Molecular Cell, Vol. 14, 637–646, June 4, 2004, Copyright ©2004 by Cell Press

# Hierarchical Recruitment of Polycomb Group Silencing Complexes

**Liangjun Wang,[1] J. Lesley Brown,[2] Ru Cao,[3,4] Yi Zhang,[3,4] Judith A. Kassis,[2] and Richard S. Jones[1,*]**

[1]Department of Biological Sciences
Southern Methodist University
Dallas, Texas 75275

[2]Laboratory of Molecular Genetics
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20892

[3]Department of Biochemistry and Biophysics
Lineberger Comprehensive Cancer Center

[4]Curriculum in Genetics and Molecular Biology
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599

## Summary

**Polycomb group (PcG) proteins maintain the transcriptional silence of target genes through many cycles of cell division. Here, we provide evidence for the sequential binding of PcG proteins at a Polycomb response element (PRE) in proliferating cells in which the sequence-specific DNA binding Pho and Phol proteins directly recruit E(z)-containing complexes, which in turn methylate histone H3 at lysine 27 (H3mK27). This provides a tag that facilitates binding by a Pc-containing complex. In wing imaginal discs, these PcG proteins also are present at discrete locations at or downstream of the promoter of a silenced target gene, *Ubx*. E(z)-dependent H3mK27 is also present near the *Ubx* promoter and is needed for Pc binding. The location of E(z)- and Pc-containing complexes downstream of the *Ubx* transcription start site suggests that they may inhibit transcription by interfering with assembly of the preinitiation complex or by blocking transcription initiation or elongation.**

## Introduction

*Drosophila* Polycomb group (PcG) and trithorax group (trxG) proteins function in an antagonistic manner to maintain the expression states of target genes. Among these are the Hox genes of the Antennapedia and bithorax gene complexes (ANT-C and BX-C, respectively). The expression patterns of Hox genes are initially established during early embryogenesis by transiently expressed activators and repressors encoded by the segmentation genes. Upon decay of these transcription factors, maintenance of the active and silenced states of homeotic genes becomes the responsibility of the trxG and PcG proteins, respectively (for review see Simon and Tamkun, 2002). For example, the wing imaginal disc is derived from a small cluster of cells at a position in the embryo at which *Ultrabithorax* (*Ubx*) is repressed (Cohen et al., 1991). These cells proliferate exponentially during larval development, resulting in a monolayer of ~50,000 epithelial cells (the wing imaginal disc) by the end of the third larval instar, which differentiates to form the adult wing and dorsal thorax during metamorphosis (Garcia-Bellido and Merriam, 1971). In order to maintain the determined state of wing imaginal disc cells, PcG proteins are required to maintain the silence of *Ubx* in this cell lineage, thus serving as a molecular memory system that heritably maintains the repressed state of a gene through many cycles of cell division.

The PcG comprises ~15 genes. Absence of any individual PcG protein results in derepression of target genes. Thus, they work in concert to maintain transcriptional silence. Two PcG protein complexes have been isolated from *Drosophila* embryos and their constituent polypeptides identified. The PRC1 complex contains Polycomb (Pc), polyhomeotic (Ph), Posterior sex combs (Psc), Zeste, dSbf1, dRing1 [also known as Sex combs extra, (Sce) (Fritsch et al., 2003)], HSC4, and five general transcription factors (Saurin et al., 2001). The Esc-E(z) complex contains the PcG proteins Extra sex combs (Esc), Enhancer of zeste [E(z)], and Suppressor 12 of zeste [Su(z)12], in addition to the histone binding NURF-55. The histone deacetylase HDAC1 (Rpd3) may be present in some forms of the complex (Czermin et al., 2002; Tie et al., 2001) but is absent from others (Müller et al., 2002). E(z) contains a SET domain [Su(var)3–9, Enhancer of zeste, Trx], a conserved domain that is present in numerous chromatin proteins that possesses histone lysine methyltransferase (HMTase) activity (Jenuwein et al., 1998; Rea et al., 2000). The E(z) SET domain preferentially methylates histone H3 at K27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002; reviewed in Cao and Zhang, 2004).

The chromo domain is an ~50 amino acid protein domain that is able to bind to histone tails methylated at specific lysine residues (for review see Eissenberg, 2001). Pc contains a chromo domain that has high affinity for histone H3 tails methylated at lysine 27 (Cao et al., 2002; Czermin et al., 2002; Fischle et al., 2003; Kuzmichev et al., 2002; Min et al., 2003). Several lines of evidence indicate that the H3mK27 HMTase activity of E(z) appears to play a role in recruiting the PRC1 complex; however, the possibility that the physical presence of Esc-E(z) complex may also contribute to PRC1 recruitment through protein-protein interactions has not been ruled out.

PcG-mediated repression requires the presence of a *cis*-acting Polycomb response element (PREs) (Chan et al., 1994; Simon et al., 1993). PREs are needed both to establish silencing and to maintain it through most of larval development (Busturia et al., 1997). This indicates that propagation of the silenced state requires the activities of proteins that bind directly to the PRE and that protein-protein interactions and/or long-lasting chromatin modifications are not sufficient. PREs contain binding sites for several sequence-specific DNA binding proteins, including Pleiohomeotic (Pho) and Pho-like (Phol), homologs of the mammalian transcription factor YY1

*Correspondence: rjones@mail.smu.edu

that have identical DNA binding specificities in vitro (Brown et al., 1998, 2003). Pho and Phol play important, albeit redundant, roles in maintaining PcG silencing during larval development.

Here, we report direct interactions of Pho with E(z) and Esc and of Phol with Esc. Using a combination of RNA interference (RNAi) and chromatin immunoprecipitation (ChIP) approaches, we provide evidence that support a hierarchical binding pathway of Pho, E(z), and Pc at the major *Ubx* PRE in Schneider line 2 (SL2) tissue culture cells. We confirm and elaborate upon this pathway in vivo, to include the role of Phol, by examining binding of these PcG proteins to the *bxd* PRE and *Ubx* promoter regions in normal and mutant wing imaginal discs using ChIP assays. Furthermore, we find that these PcG proteins also are associated with a narrowly delimited region downstream of the potential *Ubx* transcription start site in wing imaginal discs and provide evidence for the ability of H3mK27 to recruit Pc to a target site independent of the physical presence of E(z).

## Results and Discussion

### Pho Directly Interacts with E(z) and Esc Proteins

As part of a series of directed yeast two-hybrid assays to identify direct interactions between E(z) and other PcG proteins, full-length Pho was found to interact with the Cys-rich CXC domain of E(z) (aa 512–605) (Figure 1A). This interaction was confirmed using in vitro GST pull-down assays in which full-length $^{35}$S-E(z) bound to GST-Pho1-520 (Figure 1B, lane 3). $^{35}$S-E(z) bound only to GST-Pho fusion proteins that included residues 147–170 (Figure 1B, lanes 3, 6, 9, and 14), thus delimiting the E(z) binding region to the conserved Pho spacer region (residues 148–169). The Pho spacer is a 22 amino acid region that is 82% identical to human YY1 (18/22 amino acid identities) (Brown et al., 1998). Consistent with the conservation of this region, E(z) was found to also bind to a GST fusion protein that contained the human YY1 spacer region (GST-YY1-204-227) (Figure 1B, lane 13). Conserved sequence-specific DNA binding activities (Brown et al., 1998) and abilities to interact with E(z) may explain the ability of YY1 to function as a PcG protein and to rescue *pho* mutant phenotypes in *Drosophila* embryos and larvae (Atchison et al., 2003). In reciprocal GST pull-down assays, full-length $^{35}$S-Pho bound only to GST-E(z) fusion proteins that included residues 512–605 (Figure 1C, lanes 4, 5, and 9). This E(z) region includes the Cys-rich CXC domain, which contributes to chromosome binding (Carrington and Jones, 1996). These results initially were somewhat surprising, since YY1 and Pho previously were shown to interact with the human homolog of Esc, EED, but not with a human E(z) homolog, EZH2 (Satijn et al., 2001). Therefore, we tested Pho for interaction with GST fusion proteins that contain full-length EZH2 or a second human E(z) homolog, EZH1. Consistent with the previous report, we found that Pho does not interact with EZH2 (Figure 1C, lane 11); however, it does interact with EZH1 (Figure 1C, lane 10), which may indicate distinct functions of these human E(z) homologs.

