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Recruitment of *Drosophila* Polycomb-group proteins by Polycomblike, a component of a novel protein complex in larvae

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Polycomb-group (PcG) proteins are highly conserved epigenetic transcriptional repressors that play central roles in numerous examples of developmental gene regulation. Four PcG repressor complexes have been purified from *Drosophila* embryos: PRC1, PRC2, Pcl-PRC2 and PhoRC. Previous studies described a hierarchical recruitment pathway of PcG proteins at the bxd Polycomb Response Element (PRE) of the *Ultrabithorax* (*Ubx*) gene in larval wing imaginal discs. The DNA-binding proteins Pho and/or Phol are required for target site binding by PRC2, which in turn is required for chromosome binding by PRC1. Here, we identify a novel larval complex that contains the PcG protein Polycomblike (Pcl) that is distinct from PRC1 and PRC2 and which is also dependent on Pho and/or Phol for binding to the bxd PRE in wing imaginal discs. RNAi-mediated depletion of Pcl in larvae disrupts chromosome binding by E(z), a core component of PRC2, but Pcl does not require E(z) for chromosome binding. These results place the Pcl complex (PCLC) downstream of Pho and/or Phol and upstream of PRC2 and PRC1 in the recruitment hierarchy.

KEY WORDS: Chromatin, *Drosophila*, Epigenetics, Polycomb

INTRODUCTION

Drosophila Polycomb-group (PcG) genes were originally identified as negative regulators of Hox genes (Lewis, 1978). PcG-mediated silencing in *Drosophila* occurs in essentially two broadly defined stages: assumption of transcriptional repression responsibilities from gene-specific transcription factors in early embryos, followed by maintenance of the silenced state through many cycles of cell division beginning in mid-late-stage embryos and continuing throughout the remainder of development (for reviews, see Simon and Tamkun, 2002; Brock and Fisher, 2005).

Although much of the genetic analysis of PcG functions and studies of the mechanisms by which PcG proteins are targeted to specific genomic sites have focused on their activities in larval tissues, in vitro biochemical analyses have focused on PcG complexes isolated from embryos: PRC1, PRC2 and PhoRC (Czermin et al., 2002; Klymenko et al., 2006; Müller et al., 2002; Saurin et al., 2001; Shao et al., 1999). PRC1 possesses multiple chromatin modifying activities in vitro suggesting that it, among PcG complexes, might be most directly responsible for preventing transcription (Francis et al., 2004; King et al., 2002; Lavigne et al., 2004; Shao et al., 1999; Wang, H. et al., 2004). The primary functions of PhoRC and PRC2 appear to be to recruit and/or stabilize target site binding by PRC1, and potentially other PcG proteins. PhoRC includes the DNA-binding PcG protein Pleiohomeotic (Pho), which binds to sites within Polycomb Response Elements (PREs) that serve as docking platforms for PcG proteins (Brown et al., 1998; Chan et al., 1994; Simon et al., 1993). Pho directly interacts with components of both PRC1 and PRC2, and is required for recruitment of both complexes (Mohd-Sarip et al., 2002; Mohd-Sarip et al., 2005; Wang, L. et al., 2004). The E(z) subunit of PRC2 trimethylates histone H3 at lysine 27 (H3K27me3), facilitating recruitment of PRC1 (Cao et al., 2002; Czermin et al., 2002; Fischle et al., 2003; Min et al., 2003; Müller et al., 2002; Wang, L. et al., 2004).

A variant of PRC2 has recently been described that includes the PcG protein Polycomblike (Pcl) (Nekrasov et al., 2007). On the basis of gel filtration analysis of native complexes in embryo nuclear extracts and the stoichiometry of the purified Pcl-PRC2 complex, it appears that the majority of embryonic Pcl is present in Pcl-PRC2, but that the other PRC2 core subunits, E(z), Su(z)12, Esc and NURF55 (also known as Caf1 – FlyBase), predominantly are in a complex(es) lacking Pcl (Nekrasov et al., 2007; O'Connell et al., 2001; Tie et al., 2003). It has been proposed that inclusion of Pcl in PRC2 is required for high levels of H3K27me3 in vivo, although the in vitro histone methyltransferase activity of Pcl-PRC2 is indistinguishable from that of PRC2 lacking Pcl (Nekrasov et al., 2007). In this study, we identify a larval Pcl-containing complex that is distinct from PRC2 and PRC1 and show that it is required for chromosome binding by these PcG complexes.

