evidence that the in vivo functions of ESC and ESCL overlap, at least in this imaginal tissue.

Histone methyltransferase activity of complexes containing ESCL in place of ESC. A recombinant fly ESC-E(Z) complex containing ESC, E(Z), SU(Z)12, and NURF-55 has robust HMTase activity that methylates K27 of histone H3 (32). However, if the ESC subunit is singly removed, or impaired by mutation, then the resulting mutant complexes have dramatically reduced HMTase (24, 33). In agreement with this, genetic disruption of ESC yields fly embryos with little or no detectable H3 K27 methylation (24). Thus, although its biochemical mechanism is not yet clear, ESC plays a vital role in potentiating the HMTase activity of the ESC-E(Z) complex.

We reasoned that if ESCL functions molecularly in a manner similar to ESC, then it might also assemble stably into a complex with E(Z) and make key contributions to HMTase activity. To test these possibilities, we used a baculovirus system to coexpress ESCL along with E(Z), SU(Z)12, and NURF-55 and determined if these four components could be purified together as a stable complex. Purification was achieved using anti-Flag immunoaffinity using a Flag tag placed at the extreme N terminus of ESCL. Figure 6A shows that a four-subunit ESCL-E(Z) complex is obtained (lanes 3 and 4). This ESCL-E(Z) complex appears

identical in subunit composition to an ESC-E(Z) complex purified in parallel (lanes 1 and 2) except for the replacement of ESC with ESCL.

The HMTase levels of these ESC-E(Z) and ESCL-E(Z) complexes were then compared using a polynucleosome substrate. Figure 6B demonstrates that the two recombinant complexes display similar levels of H3 methylation activity. Since single loss of ESC from its complex leads to at least a 25- to 50-fold reduction in HMTase in vitro (24, 33), this result indicates that ESCL can potentiate the enzyme activity in a manner similar to ESC.

Edman degradation analysis of nucleosomes methylated in vitro and studies using *E(z)* and *esc* mutants in vivo indicate that the primary specificity of the fly ESC-E(Z) HMTase is for K27 of histone H3 (5, 24, 32). In addition, methylation of histone substrates bearing lysine substitutions confirmed that the recombinant four-subunit ESC-E(Z) complex displays this K27 specificity (32). We used this assay here to compare the lysine specificity of the ESCL-E(Z) complex to that of the ESC-E(Z) complex (Fig. 6C). Histone H3/H4 tetramers containing wild-type H3 or mutant forms of H3 bearing a substitution at K9 or K27 were used as the substrate in HMTase assays. Figure 6C shows that the ESCL-E(Z) complex displays a similar marked preference for methylation of K27. Taken together, these in vitro assays show that ESCL can functionally substitute for ESC in the four-subunit HMTase complex.

FIG. 6. Histone methyltransferase activities of recombinant E(Z) complexes containing either ESC or ESCL. (A) Subunit compositions of complexes purified after coexpression of E(Z), SU(Z)12, and NURF-55 with either ESC (leftmost lanes) or ESCL (rightmost lanes). Complexes were purified using Flag tags on either ESC or ESCL. The second lane of each pair shows 40% as much material loaded. (B) Comparison of HMTase activities of four-subunit ESC-E(Z) and ESCL-E(Z) complexes. Numbers indicate concentrations of complexes (in nanomolar) in HMTase reactions using 1 μ g HeLa polynucleosomes as the substrate. (C) Comparison of lysine specificities of four-subunit ESC-E(Z) and ESCL-E(Z) complexes. HMTase reactions were performed using each complex at a concentration of 125 nM and H3/H4 tetramers containing either wild-type H3 or the indicated H3 mutant. Pairs of lanes show reactions using 50 and 200 ng of the indicated histone substrate.

DISCUSSION

ESC and E(Z), and their homologs, are functional partners in the chromatin of plants, invertebrates, and mammals. Working together, they control a diverse array of developmental processes, including flower and seed differentiation in *Arabidopsis thaliana* (19, 49, 61), germ line development in *Caenorhabditis elegans* (21, 25), X chromosome inactivation in mice (37, 44, 59), and *Hox* gene repression in flies and mammals (23, 42, 46, 52). Recent studies show that this partnership reflects a requirement for ESC in potentiating the histone methyltransferase activity of E(Z) (24, 31, 33).