Since EED has been shown to interact with YY1 (Satijn et al., 2001), reciprocal GST-pull-down assays were performed to test for interaction of Esc with Pho. $^{35}$S-Pho bound to full-length GST-Esc (Figure 1D, lane 12) and full-length $^{35}$S-Esc interacted most strongly with the Pho C-terminal (GST-Pho-357-520) and spacer regions (GST-

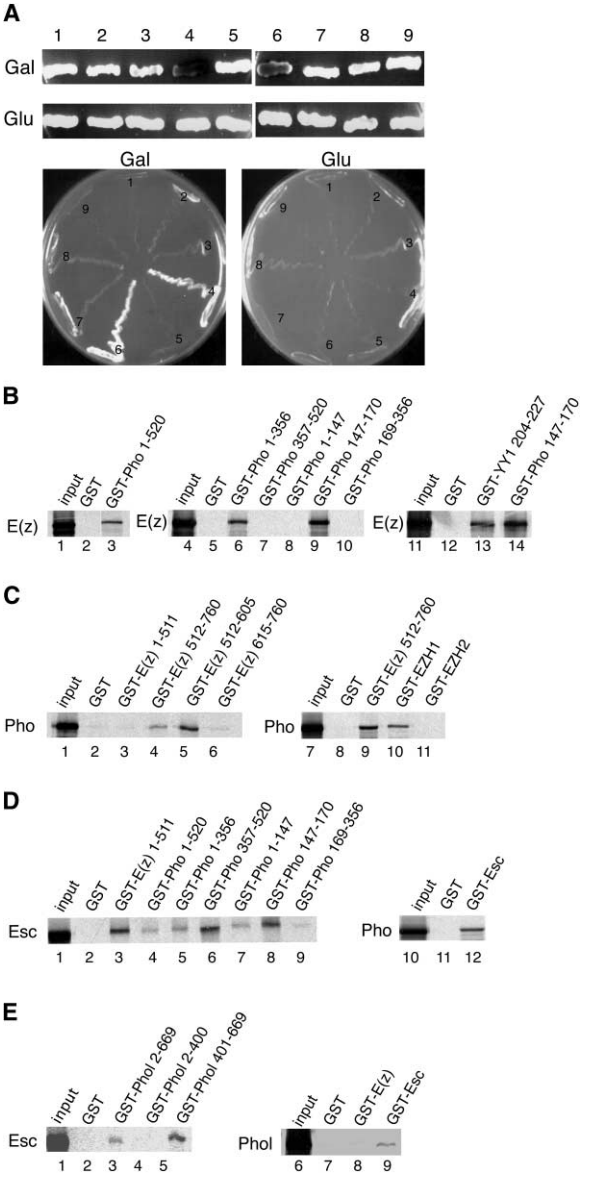


Figure 1. Pho Interacts with E(z) and Esc; Phol Interacts with Esc but Not E(z)

(A) Yeast two-hybrid assays of EGY48 cells containing the following plasmids: (1) pJG4-5 and pEG202, (2) pJG4-5-Pho and pEG202-E(z)1-511, (3) pJG4-5 and pEG202-E(z)1-511, (4) pJG4-5-Pho and pEG202-E(z)512-760, (5) pJG4-5 and pEG202-E(z)512-760, (6) pJG4-5-Pho and pEG202-E(z)512-605, (7) pJG4-5 and pEG202-E(z)512-605, (8) pJG4-5-Pho and pEG202-E(z)615-760, (9) pJG4-5 and pEG202-E(z)615-760. Expression of pJG4-5 constructs was induced on medium containing galactose (Gal) but not on glucose-containing (Glu) medium. (Top) Expression of β-gal expression from lacZ reporter plasmid. (Bottom) Growth assays on medium lacking leucine. (B–E) Autoradiographs of SDS gels from GST pull-down assays. Radiolabeled proteins (to the left of each panel) were tested for binding in vitro to the indicated GST-fusion proteins or to GST alone. The input lanes contain 20% of the amount of radiolabeled protein used in each assay.

Pho-147-170) (Figure 1D, lanes 6 and 8, respectively). These are homologous to the YY1 regions that interact with EED (Satijn et al., 2001).

**Pho-like Directly Interacts with Esc but Not with E(z)**

In light of the functional redundancy of *pho* and *phol* (Brown et al., 2003), we tested for potential interactions of Phol with Esc and E(z). Pho and Phol share 80% sequence identity in their C-terminal zinc finger regions. Outside of this region the two proteins are much less similar; their 22 amino acid spacer regions are only identical at 10 positions. In GST pull-down assays, $^{35}$S-Phol bound to GST-Esc but not to GST-E(z) (Figure 1E, lanes 9 and 8, respectively). In reciprocal assays, full-length $^{35}$S-Esc bound to GST-Phol401-669 (Figure 1E, lane 5), which includes the conserved zinc fingers region but not to GST-Phol2-400 (Figure 1E, lane 4), which includes the spacer region. The conserved abilities of the C termini of Pho and Phol to interact with Esc and the inability of Phol spacer region to interact with Esc or E(z) are consistent with their respective levels of sequence conservation.

**Pho Is Required for PRE Binding by E(z)**

Even though neither Pho nor Phol appear to be stable components of Esc-E(z) complexes (Czermin et al., 2002; Müller et al., 2002), multiple observations, including direct interactions with E(z) and/or Esc (Figure 1), coimmunoprecipitation of Pho with Esc and E(z) (Poux et al., 2001), and strong genetic interaction between *pho* and *E(z)* mutant alleles (Bajusz et al., 2001), collectively suggest a close functional interaction between Pho and possibly Phol and Esc-E(z) complexes. Pho binding sites have been identified in multiple PREs and are required for PRE function (Fritsch et al., 1999; Busturia et al., 2001; Mishra et al., 2001). Therefore, we reasoned that Pho might directly recruit Esc-E(z) complexes to PREs. The major *Ubx* PRE, located in the *bxd* region, has been delimited to a 567 bp fragment ($PRE_D$), which contains multiple Pho binding sites (Fritsch et al., 1999). E(z) previously has been shown to bind to the $PRE_D$ region and to methylate histone H3 at lysine 27 at this site in SL2 tissue culture cells and wing imaginal discs (Cao et al., 2002). To determine the localization of Pho and to examine the possible colocalization of Pho, E(z), and Pc, ChIP assays using anti-Pho and anti-E(z) antibodies were performed in the *Ubx* promoter region and *bxd* region that encompasses the $PRE_D$. Locations of the regions amplified by PCR in the ChIP assays are shown in Figure 2A. This analysis revealed colocalization of Pho, E(z), and Pc at $PRE_D$ (Figure 2B). Interestingly, although neither Pho nor E(z) were detected in the promoter region, Pc binding in the promoter region was detected (Figure 2B), consistent with previous observations (Breiling et al., 2001).