MATERIALS AND METHODS

Drosophila stocks and genetic crosses

Strains are described at the Bloomington *Drosophila* Stock Center website (http://flystocks.bio.indiana.edu) unless otherwise specified. pUAST-R57-*Pcl* was provided by the NIG Stock Center (stock number 5109R-1) and a description of the stock is at http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp. Unless otherwise specified, all crosses were performed at 25°C. $phol^{81A}$; pho^{1} larvae were selected as previously described (Wang, L. et al., 2004).

Generation and testing of pWIZ-*Pcl* germline transformants

Pcl sequence from 131 bp upstream to 628 bp downstream of the ATG was ligated in inverted orientation into the pWIZ vector (Lee and Carthew, 2003). Germline transformants were generated in a *y* $Df(1)w^{67c23}$ genetic background. Tests for transgene activity were performed by crossing to P{GAL4-da.G32} at 25°C and examining phenotypes of the progeny. One line, which on the basis of eye color was determined to contain multiple inserts on the third chromosome, produced early pupal lethality in combination with P{GAL4-da.G32} and was used in all subsequent experiments. To confirm Pcl knockdown, wing discs from Oregon R, P{GAL4-da.G32}/+ and pWIZ-*Pcl*/P{GAL4-da.G32} larvae were dissected

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in PBS, pelleted, resuspended in 1× SDS sample buffer, run on an 8% SDS-PAGE gel, and the resulting western blot probed sequentially with anti-Pcl and anti-E(z) antibodies.

Preparation of nuclear extracts

Third instar Oregon R larvae were suspended in 50% sucrose at room temperature, washed thoroughly with ice-cold water (all subsequent steps were performed on ice or at 4°C), resuspended in nuclear isolation buffer (Ng et al., 2000) and passed through a Yamato LSC Homogenizer LH-22. The homogenate was then briefly dounced with the B-pestle and nuclear pellets prepared and nuclear proteins extracted as previously described (Ng et al., 2000). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce) and aliquots stored at –80°C.

Gel filtration chromatography and analysis of native protein complexes

Chromatography was performed as previously described (O'Connell et al., 2001). Larval nuclear extract (0.5 mg) was loaded onto a Superose 6 column (Amersham Pharmacia Biotech) and 0.5 ml fractions collected. Proteins from even number fractions were concentrated by trichloroacetic acid/deoxycholate precipitation. Equal amounts of each sample were run on duplicate 8% SDS-PAGE gels and transferred to nitrocellulose filters. Duplicate western blots were probed with affinity-purified rabbit anti-E(z) (Carrington and Jones, 1996) or rabbit anti-Pcl (O'Connell et al., 2001) antibodies.

Chromatin immunoprecipitation (ChIP)

ChIP assays of wing imaginal discs were performed as previously described (Wang, L. et al., 2004), except that imaginal discs were dissected in PBS and fixed by incubating in 1.5 mM ethylene glycol-bis (succinimidylsuccinate) (EGS) in PBS for 20 minutes at room temperature followed by addition of formaldehyde to a final concentration of 1% and continued incubation at 37°C for 10 minutes (Zeng et al., 2006). Fixation was quenched by addition of glycine to a final concentration of 50 mM. Chromatin was immunoprecipitated using 10 μl anti-Pcl, 20 μl anti-E(z), or 25 μl anti-Phol antibodies. Quantitative PCR was performed using the Platinum SYBR Green Kit (Invitrogen) in the Rotor Gene RG3000 thermocycler (Corbett Research). The equivalent of one wing disc per reaction was used. Sequences of primers are available upon request. For each ChIP experiment, reactions were performed in triplicate. Data were obtained by taking the average of six PCR reactions per region from two independent ChIP experiments.

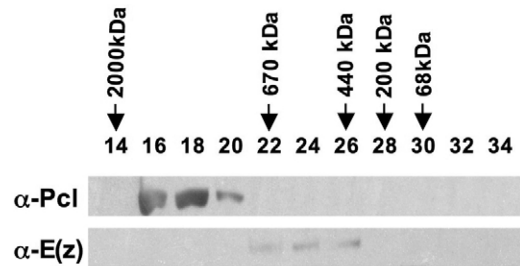

Fig. 1. Gel filtration analysis of Pcl and E(z) proteins in *Drosophila* larval nuclear extracts. Duplicate western blots of larval proteins fractionated on a Superose 6 column were probed with anti-Pcl (top) or anti-E(z) (bottom) antibodies. Elution positions of molecular mass standards are indicated above the appropriate fraction numbers.