In light of this functional interdependence, a paradox is presented by developmental studies in *Drosophila melanogaster*, which show that ESC is primarily needed during early embryogenesis, whereas E(Z) is required throughout embryonic, larval, and pupal development. Our analysis of ESCL, which can replace ESC in E(Z) HMTase complexes in vitro, provides a plausible solution to this puzzle. ESCL expression is largely complementary to that of ESC, peaking during later developmental stages, and our functional studies show that

ESCL is partially redundant with ESC in imaginal tissues. These results, together with prior genetic data that address *esc* time of action (see below), indicate that ESC predominates in embryos, whereas both ESCL and ESC make functional contributions during postembryonic development.

Developmental times of ESC and ESCL function in PcG repression. Phenotypic analyses of *esc* loss-of-function mutants provided the original evidence that the primary time of ESC action is during embryogenesis. Although complete loss of *esc*⁺ product is embryonic lethal and yields wholesale misexpression of *Hox* genes (46, 52), it was shown that maternally provided *esc*⁺ product provides sufficient function during embryogenesis to enable zygotically null *esc*⁻ animals to survive to adulthood (50, 51). These *esc*⁻ adults are fertile, healthy, and phenotypically normal except for minor homeotic transformations such as extra sex combs on the meso- and metathoracic legs. In contrast, animals that are zygotically null for any other PcG subunit of the ESC-E(Z) complex or PRC1 fail to survive beyond early pupal stages, with most dying by the embryonic/L1 stage.

Additional experiments with a conditional *esc* allele further delimited the main time of ESC function to a period of mid-embryogenesis extending from about the onset of gastrulation (about 3 h at 25°C) until germ band shortening (approximately 9 to 12 h) (53). An independent study that measured phenotypic rescue by a heat-inducible *esc*⁺ transgene confirmed that the time of ESC action begins at about 3 h of embryogenesis (45). These genetically determined times of *esc*⁺ function coincide with the accumulation of ESC protein, which peaks during mid-embryogenesis and declines by the end of embryogenesis (17, 20, 34).

However, full consideration of the genetic evidence also indicates that ESC does contribute to postembryonic PcG repression, particularly in imaginal tissues. Analysis of *esc*⁻ larvae showed modest defects in *Hox* gene repression in imaginal discs as well as in the central nervous system (18). In particular, this study attributed the extra sex combs phenotype of *esc*⁻ larvae to misexpression of the *Scr Hox* gene in the T2 and T3 leg discs. In addition, production of extra sex combs from patches of *esc*⁻ tissue generated by somatic recombination during larval development indicates that the time of ESC action extends into the larval period, at least in leg discs (56).

A postembryonic role is consistent with the detection of ESC on the *Ubx* gene in wing discs (Fig. 4B and C) and with the overlapping roles of ESC and ESCL in *Ubx* repression in discarded MCW12 cells (Fig. 5E). This result might explain why *esc*⁻ wing discs did not produce homeotic phenotypes even after sufficient passage to ensure depletion of maternal *esc*⁺ product (53); presumably, both ESC and ESCL would need to be disrupted in this tissue to yield robust *Hox* misexpression. Finally, although it is much less abundant at late developmental times than in embryos, ESC is detected by Western blotting in larval and pupal extracts (34). Thus, the genetic and molecular data together indicate that ESC does function during postembryonic stages, albeit with a more modest overall contribution than its critical role in embryos.

These considerations imply that the developmental division of labor between ESC and ESCL is not simply that ESC functions only in embryos and ESCL takes over for subsequent stages. Rather, although ESC does predominate early,

as evidenced by the global loss of H3 K27 methylation in *esc*⁻ embryos (24), postembryonic development appears to involve both ESC and ESCL. We note that Struhl and Brower originally hypothesized that late developmental functions of the *esc* locus might be executed by an *esc*⁺ isoform distinct from the embryonic version (53). Our data confirm that multiple ESC-related proteins do operate during fly development, with a late-acting version supplied by a second copy of the *esc* gene.

The functional context for ESCL during postembryonic development is presumably as a subunit in E(Z)-containing complexes with histone methyltransferase activity. The fact that ESCL can assemble in place of ESC and restore HMTase activity to a reconstituted E(Z) complex (Fig. 6) indicates that the biochemical roles of ESCL and ESC are similar. ESCL/ESC functional overlap could reflect a mixture of postembryonic E(Z) complexes, with some containing ESCL and others containing ESC. The simplest version of this scenario would entail four-subunit postembryonic HMTase complexes similar to the embryonic core complex of E(Z), SU(Z)12, NURF-55, and ESCL or ESC. However, postembryonic E(Z) complexes have yet to be purified, so their molecular compositions are not yet known. In fact, there is evidence that larval E(Z) complexes may differ from embryonic E(Z) complexes in features besides the ESCL/ESC subunit (16, 17). For example, the SIR2 histone deacetylase has been reported to associate with larval but not embryonic E(Z) complexes (16). Much remains to be determined about postembryonic E(Z) complexes, including subunit compositions and characterization of presumed HMTase activity.