To test for E(z) dependence on Pho for $PRE_D$ binding, duplicate samples of SL2 cells were transfected with either *pho* or GFP dsRNA. The latter served as a negative control in all RNAi experiments. Western analysis revealed effective depletion of Pho protein in the *pho* RNAi-treated cells but no effect on the level of E(z) protein (Figure 3A). Depletion of Pho resulted in loss of PRE binding by E(z) (Figure 3B), demonstrating dependence

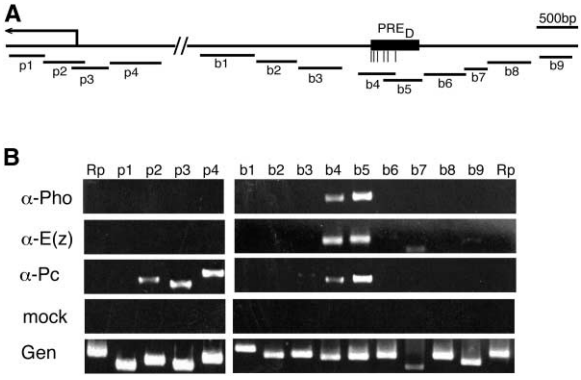


Figure 2. Distribution of Pho, E(z), and Pc Proteins at the *Ubx* Promoter and $PRE_D$ Regions in SL2 Cells

(A) Schematic representation of the *Ubx* promoter and *bxd* PRE regions. The regions amplified by PCR in these and subsequent ChIP assays, p1–p4 and b1–b9, are depicted as horizontal lines below. Vertical lines represent Pho binding sites.

(B) ChIP assays showing the distribution of Pho, E(z), and Pc in SL2 cells. Antibodies used in immunoprecipitations and the PCR amplified regions are indicated to the left and above, respectively. Rp, RpII140 promoter; mock, crude rabbit antiserum; Gen, genomic DNA.

of E(z) on Pho for $PRE_D$ binding in SL2 cells. ChIP assays using anti-Phol antibodies did not detect the presence of Phol at this PRE region in SL2 cells (data not shown). Therefore, although Phol contributes to recruitment of PcG proteins to $PRE_D$ in other cells (as described below), it does not play such a role in SL2 cells.

Possible reciprocal dependency of Pho on E(z) for PRE binding was tested by RNAi depletion of E(z) protein (Figure 3A) followed by ChIP analysis using anti-Pho antibodies. As shown in Figure 3C, depletion of E(z) had no effect on $PRE_D$ binding by Pho. These observations are consistent with independent binding of Pho to $PRE_D$ and subsequent recruitment of Esc-E(z) complex and provide further evidence that Pho is not in the Esc-E(z) complex.

**Pc Requires Both Pho and E(z) for PRE Binding in SL2 Cells**

Depletion of Pho also resulted in loss of binding by Pc to the PRE region (Figure 3B). Thus, Pho is required for $PRE_D$ binding by both the Esc-E(z) and Pc-containing (e.g., PRC1) complexes in SL2 cells. Previous studies (Mohd-Sarip et al., 2002) indicate direct interaction between Pho and two components of the PRC1 complex, Pc and Ph. Consequently, it was suggested that Pho directly recruits the PRC1 complex to target sites through these interactions (Mohd-Sarip et al., 2002). However, depletion of E(z) resulted in loss of Pc binding, even though Pho remained at the PRE (Figure 3C). This is consistent with our previous observations that binding of Pc to the $PRE_D$ region requires functional Esc-E(z) complex (Cao et al., 2002) and shows that Pho alone is not sufficient to stably recruit the Pc-containing complex in SL2 cells.

In order to more thoroughly define the binding hierarchy of these protein complexes, Pc was depleted by RNAi (Figure 3A) and the potential effects on E(z) and

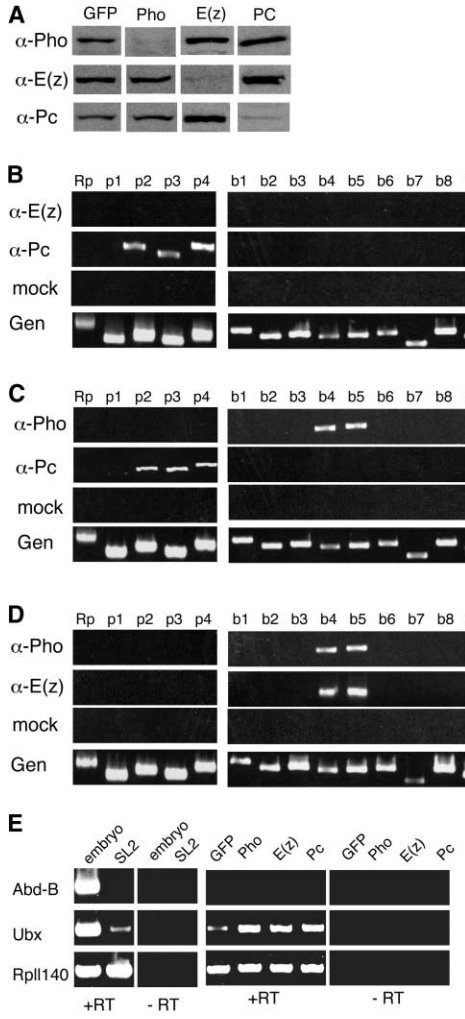


Figure 3. Hierarchical Binding of Pho, E(z), and Pc to the *Ubx* $PRE_D$ Region in SL2 Cells

(A) Western blots of proteins from cells transfected with each of the dsRNAs indicated above and probed with the antibodies indicated to the left of each panel.

(B–D) ChIP assays showing the effects of RNAi depletion of (B) Pho, (C) E(z), or (D) Pc on binding by the proteins indicated to the left of the panels.

(E) RT-PCR analysis of *Abd-B*, *Ubx*, and *RpII140* expression in (left) embryos and untreated SL2 cells and (right) SL2 cells transfected with GFP or *pho*, *E(z)*, or *Pc* dsRNA, as indicated above. RNA was extracted from the same cells used in the ChIP assays in (B)–(D), respectively. +RT, reverse transcriptase added; −RT, reverse transcriptase not added to reverse transcription reaction.

Pho binding were examined. Depletion of Pc had no effect on PRE binding by either E(z) or Pho (Figure 3D). Although Pc is a core component of PRC1, it is possible that depletion of Pc may not completely eliminate the activities of Pc-containing complexes. Nevertheless, collectively, these data suggest an assembly pathway of PcG proteins at $PRE_D$ in SL2 cells in which Pho binds directly to its sites within the PRE and recruits the Esc-E(z) complex through direct interaction with E(z) and/or Esc. As previously described (Cao et al., 2002), the Esc-E(z) complex then methylates histone H3 at K27 in the $PRE_D$ region, providing a tag to which the Pc chromo domain binds and facilitating the recruitment of Pc-containing complexes, such as PRC1.

Consistent with a previous study (Breiling et al., 2001), we observed Pc binding to the p2-p4 regions, encompassing the *Ubx* transcription start site extending from approximately +396 to −970 (the distal ends of regions p1 and p4, respectively, Figure 2B). We could not detect the presence of either Pho or E(z) at the *Ubx* promoter in these same cells (Figure 2B). Nevertheless, it remained possible that Pho and/or E(z) might indirectly assist in recruitment of a Pc-containing complex to the *Ubx* promoter. However, depletion of either Pho or E(z) had no effect on Pc binding to the *Ubx* promoter region (Figures 3B and 3C, respectively). Thus, neither Pho nor the Esc-E(z) complex appears to be required for Pc binding to the *Ubx* promoter in SL2 cells under these conditions. The mechanism by which Pc-containing complexes are recruited to the *Ubx* promoter is not clear; however, it has been recently reported that components of the PRC1 complex may recruit chromatin templates in *trans*, which may allow a Pc-containing complex assembled at a PRE to form a bridge with target promoters (Lavigne et al., 2004). It is also possible that the DNA binding activities of ZESTE or GAGA factor may contribute to promoter region binding (Mulholland et al., 2003).