Immunostaining of polytene chromosomes

Salivary gland chromosomes from third instar larvae were fixed for 5 minutes in 3.7% formaldehyde, 50% acetic acid and stained as previously described (Zink and Paro, 1989). Antibodies were used at the following dilutions: anti-E(z), 1:50; anti-Pcl, 1:200; anti-RNA polymerase IIo^{Ser2} H5 monoclonal antibody, 1:50 (Covance). E(z) and Pcl signals were detected with goat anti-rabbit-Cy3. IIo^{Ser2} signal was detected with goat anti-mouse-Cy2 (Jackson ImmunoResearch Laboratories). Images were captured on an Eclipse TE2000-U microscope (Nikon) using Metamorph software (Universal Imaging).

RESULTS AND DISCUSSION

Pcl is in a distinct complex in larvae

In order to examine potential differences between embryonic and larval stage PcG complexes, we fractionated larval nuclear extracts over a Superose 6 gel filtration column and probed western blots of

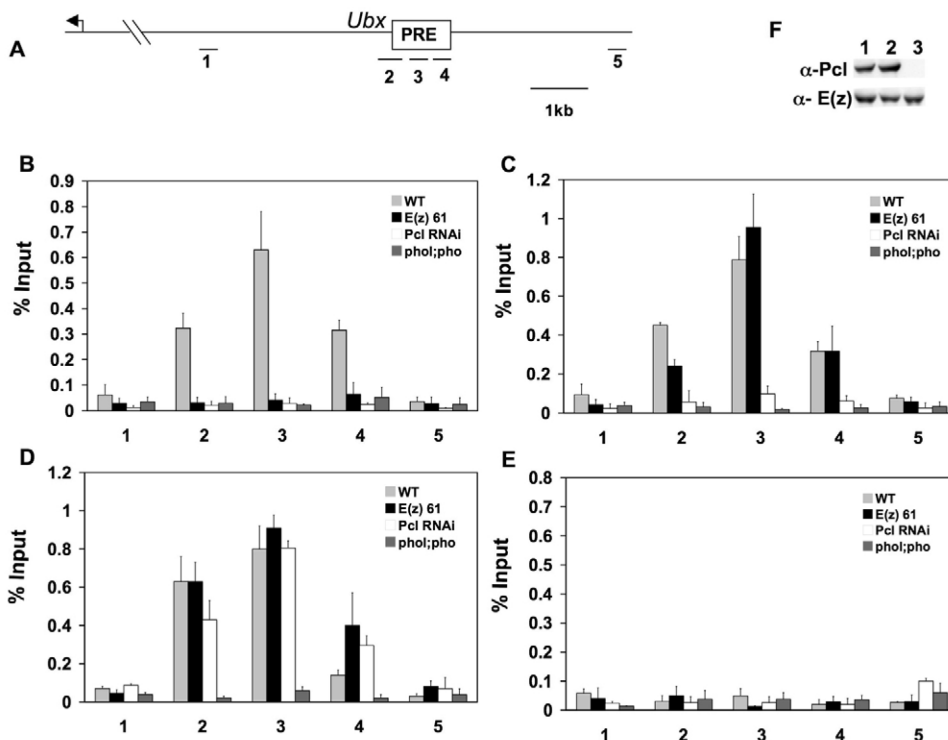

Fig. 2. Non-reciprocal interdependence of E(z), Pcl and Phol for PRE binding. (**A**) Schematic of the *bxd* regulatory region of the *Ubx* gene. PCR-amplified regions are indicated below. (**B-E**) Quantitative ChIP results using (B) anti-E(z), (C) anti-Pcl, or (D) anti-Phol antibodies, or (E) no antibodies. Wing imaginal discs were dissected from the following larvae: Oregon R (WT); $E(z)^{61}$ homozygotes [E(z)61]; PWIZ-Pcl/P{GAL4-da.G32} (*Pcl* RNAi); $phol^{81A}$; pho^{1} homozygotes (phol; pho). (**F**) Western blot showing Pcl and E(z) levels in Oregon R (1), P{GAL4-da.G32}/+ (2) and PWIZ-*Pcl*/P{GAL4-da.G32} (3) wing imaginal discs.