Contributions of the ESC/ESCL subunit to histone methyltransferase complexes. Although the catalytic subunit E(Z) contains the conserved SET domain, studies on fly, worm, and mammalian homologs reveal that the ESC subunit is also critical for HMTase function. The single loss of ESC from the fly complex or loss of its homolog, MES-6, from the *C. elegans* complex yields subcomplexes with little or no HMTase activity in vitro (24, 33). In agreement with this, genetic removal of ESC eliminates most or all methyl-H3 K27 in fly embryos (24), loss of MES-6 eliminates most or all methyl-H3 K27 in worm germ lines and early embryos (1), and loss of EED removes most or all methyl-H3 K27 from embryonic mouse cells (31, 44). The mechanism by which ESC and its relatives potentiate the activity of HMTase complexes is not known. An in vitro study argues against a role for fly ESC in mediating stable contacts with nucleosome substrate (33). On the other hand, loss of ESC by RNA interference in fly S2 cells leads to dissociation of E(Z) from chromatin targets (5).

A biochemical analysis of the human EED-EZH2 complex (also called PRC2) has revealed an intriguing difference in the HMTase depending upon the subtype of EED subunit present in the complex (26). Multiple isoforms of EED are expressed in HeLa cells which differ in the extents of their N-terminal tails through use of alternative translation start sites. Incorporation of particular EED isoforms into EZH2 complexes can shift the enzyme specificity so that K26 of histone H1 is methylated in addition to H3 K27 (26). Taken together with other studies, this suggests that EED is a regulatory subunit that can influence both substrate specificity and catalytic efficiency of the HMTase.

In light of this finding, it seems possible that ESCL-E(Z) complexes might also have HMTase activity with altered lysine specificity. However, both ESCL-E(Z) and ESC-E(Z) recombinant complexes showed similar specificities for H3 K27 in H3/H4 tetramers (Fig. 6C) and we were unable to detect methylation of mammalian histone H1 by either of these recombinant complexes in vitro (data not shown). We note that the human H1 K26 methylation site is embedded in an ARKS sequence, which is also present surrounding H3 K27. This sequence is not conserved in *Drosophila* histone H1, suggesting that the ability of certain EZH2 complexes to methylate H1 may not be conserved in the fly system. However, there may well be other relevant methylation substrates besides histone H3 and it remains possible that alternative ESC isoforms could alter lysine specificities for these other substrates.

Functional relationship between ESC and ESCL proteins. Based upon their temporal expression profiles, it seems clear that *esc* and *escl* have distinct functions in a developmental context. Their temporal division of labor is most clearly demonstrated by *esc*⁻ *escl*⁺ embryos, which show extreme homeotic transformations (50) accompanied by dramatically reduced levels of methylated H3 K27 (24). This division could be entirely a consequence of differential transcriptional controls built into their divergent promoters. That is, ESC and ESCL could be functionally identical proteins that are just expressed at peak levels at different times. Alternatively, the two proteins may possess intrinsic differences that are also important during development but are not revealed by the assays we have applied so far (i.e., Fig. 6). One possibility, as mentioned above, is that ESC and/or ESCL may play a role in methylation of nonhistone proteins. The only nonhistone proteins yet identified that fly E(Z) complexes can methylate are two subunits of the core complex itself, E(Z) and SU(Z)12 (32). It is not clear if this self-methylation is functionally relevant and, in any case, it occurs at comparable levels with the ESC- and ESCL-containing recombinant complexes (data not shown).

It is also possible that ESC and ESCL could differ in contributions to E(Z) complexes besides HMTase activity. These other functions could include interacting with and recruiting histone deacetylases (55, 57), mediating physical interactions with PRC1 components, recruiting E(Z) complexes to target loci, and influencing the way E(Z) complexes interact with other (non-K27) histone tail modifications. Indeed, there is evidence for differential association of histone deacetylases with E(Z) complexes at embryonic versus larval stages (16), which parallels temporal changes in ESC and ESCL abundance. At the same time, ESC and ESCL functions must overlap enough to account for the sufficiency of either one to maintain *Ubx* repression in at least some postembryonic cells (Fig. 5E).

We envision that definitive answers will require promoter swap experiments in which ESCL is placed under control of the ESC promoter and vice versa, to determine which combinations provide genetic rescue of *esc* and *escl* mutations in vivo. Along with this approach, a complete understanding of the developmental role of ESCL will require generation of *escl* mutant alleles and systematic analysis of the phenotypic consequences of *escl* loss of function.

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