Derepression of *Ubx* in SL2 cells following RNAi-mediated depletion of Pc has been previously described (Breiling et al., 2001). The SL2 cells used in their studies normally express the Hox gene *Abd-B* but not *Ubx*. However, the SL2 cells used in our studies do not express *Abd-B* but do express *Ubx* at low levels (Figure 3E). Low and relatively uniform Ubx expression in these cells was confirmed by immunofluorescent staining with anti-Ubx antibody (data not shown). Since little is known about the factors responsible for regulation of Hox genes in SL2 cells, we cannot fully explain these observed differences. However, since *Ubx* is negatively regulated by Abd-B in embryos (Struhl and White, 1985), the lower basal levels of *Ubx* reported by Breiling et al. (2001) might be due to repression of *Ubx* by Abd-B. If this is the case, the presence or absence of a single activator of *Abd-B* in different SL2 sublines might explain these discrepancies. Depletion of Pc, E(z), or Pho resulted in increased levels of *Ubx* expression (Figure 3E). It is worth noting that increased expression of *Ubx* in Pho- and E(z)-depleted cells occurred even in the continued presence of Pc at the *Ubx* promoter. Thus, although *Ubx* is negatively regulated by the PcG in SL2 cells, it does not appear to be completely silenced as is the case in wing imaginal disc cells (Cabrera et al., 1985; Glicksman and Brower, 1988a).

### Pho Is Redundant with Phol for Recruitment of PcG Complexes in Wing Imaginal Discs

In order to examine the recruitment and distribution of PcG proteins in cells in which they actually silence *Ubx* transcription, we performed ChIP assays using wing imaginal discs. Although depletion of Pho alone is sufficient to eliminate PRE binding by E(z) and Pc in SL2 cells, *pho* functions in a redundant fashion with *phol* in wing imaginal discs to maintain *Ubx* transcriptional silence (Brown et al., 2003). Therefore, we reasoned that both Pho and Phol might contribute to recruitment of

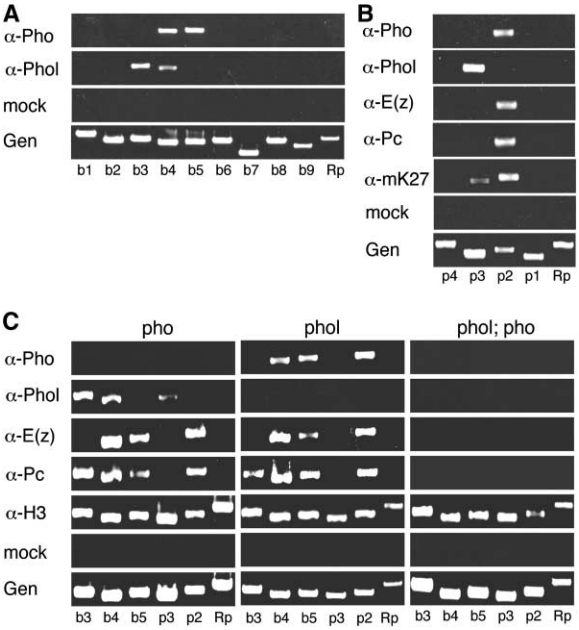


Figure 4. Pho and Phol Are Redundant for Recruitment of E(z) and Pc to the *Ubx* Transcription Start Site and PRE Regions in Wing Imaginal Discs

(A) ChIP assays showing distribution of Pho and Phol in the PRE region. (B) ChIP assays showing distribution of Pho, Phol, E(z), Pc, and H3mK27 near the *Ubx* transcription start site. (A and B) Wing imaginal discs were dissected from *E(z)*$^{61}$ larvae reared continuously at 18°C. (C) ChIP assays of wing imaginal discs dissected from (left) *pho*$^{1}$, (middle) *phol*$^{81A}$, and (right) *phol*$^{81A}$; *pho*$^{1}$ larvae. α-H3, anti-histone H3 was used as a positive control in these assays and those in Figure 5.

E(z) and Pc complexes in these cells. This possibility was tested by first examining the distributions of Pho and Phol at the *Ubx* $PRE_D$ region in wing imaginal discs dissected from third instar larvae that were wild-type for *pho* and *phol*. Distributions of E(z) and Pc at the $PRE_D$ region previously have been shown to be nearly identical in wing imaginal discs and SL2 cells, associating with the b4 and b5 regions in both (Cao et al., 2002). H3mK27 is also colocalized with and dependent on the presence of E(z) at $PRE_D$ (Cao et al., 2002). As in SL2 cells, Pho colocalizes with E(z) and Pc at the $PRE_D$ region in wing discs, crosslinking to regions b4 and b5 (Figure 4A). Phol is also present at the $PRE_D$ region in wing discs but appears to be slightly offset from the other PcG proteins, crosslinking to regions b3 and b4 (Figure 4A). It is curious that, in spite of their identical sequence-specific DNA binding activities in vitro, Pho and Phol appear to bind to slightly different $PRE_D$ regions in vivo. Both bind to b4, which encompasses the part of $PRE_D$ that contains Pho/Phol binding sites. b3 does not include Pho/Phol binding sites and b5 includes just one site that lies near the middle of $PRE_D$. Therefore, they may both bind directly to sites in the b4 region and detection of Pho in b5 and Phol in b3 may be due to crosslinking of Pho and Phol with different unidentified proteins that bind to these flanking regions. Alternatively, these slightly different distributions may be due to their different responses to the chromatin context of their binding sites in vivo.

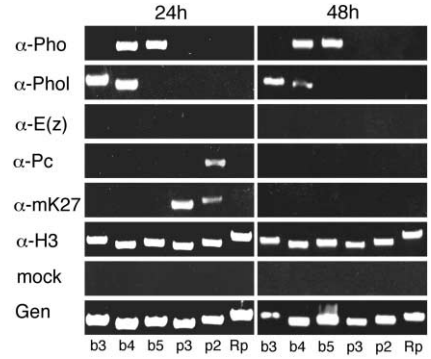


Figure 5. Role of E(z) and H3mK27 in Recruitment of PcG Proteins

ChIP assays of wing imaginal discs from *E(z)*$^{61}$ larvae shifted from 18°C to 29°C (left) 24 hr or (right) 48 hr prior to dissection.

Distribution of PcG proteins at the *Ubx* promoter region in wing imaginal discs is different from their distribution in SL2 cells. Pc is less broadly distributed and is only detected at the p2 region, just downstream of the transcription start site (Figure 4B). p2 extends from +409 to −101 and overlaps slightly with p1, which extends from +834 to +396, and more extensively with p3, which extends from +71 to −382. Since Pc was only detected in the p2 region, we conclude that it is associated with the +396 to +71 interval. E(z) and Pho also are associated with this same p2 region in wing imaginal discs (Figure 4B), whereas we were unable to detect their presence near the *Ubx* promoter in SL2 cells (Figure 2B). Phol is also detected at the *Ubx* promoter but crosslinks to region p3, not to p2 (Figure 4B). Three sites that closely resemble the consensus Pho/Phol binding sequence are located in the p4 region and one is located in the p2 region. Since neither Pho nor Phol are detected in the p4 region, it seems unlikely that these are functional sites. The site within the p2 region is at +219 (GCCATAGT). It is not known whether Pho or Phol are capable of binding to this site or whether a single site might be sufficient for stable binding in vivo. As described below (Figure 5), it seems more likely that detection of Pho and Phol in these regions is due to crosslinking with other proteins. If this is the case, as suggested above to explain their different patterns in the $PRE_D$ region, detection of Pho and Phol at the p2 and p3 regions, respectively, also may be due to interactions with different proteins located near the *Ubx* transcription start site.