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the fractions with anti-E(z) and anti-Pcl antibodies. Larval E(z)-containing complexes have a relative mass of ~500 to 600 kDa, similar to that of embryonic PRC2 complexes that lack Pcl (Ng et al., 2000; Tie et al., 2003). However, Pcl was undetectable in E(z)-containing fractions and appeared to be in a complex with a relative mass of ~1500 kDa (Fig. 1). This is different from the fractionation profile of Pcl from embryo extracts, in which it co-fractionates with E(z) in native complexes with relative mass estimates in the range of ~650 kDa (O'Connell et al., 2001) to 1000 kDa (Tie et al., 2003), suggesting that, unlike its association with a subset of PRC2 complexes in embryos, Pcl functions as a component of a distinct complex in larvae, which we will refer to as the Pcl-Complex (PCLC).

Role of Pcl in the hierarchical assembly of PcG repressive complexes at the bxd PRE

In order to further investigate the relationship of Pcl with other PcG proteins and its role in PcG-mediated silencing in larvae, chromatin immunoprecipitation (ChIP) assays were performed on wing imaginal discs. The PcG maintains the transcriptional silence of the Hox gene *Ultrabithorax* (*Ubx*) in the epithelial cells of wing discs (Beuchle et al., 2001). Other PcG proteins, including the DNA-binding proteins Pho and Phol and components of the PRC1 and PRC2 complexes, have previously been shown to be present at the major PRE in the *Ubx* cis-regulatory *bxd* region in this tissue (Cao et al., 2002; Papp and Müller, 2006; Wang, H. et al., 2004; Wang, L. et al., 2004). Consistent with a previous report, Pcl also was detected at the *bxd* PRE, and appears to largely colocalize with E(z) and Phol (Fig. 2) (Papp and Müller, 2006).

We previously described a hierarchical relationship among PcG proteins at the *bxd* PRE in which Pho and/or Phol are required, but are not necessarily sufficient, for recruitment of PRC2, which in turn facilitates recruitment of PRC1 (Wang, L. et al., 2004). In order to determine how Pcl might fit into this recruitment pathway, ChIP assays were performed on *E(z)* mutant wing imaginal discs. $E(z)^{61}$ is a temperature-sensitive allele that displays nearly wild-type activity at 18°C, but strongly reduced activity at 29°C (Jones and Gelbart, 1990). Following shift from 18°C to 29°C, *bxd* PRE binding by $E(z)^{61}$ protein is rapidly lost and along with it the detection of H3K27me3 and Pc in this region (Cao et al., 2002; Wang, L. et al., 2004). ChIP assays of wing discs dissected from $E(z)^{61}$ larvae 24 hours following shift from 18° to 29°C confirmed loss of E(z) from the PRE (Fig. 2B), but revealed no effect on Pcl and Phol binding to PRE fragments 3 and 4, but a slight decrease of both proteins at the 2 fragment (Fig. 2C,D). We speculate that Pcl and Phol signals at this proximal edge of the PRE are partly due to protein-protein cross-links, which might be reduced in the absence of PRC2. Retention of Pcl at the PRE in the absence of E(z) and by extension absence of PRC1, which requires PRC2 for binding to this region, confirms that Pcl is not a stable subunit of larval versions of either PRC1 or PRC2 and is consistent with its inclusion in a distinct complex.

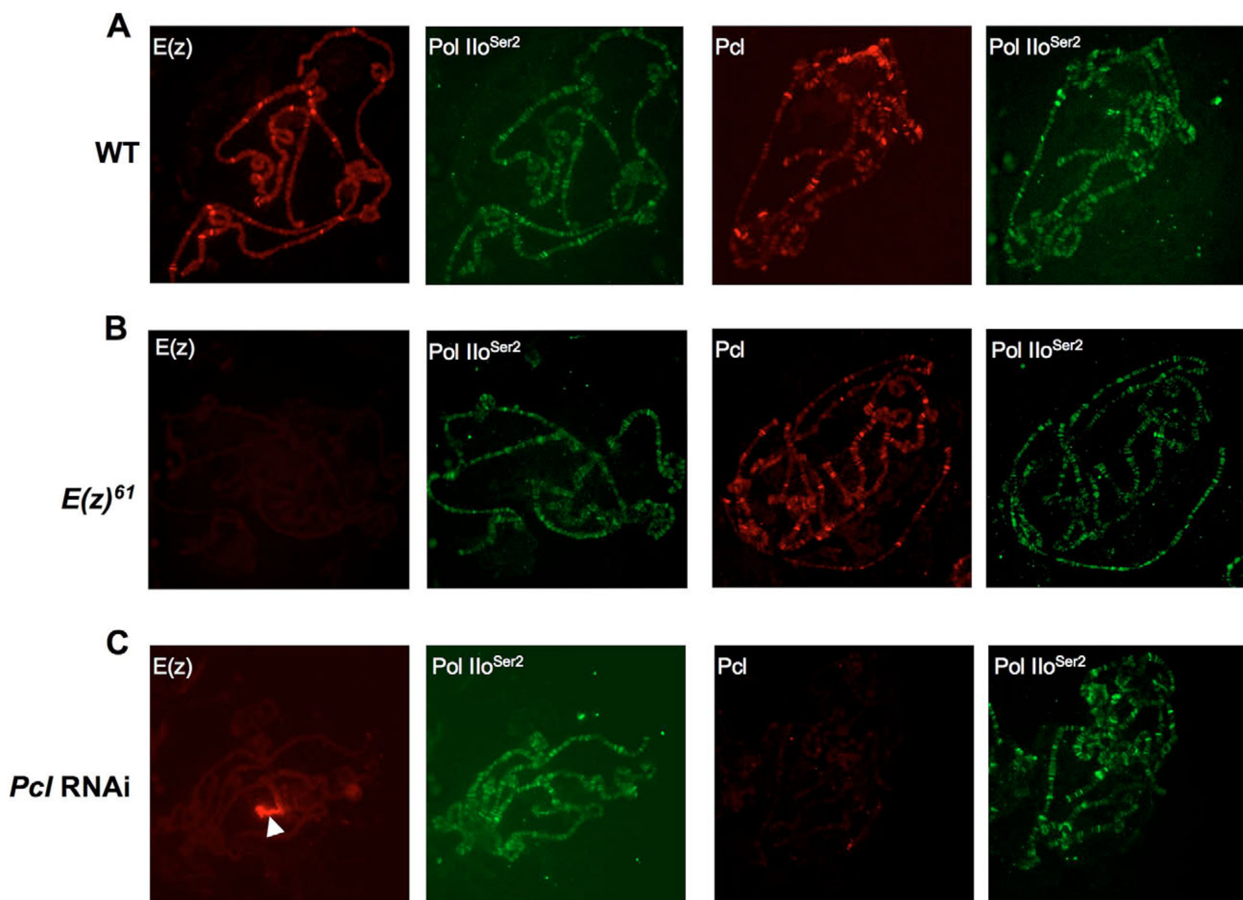

Fig. 3. Non-reciprocal genome-wide dependence of E(z) on Pcl for chromosome binding. Polytene chromosomes from (**A**) Oregon R, (**B**) $E(z)^{61}$, or (**C**) pUAST-R57-Pcl/P{GawB}c729 larvae were stained with anti-E(z) or anti-Pcl (red) and anti-IIo^{Ser2} (green). (A) Wild-type distributions of E(z) and Pcl. (B) Inactivation of $E(z)^{61}$ does not affect chromosome binding by Pcl. (C) Pcl knockdown results in loss of chromosome binding by E(z) (background debris signal is indicated by an arrowhead).

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Flies that are homozygous for null *Pcl* alleles die as embryos and no conditional *Pcl* alleles exist, precluding reciprocal experiments on *Pcl* mutant larvae. Therefore, we generated transgenic fly lines that contain inserts of a pWIZ-Pcl construct, which expresses Pcl shRNA under the control of Gal4, permitting inducible RNAi-mediated knockdown of Pcl in combination with *Gal4* drivers. Individuals that contain both pWIZ-Pcl and P{GAL4-da.G32}, which constitutively expresses Gal4, died as early pupae (data not shown) and exhibited dramatically reduced levels of Pcl in wing imaginal discs (Fig. 2F). E(z) levels were not affected (Fig. 2F). ChIP assays of these Pcl-depleted wing discs confirmed reduced Pcl levels at the *bxd* PRE and revealed commensurate loss of E(z) (Fig. 2B,C). Thus, although Pcl does not require PRC2 for PRE binding, Pcl, presumably functioning as a subunit of PCLC, is needed for stable binding of PRC2 to the bxd PRE. Phol remains at the PRE in the absence of Pcl (Fig. 2D).