H3mK27 is also detected in p2, colocalized with E(z); however, it is also detected in p3. Indeed, in the majority of assays that we have performed the H3mK27 signal has been even stronger in p3 than in p2 (data not shown), which may be due in part to slightly greater efficiency of the p3 PCR primers relative to the p2 primers (note the relative p2 and p3 signals for genomic DNA in Figure 4B; data not shown). Thus, unlike their distributions in the $PRE_D$ region, the distribution of H3mK27 near the transcription start site appears to overlap the distribution of E(z) but not to precisely colocalize with E(z). It is possible that the H3mK27 signal in p3 may be due to crosslinking of the modified nucleosome(s) with a protein located further upstream. Alternatively, this may be

due to transient interaction with and modification of an upstream nucleosome by the E(z)-containing complex or mobility of H3mK27 modified nucleosome(s). Although we cannot resolve this puzzle at this time, as described below (Figure 5), methylation of H3 at K27 in both the p2 and p3 regions is dependent on E(z).

We then examined Pho, Phol, E(z), and Pc binding to the *Ubx* $PRE_D$ and near the transcription start site in wing imaginal discs dissected from $pho^1$ homozygotes, $phol^{81A}$ homozygotes, or $phol^{81A}$; $pho^1$ double homozygotes (Figure 4C). Binding by neither Phol nor Pho was altered in the absence of the other. Likewise, no effect on E(z) or Pc binding at either the PRE or downstream of the transcription start site is observed in $pho^1$ or $phol^{81A}$ homozygotes. However, binding by both E(z) and Pc is eliminated at both sites in $phol^{81A}$; $pho^1$ double homozygotes. Thus, either Pho or Phol appears to be capable of recruiting E(z)- and Pc-containing complexes in this tissue. This is consistent with the lack of *Ubx* derepression in *phol* homozygotes, very weak derepression of *Ubx* in *pho* homozygotes, but extensive derepression of *Ubx* in *phol*; *pho* double homozygotes (Brown et al., 2003).

It is striking that, in contrast to the requirement of Pho or Phol for E(z) and Pc binding at $PRE_D$ and near the *Ubx* transcription start site in wing imaginal discs, binding of E(z) and Pc to polytene chromosomes is little affected in *phol*; *pho* double mutants (Brown et al., 2003). A possible explanation for these apparently contradictory results is that polytene chromosomes are nonmitotic and are the products of multiple cycles of DNA endoreplication. Thus, Pho and/or Phol appear to be specifically required for chromosome binding by E(z)- and Pc-containing complexes in mitotic cells, such as SL2 and wing imaginal disc cells, but not in cells that do not undergo mitosis. Several components of the PRC1 complex dissociate from chromosomes during mitosis (Buchenau et al., 1998). Therefore, a mechanism must exist for rerecruitment of these proteins following mitosis. We suggest that the primary role of Pho and Phol in larvae is to fulfill this function of repeatedly recruiting PcG complexes following mitosis.

### E(z) and H3mK27 Are Required for Pc Binding to Both the *Ubx* PRE and Downstream of the Transcription Start Site; Pho and Phol Binding Require E(z) at the *Ubx* Transcription Start Site but Not at $PRE_D$

In order to further compare the binding hierarchy of these PcG proteins in wing imaginal discs to that in SL2 cells, ChIP assays were performed on wing discs dissected from larvae homozygous for the temperature-sensitive $E(z)^{61}$ allele following shifts to restrictive temperature. At 18°C (permissive temperature), $E(z)^{61}$ behaves as a wild-type allele, but at 29°C (restrictive temperature), chromosome binding by $E(z)^{61}$ protein is disrupted and methylation of H3 at K27 is lost (Jones and Gelbart, 1990; Carrington and Jones, 1996; Cao et al., 2002). $E(z)^{61}$ larvae were shifted from 18°C to 29°C at mid or early third instar stages, wing discs were dissected after 24 or 48 hr at restrictive temperature, respectively, and ChIP assays were performed to determine the effects on binding by PcG proteins and H3mK27. After 24 hr at 29°C, $E(z)^{61}$ protein was no longer detected at either $PRE_D$ or downstream of the transcription start site (Figure 5). As in SL2 cells, Pho remained at the PRE in the absence of E(z), as did Phol. However, both Pho and Phol were no longer detectable near the transcription start site (Figure 5). Therefore, although binding of Pho and Phol to the $PRE_D$ region does not require E(z), their presence near the *Ubx* transcription start site region is E(z) dependent. This reinforces the idea that Pho and Phol do not directly bind to DNA near the transcription start site but rather are detected at p2 and p3, respectively, due to crosslinking with other proteins. These could include an E(z)-containing complex and/or other protein(s) whose association with this region requires E(z) activity. It may be that an E(z)-containing complex facilitates the formation of a chromatin loop that brings the PRE region close to the *Ubx* promoter.

After 24 hr at 29°C, H3mK27 was no longer detectable at the PRE but remained at the p2 and p3 regions (Figure 5). Likewise, Pc was lost from the PRE region but remained at p2. After 48 hr at 29°C, both H3mK27 and Pc were no longer detectable near the *Ubx* transcription start site (Figure 5). Although we cannot make quantitative conclusions based on these data, slightly weaker Pc and H3mK27 signals near the transcription start site at the 24 hr time point would be consistent with dilution of H3mK27 and Pc binding due to replication and cell division. Alternatively, kinetics of Pc loss in the absence of an E(z)-containing complex may be relatively slower at the p2 region due to stabilization of Pc binding by other unidentified factors near the transcription start site but not at the PRE. However, correlation of Pc binding with the presence of H3mK27 but not continued presence of E(z) appears to be most consistent with the model in which H3mK27 provides a tag to which the Pc chromo domain binds, facilitating recruitment of Pc-containing complexes, and suggests that binding of Pc to a target site does not require the physical presence of an E(z)-containing complex. Recent studies have revealed the structural basis for the specific recognition of H3mK27 by the Pc chromo domain (Fischle et al., 2003; Min et al., 2003). It is interesting to compare these results with previous observations of the differences in durations of time required to observe initial *Ubx* derepression following induction of somatic clones of PcG mutant alleles in wing imaginal discs (Beuchle et al., 2001; Müller et al., 2002). *Ubx* derepression was first observed 48 hr after induction of *Pc* clones (Beuchle et al., 2001) but not until 72 hr after induction of *E(z)* clones (Müller et al., 2002). This 24 hr difference is consistent with the approximately 24 hr perdurance of H3mK27 and Pc near the *Ubx* promoter following inactivation of $E(z)^{61}$ (Figure 5).

These data also show that the dynamics of histone modifications are different at the *Ubx* $PRE_D$ and transcription start site regions. Although $E(z)^{61}$ protein appears to be simultaneously lost from both regions within the first 24 hr at 29°C, H3mK27 signal is lost along with E(z) from the PRE but retained near the transcription start site for an additional approximately 24 hr. Since the estimated duration of the cell cycle in wing imaginal discs at this stage is 8–10 hr (Garcia-Bellido and Merriam, 1971), the slow rate at which H3mK27 signal is lost near the transcription start site may reflect dilution of relatively stable H3mK27 by chromatin replication. However, the more rapid loss of H3mK27 signal at the

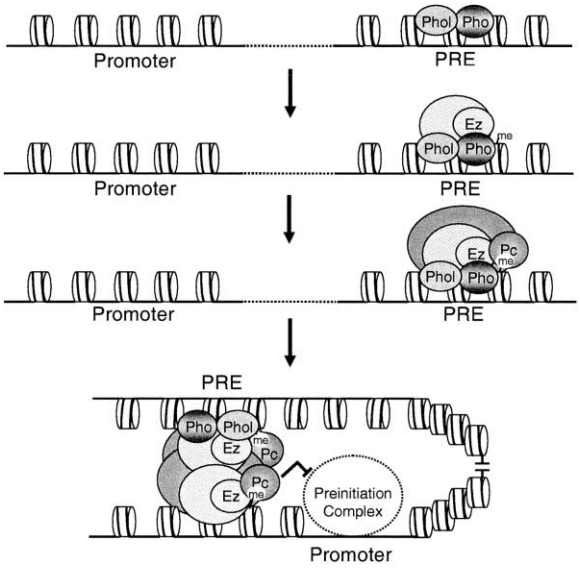


Figure 6. Model for Hierarchical Binding Pathway of PcG Proteins at the *Ubx* $PRE_D$ and Promoter Regions

Histone H3 tails methylated at K27 are depicted as "me."