In order to determine whether Pcl, like components of PRC1 and PRC2, requires Pho and/or Phol for PRE binding, ChIP assays were performed using wing imaginal discs from $phol^{81A}$; pho^1 larvae. Consistent with their role in recruiting other PcG proteins (Mohd-Sarip et al., 2002; Mohd-Sarip et al., 2005; Wang, L. et al., 2004), Pcl was lost from the *bxd* PRE in the absence of Pho and Phol (Fig. 2C).

Genome-wide requirement of Pcl for E(z) chromosome binding

In order to determine whether this non-reciprocal relationship between Pcl and E(z) occurs at other genomic sites, polytene chromosomes from either wild-type larvae or $E(z)^{61}$ larvae, which had been shifted to 29°C 24 hours prior to dissection, were stained with anti-E(z) or anti-Pcl antibodies. As a positive control, chromosomes were double stained with an antibody against RNA polymerase II phosphorylated at the Ser2 position in the C-terminal domain (IIo^{Ser2}). Consistent with previous studies (Carrington and Jones, 1996), $E(z)^{61}$ protein was largely lost from polytene chromosomes following shift to restrictive temperature; however, chromosome binding by Pcl was unchanged (Fig. 3B). Although induced expression of pWIZ-Pcl significantly knocks down Pcl in wing discs, an alternative shRNA-expressing construct, pUAST-R57-Pcl, was found to be more effective in salivary glands. Polytene chromosomes from larvae heterozygous for pUAST-R57-Pcl and P{GawB}c729, which expresses Gal4 in salivary glands, exhibited significantly diminished Pcl signals and lacked detectable E(z) bands (Fig. 3C). Thus, our observations at the bxd PRE appear to generally apply to PcG-binding sites throughout the genome.

These results demonstrate the existence of a distinct Pcl protein complex in larvae that is required for recruitment of PRC2 to chromosomal target sites and/or to stabilize its binding. As previously described, E(z), as a core subunit of PRC2, is required for target site binding by PRC1 (Cao et al., 2002; Platero et al., 1996; Rastelli et al., 1993; Wang, L. et al., 2004). Therefore, Pcl is indirectly required for chromosome binding by PRC1 as well, although direct interaction with PRC1 cannot be ruled out, similar to the way in which Pho may contribute to target site binding by PRC1 by interacting both with PRC2 subunits (Wang, L. et al., 2004) and with Pc, a core subunit of PRC1 (Mohd-Sarip et al., 2002; Mohd-Sarip et al., 2005).

In vitro histone methyltransferase assays of Pcl-PRC2 show that its activity and specificity for methylation of H3K27 are essentially indistinguishable from that of PRC2 complexes lacking Pcl. ChIP analysis of *Pcl* mutant embryos has shown that Pcl does not seem to be required for target site binding by other PRC2 subunits, but that it may be needed for high levels of trimethylation of H3K27 (Nekrasov et al., 2007). One explanation for these observations is that the contribution of Pcl to Pcl-PRC2 in embryos might be to mediate interaction with other proteins that are yet to be identified. In larvae, Pcl exists as a subunit of a distinct complex. Given the ability of Pcl to directly interact with several PRC2 subunits (Nekrasov et al., 2007; O'Connell et al., 2001), colocalization of Pcl and E(z) at the PRE (Fig. 2) (Papp and Müller, 2006), and dependence of E(z) on Pcl for binding to the *bxd* PRE and other genomic sites (Fig. 2B, Fig. 3C), it is likely that PCLC is closely associated with PRC2 at target sites in larvae. In both embryos and larvae, some of the activities attributed to Pcl might, upon further inspection, be due to the activities of other Pcl-associated proteins, the close apposition of which with PRC2 and other PcG complexes may be mediated by Pcl. The differential deployment of Pcl as a subunit of PRC2 and as a subunit of PCLC at distinct developmental stages is intriguing and might reflect the different molecular activities needed for establishment of silencing in embryos and maintenance of the silenced state in larval tissues. A more detailed understanding of the mechanisms by which Pcl contributes to PcG silencing will require identification of the other proteins contained within the larval PCLC complex and the potential biochemical activities of the complex.

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