PRE suggests a replication-independent mechanism. Indeed, we have observed loss of the H3mK27 signal within an hour of loss of E(z)[61] from the $PRE_D$ region (data not shown). Possible mechanisms include removal of the methyl groups by a histone demethylase, replication-independent replacement of H3mK27 by histone variant H3.3 (Ahmad and Henikoff, 2002), or masking of the anti-H3mK27 epitope by additional modifications, such as phosphorylation of Ser28.

### Model for Hierarchical Binding Pathway of PcG Proteins

PREs are required to establish silencing in embryos and to maintain silencing throughout much of larval development. We propose that this is due to the requirement of PRE binding proteins Pho and Phol and possibly additional PRE DNA binding proteins to repeatedly recruit E(z)-containing complexes to the PRE, which in turn methylates H3mK27, facilitating recruitment of Pc-containing complexes to the PRE (Figure 6). By a mechanism not yet understood, E(z) would then be brought into proximity with the promoter region, possibly involving the formation of a loop between PRE and promoter regions. This would allow E(z) to add H3mK27 tags to this region as well, enabling Pc-containing complexes to bind just downstream of the potential transcription start site (the p2 region). However, we must consider why Pc does not also bind to the p3 region. If the H3mK27 signal in p3 is simply due to crosslinking of the H3mK27-containing nucleosome(s) with one or more other proteins located further upstream, then Pc would be expected to only bind to the tagged nucleosome(s) in p2. Alternatively, Pc-containing complexes may be excluded from the p3 region due to the presence of other proteins or protein complexes. It has been proposed that Pc chromo domains dimerize and bind to methylated K27 residues of two separate nucleosomes (Min et al., 2003). Although this may allow Pc-containing complexes to form a link between adjacent nucleosomes, it is also possible that such dimers may simultaneously bind to nucleosomes in the $PRE_D$ region and downstream of the transcription start site, contributing to stability of a loop between these regions. Due to greater stability of the H3mK27 tag near the transcription start site, target gene silence may be maintained for a few cell cycles following loss of the PRE and proteins such as Pho, Phol, and E(z) that are involved in recruitment but which may not directly repress transcription. This model is consistent with the reduced requirement of the *Mcp* PRE for maintenance of reporter gene repression in later larval stages (Busturia et al., 1997) and with the greater duration of time required to observe *Ubx* derepression following loss of *E(z)* compared to loss of *Pc* (Beuchle et al., 2001; Müller et al., 2002).

### Implications for the Mechanisms by which PcG Proteins Repress Transcription

It was somewhat surprising to find Pc and E(z) complexes associated with a very narrow region downstream of the potential *Ubx* transcription start site. In vitro studies indicate several possible mechanisms by which a Pc-containing complex, such as PRC1, positioned at this site may repress transcription. Either a complete PRC1 or a recombinant core complex, PCC, containing only the four PcG proteins Pc, Ph, Psc, and dRing1, is able to inhibit nucleosome remodeling by human SWI/SNF (Shao et al., 1999; Francis et al., 2001). Several trxG proteins are components of the Brahma (BRM) complex. BRM is a member of the SWI/SNF family (Papoulas et al., 1998; Kal et al., 2000; Crosby et al., 1999; Vazquez et al., 1999; Collins et al., 1999). PRC1 inhibition of BRM activity may interfere with assembly of the preinitiation complex in the flanking promoter region. PRC1 and PCC also inhibit transcription by RNA polymerase II or T7 RNA polymerase in vitro (King et al., 2002). This activity appears not to involve prevention of activator binding but rather a selective block of the transcription process, possibly involving interference with assembly of the preinitiation complex or interaction with the template to prevent initiation or elongation of transcription. It has been recently reported that the trxG TAC1 complex contributes to full expression of heat shock gene *hsp70* by associating with and modifying histones in the 5′ coding region, not upstream of the transcription start site (Smith et al., 2004). Therefore, TAC1 appears to facilitate elongation but not initiation of transcription by RNA polymerase II, in this case. This suggests an intriguing possibility in which TAC1 and PcG complexes, such as PRC1, may play antagonistic roles in a similar step in transcription of Hox genes, such as *Ubx*.

## Experimental Procedures

### Yeast Two-Hybrid Assays

*E(z)* yeast two-hybrid constructs were made by PCR amplification of the indicated coding regions and ligation of the PCR products into the EcoRI-XhoI sites of the pEG202 vector. pJG4-5-Pho contains the full-length *pho* coding sequence (generously provided by Dr. Jeff Simon). LexA-fusion proteins are constitutively expressed from pEG202, whereas expression of AD-fusion proteins from pJG4-5 is induced by growing cells on medium containing 2% galactose and

1% raffinose (Golemis et al., 1994). The EGY48 host strain, which contained the pSH18-34 lacZ reporter plasmid, was cotransformed with pEG202 and pJG4-5 constructs and potential interactions were tested by streaking transformants in replica on medium lacking leucine to test for leucine prototrophy and expression of β-gal by growing cells on X-gal-containing medium, as previously described (Jones et al., 1998). Due to transcriptional activation by LexA-Pho, reciprocal tests were not possible.

### In Vitro Protein-Protein Interaction Assays

All pGEX constructs were made by PCR amplification of the indicated coding regions and ligated into the BglII-XhoI sites of the pGEX-BgRP3i vector (Jones et al., 1998). All pGEX-E(z) constructs and pGEX-Esc and pGEX-EZH1 have been previously described (O'Connell et al., 2001; Wang et al., 2002). pGEX-Pho and pGEX-Phol constructs were derived from pJG4-5-Pho and pET28a-*phol* cDNA clones, respectively. The inserts of pGEX-YY1-204-227 and pGEX-EZH2 were amplified from cDNA clones kindly provided by Dr. Michael Atchison and Dr. Haiming Chen, respectively. Expression and purification of GST-fusion proteins and in vitro pull-down assays were performed as previously described (Jones et al., 1998) (O'Connell et al., 2001). Radiolabeled full-length E(z), Pho, and Phol proteins were synthesized by in vitro transcription-translation of *E(z)* cDNA clone e32-55.26 (Jones et al., 1998), the *pho* cDNA clone T7-link-*pho* (Fritsch et al., 1999), and the *phol* cDNA clone pET28a-*phol*, respectively, using T7 RNA polymerase with the TNT-coupled reticulocyte lysate system (Promega) and [$^{35}$S]methionine. SP6 RNA polymerase was used to produce $^{35}$S-Esc from *esc* clone e2 (Simon et al., 1995).

### Antibodies

Affinity-purified rabbit anti-E(z) and anti-histone H3-trimethyl-K27 (anti-H3mK27) antibodies have been previously described (Carrington and Jones, 1996; Plath et al., 2003, respectively). Rabbit anti-Pc antisera was raised against a $His_6$-fusion protein containing Pc residues 191–354 (injections and bleeds were performed at Covance Research Products, Inc.). Anti-Pc antibodies were affinity purified using the same fusion protein. Rabbit anti-Phol antisera was raised against a $His_6$-fusion protein containing Phol residues 1–196. Anti-Phol antibodies were affinity purified using the same fusion protein. Anti-Pho antibodies were affinity purified from previously described crude rabbit antisera (Brown et al., 2003) using a GST-fusion protein containing Pho residues 1–356. All affinity purifications were performed essentially as previously described (O'Connell et al., 2001), except that anti-Pc and anti-Phol antisera were first preadsorbed against total *E. coli* proteins that included $His_6$-dSAP18 (Wang et al., 2002) and anti-Pho antisera was first preadsorbed against total *E. coli* proteins that included GST, respectively, attached to CNBr-activated Sepharose (Pharmacia). Rabbit anti-histone H3 antibodies were purchased from Upstate Biotechnology, Inc.

### Tissue Culture and RNA Interference

SL2 cells (Invitrogen) were grown in SD medium supplemented with 10% fetal bovine serum (Gibco). For Western blots, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 10 mM NaF, 1% NP40, 0.1% SDS, 0.4 mM EDTA, 10% glycerol [pH 8.0]), the lysate centrifuged for 20 min, and the protein concentration in the supernatant determined using the BCA protein assay reagent kit (Pierce). We loaded 50 μg of protein in each lane of 8% SDS-PAGE gels. For RT-PCR, RNA was extracted from cells using Trizol Reagent (Invitrogen). We used 5 μg of total RNA for first strand cDNA synthesis using Superscript II (Invitrogen) and oligo(dT) primers. Following heat inactivation of reverse transcriptase, equal aliquots (1/20 of total sample) were used as templates for PCR with Taq polymerase (Promega) and the following gene-specific primers: *Ubx*, 5′-CGAG GAAATCCGTCAGCAGAC-3′ and 5′-CAGAGTAACCAATTTGTTTTT CAC-3′; *Abd-B*, 5′-CTCCCCTCGCAATTACCAAAGG-3′ and 5′-TGC CGTGTGCCGCTTGACCG-3′; *RpII140*, 5′-CCTGCTGGATCGTGAT TAACGC-3′ and 5′-GTTGATGATGAAGTAGCCACCG-3′. These are identical to primers used in a previous study (Breiling et al., 2001; A. Breiling, personal communication). PCR protocol: 94°C for 2 min, once; 50°C for 30 s, 72°C for 60 s, 94°C for 30 s, 30 times; 72°C for 5 min, once.

### RNAi

The templates for synthesizing *pho*, *E(z)*, and *Pc* dsRNA were produced by RT-PCR amplification of transcripts from 0–24 hr *Drosophila* embryos. The template for synthesizing GFP dsRNA was produced by PCR amplification of GFP coding sequence from pcDNA3.1-GFP (Invitrogen). Gene-specific primers, each of which included T7 promoter sequences at their 5′ ends, were used to amplify the following exonic sequences: *pho*, 801 bp extending from 116 bp upstream of the ATG to 685 bp downstream; *E(z)*, 650 bp extending from 28 to 678 bp downstream of the ATG; *Pc*, 935 bp extending from 120 to 955 bp downstream of the ATG; GFP, 662 bp extending from 25 to 687 bp downstream of the ATG. Synthesis of dsRNA and transfections were performed as previously described (Cao et al., 2002).

### *Drosophila* Cultures

Homozygous $E(z)^{61}$ stocks were maintained at 18°C; all others were maintained at 25°C. All stocks were brooded daily. For temperature shift experiments, bottles containing $E(z)^{61}$ larvae were transferred to a 29°C incubator 24 hr or 48 hr prior to dissection. *pho* homozygous (*y*) larvae were selected from a *y* $Df(1)w^{67c23}$; $pho^{1}/Dp(1;4)1021$ stock. *phol* homozygous ($Tb^{+}$) larvae were selected from a $phol^{81A}/TM6B,Tb$ stock. *phol pho* double mutants ($Tb^{+}$ *y*) were selected from a *y* $Df(1)w^{67c23}$; $phol^{81A}/TM6B,Tb$; $pho^{1}/Dp(1;4)1021$, $y^{+}$ *spa* stock. The genotypes of $pho^{1}$, $phol^{81A}$, and $phol^{81A}$; $pho^{1}$ wing imaginal discs used in ChIP assays were confirmed by PCR amplification of DNA from lysed pooled discs (following cell lysis but prior to the sonication step in ChIP assays) using allele-specific primers (Brown et al., 2003).

### ChIP Assays

Immunoprecipitations of formaldehyde-crosslinked chromatin from SL2 cells and wing imaginal discs were performed using the ChIP assay kit (Upstate Biotechnology) essentially as previously described (Cao et al., 2002). Wing imaginal discs were dissected in serum-free SS3 medium, pooled, and stored on ice prior to fixation. The equivalent of one imaginal disc was used per PCR reaction per antibody, including equal aliquots for mock ChIPs and genomic DNA controls. In order to reduce nonspecific background and thus improve sensitivity in wing disc ChIP assays, affinity-purified antibodies or crude rabbit preimmune antisera (for mock immunoprecipitations) were preadsorbed to protein A agarose prior to incubation with sonicated lysates. The storage buffer, including sonicated salmon sperm DNA, was removed from the protein A agarose and reserved. Antibodies or preimmune antisera were incubated with protein A agarose in PBS for 1 hr at 4°C, thoroughly washed with PBS, and the original salmon sperm DNA-containing storage buffer replaced prior to use. Antibody-bead conjugates were incubated with sonicated lysates overnight at 4°C, then washed and processed as usual. For experiments involving 6 or fewer PCR reactions per antibody, 20 μl of the protein A agarose suspension was used per antibody. For experiments involving more than 6 PCR reactions per antibody, 30 μl of the protein A agarose suspension was used. The following amounts of antibodies were incubated with 20 or 30 μl of protein A agarose beads, respectively: anti-E(z), 10 or 20 μl; anti-Pho, 10 or 20 μl; anti-Pc, 10 or 20 μl; anti-Phol, 15 or 30 μl; anti-H3mK27, 5 μl (all experiments); preimmune antisera, 5 or 10 μl. Pairs of PCR primers (each 20 nt in length) were used to amplify the following segments of the region encompassing the *Ubx* transcription start site (p) and the *bxd* region (b) that includes $PRE_D$ (coordinates refer to nucleotide positions presented in Martin et al. [1995]); Genbank entry U31961): p1, 243702–243264; p2, 243277–242767; p3, 242939–242486; p4, 242524–241898; b1, 221467–220797; b2, 220772–220275; b3, 220290–219758; b4, 219588–219125; b5, 219283–218799; b6, 218818–218319; b7, 218351–218070; b8, 218094–217555; b9, 217459–217050. Based on previous studies, the *Ubx* transcription start site is positioned at coordinate 242868 (Saari and Bienz, 1987) and $PRE_D$ extends from 219375 to 218818 (Fritsch et al., 1999).

### Acknowledgments

We thank Mark Mortin for helpful comments on the manuscript and we thank Judith Benes for excellent technical assistance. We thank

Jeff Simon, Michael Atchison, Haiming Chen, and Rob Harrod for providing plasmids and we thank Rob White for providing anti-Ubx antibody. We also thank Rob Harrod for assistance with confocal microscopy. This work was supported by NIH grants GM46567 (to R.S.J.) and GM068804 (to Y.Z.).

Received: March 11, 2004
Revised: April 28, 2004
Accepted: May 3, 2004
Published: June 3, 2004

## References

Ahmad, K., and Henikoff, S. (2002). The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol. Cell *9*, 1191–1200.

Atchison, L., Ghias, A., Wilkinson, F., Bonini, N., and Atchison, M.L. (2003). Transcription factor YY1 functions as a PcG protein in vivo. EMBO J. *22*, 1347–1358.

Bajusz, I., Sipos, L., Gyorgypal, Z., Carrington, E.A., Jones, R.S., Gausz, J., and Gyurkovics, H. (2001). The *Trithorax-mimic* allele of *Enhancer of zeste* renders active domains of target genes accessible to Polycomb-group dependent silencing in *Drosophila melanogaster*. Genetics *159*, 1135–1150.

Beuchle, D., Struhl, G., and Müller, J. (2001). Polycomb group proteins and heritable silencing of *Drosophila* Hox genes. Development *128*, 993–1004.

Breiling, A., Turner, B.M., Bianchi, M.E., and Orlando, V. (2001). General transcription factors bind promoters repressed by Polycomb group proteins. Nature *412*, 651–655.

Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.-L., and Kassis, J.A. (1998). The *Drosophila* Polycomb group gene *pleiohomeotic* encodes a sequence-specific DNA binding protein with homology to the multifunctional mammalian transcription factor YY1. Mol. Cell *1*, 1057–1064.

Brown, J.L., Frisch, C., Müller, J., and Kassis, J.A. (2003). The *Drosophila pho-like* gene encodes a YY1-related DNA binding protein that is redundant with *pleiohomeotic* in homeotic gene silencing. Development *130*, 285–294.

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

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

Busturia, A., Lloyd, A., Bejarano, F., Zavortink, M., Xin, H., and Sakonju, S. (2001). The MCP silencer of the *Drosophila Abd-B* gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression. Development *128*, 2163–2173.

Cabrera, C.V., Botas, J., and Garcia-Bellido, A. (1985). Distribution of *Ultrabithorax* proteins in mutants of *Drosophila bithorax* complex and its transregulatory genes. Nature *318*, 569–571.

Cao, R., and Zhang, Y. (2004). The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr. Opin. Genet. Dev. *14*, 155–164.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043.

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

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

Cohen, B., Wimmer, E.A., and Cohen, S.M. (1991). Early development of leg and wing primordia in the *Drosophila* embryo. Mech. Dev. *33*, 229–240.

Collins, R.T., Furukawa, T., Tanese, N., and Treisman, J.E. (1999). Osa associates with the Brahma chromatin remodeling complex and promotes the activation of some target genes. EMBO J. *18*, 7029–7040.

Crosby, M.A., Miller, C., Alon, T., Watson, K.L., Verrijzer, C.P., Goldman-Levi, R., and Zak, N.B. (1999). The trithorax group gene *moira* encodes a brahma-associated putative chromatin-remodeling factor in *Drosophila melanogaster*. Mol. Cell. Biol. *19*, 1159–1170.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). *Drosophila* enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185–196.

Eissenberg, J.C. (2001). Molecular biology of the chromo domain: an ancient chromatin module comes of age. Gene *275*, 19–29.

Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev. *17*, 1870–1881.

Francis, N.J., Saurin, A.J., Shao, Z., and Kingston, R.E. (2001). Reconstitution of a functional core Polycomb repressive complex. Mol. Cell *8*, 545–556.

Fritsch, C., Brown, J.L., Kassis, J.A., and Müller, J. (1999). The DNA-binding Polycomb group protein Pleiohomeotic mediates silencing of a *Drosophila* homeotic gene. Development *126*, 3905–3913.

Fritsch, C., Beuchle, D., and Müller, J. (2003). Molecular and genetic analysis of the Polycomb group gene *Sex combs extra/Ring* in *Drosophila*. Mech. Dev. *120*, 949–954.

Garcia-Bellido, A., and Merriam, J.R. (1971). Parameters of the wing imaginal disc development of *Drosophila melanogaster*. Dev. Biol. *24*, 61–87.

Glicksman, M.A., and Brower, D.L. (1988a). Misregulation of homeotic gene expression in *Drosophila* larvae resulting from mutations at the *extra sex combs* locus. Dev. Biol. *126*, 219–227.

Golemis, E.A., Gyuris, J., and Brent, R. (1994). Two hybrid systems/interaction traps. In Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J.A. Smith, and K. Struhl, eds. (New York: John Wiley & Sons), pp. 13.14.11–13.14.17.

Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998). SET domain proteins modulate chromatin domains in eu- and heterochromatin. Cell. Mol. Life Sci. *54*, 80–93.

Jones, R.S., and Gelbart, W.M. (1990). Genetic analysis of the *Enhancer of zeste* locus and its role in gene-regulation in *Drosophila melanogaster*. Genetics *126*, 185–199.

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

Kal, A.J., Mahmoudi, T., Zak, N.B., and Verrijzer, C.P. (2000). The *Drosophila* Brahma complex is an essential coactivator for the trithorax group protein Zeste. Genes Dev. *14*, 1058–1071.

King, I.F.G., Francis, N.J., and Kingston, R.E. (2002). Native and recombinant Polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro. Mol. Cell. Biol. *22*, 7919–7928.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. *16*, 2893–2905.

Lavigne, M., Francis, N.J., King, I.F.G., and Kingston, R.E. (2004). Propagation of silencing: recruitment and repression of naive chromatin in *trans* by Polycomb repressed chromatin. Mol. Cell *13*, 415–425.

Martin, C.H., Mayeda, C.A., Davis, C.A., Ericsson, C.L., Knafels, J.D., Mathog, D.R., Celniker, S.E., Lewis, E.B., and Palazzolo, M.J. (1995). Complete sequence of the bithorax complex of *Drosophila*. Proc. Natl. Acad. Sci. USA *18*, 8398–8402.

Min, J., Zhang, Y., and Xu, R.-M. (2003). Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. Genes Dev. *17*, 1823–1828.

Mishra, R.K., Mihaly, J., Barges, S., Spierer, A., Karch, F., Hagstrom, K., Schweinsberg, S.E., and Schedl, P. (2001). The *iab-7* polycomb response element maps to a nucleosome-free region of chromatin and requires both GAGA and pleiohomeotic for silencing activity. Mol. Cell. Biol. *21*, 1311–1318.

Mohd-Sarip, A., Venturini, F., Chalkley, G.E., and Verrijzer, C.P. (2002). Pleiohomeotic can link Polycomb to DNA and mediate transcriptional repression. Mol. Cell. Biol. *22*, 7473–7483.

Mulholland, N.M., King, I.F.G., and Kingston, R.E. (2003). Regulation of Polycomb group complexes by the sequence-specific DNA binding proteins Zeste and GAGA. Genes Dev. *17*, 2741–2746.

Müller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a *Drosophila* Polycomb group repressor complex. Cell *111*, 197–208.

O'Connell, S., Wang, L., Robert, S., Jones, C.A., Saint, R., and Jones, R.S. (2001). Polycomblike PHD fingers mediate conserved interaction with Enhancer of zeste protein. J. Biol. Chem. *276*, 43065–43073.

Papoulas, O., Beek, S.J., Moseley, S.L., McCallum, C.M., Sarte, M., Shearn, A., and Tamkun, J.W. (1998). The *Drosophila* trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. Development *125*, 3955–3966.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131–135.

Poux, S., Melfi, R., and Pirrotta, V. (2001). Establishment of Polycomb silencing requires a transient interaction between PC and ESC. Genes Dev. *15*, 2509–2514.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.-W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., et al. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593–599.

Saari, G., and Bienz, M. (1987). The structure of the *Ultrabithorax* promoter of *Drosophila melanogaster*. EMBO J. *6*, 1775–1779.

Satijn, D.P.E., Hamer, K.M., den Blaauwen, J., and Otte, A.P. (2001). The Polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in *Xenopus* embryos. Mol. Cell. Biol. *21*, 1360–1369.

Saurin, A.J., Shao, Z., Erdjument-Bromage, H., Tempst, P., and Kingston, R.E. (2001). A *Drosophila* Polycomb group complex includes Zeste and dTAFII proteins. Nature *412*, 655–660.

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C.T., Bender, W., and Kingston, R.E. (1999). Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell *98*, 37–46.

Simon, J.A., and Tamkun, J.W. (2002). Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. Curr. Opin. Genet. Dev. *12*, 210–218.

Simon, J., Chiang, A., Bender, W., Shimell, M.J., and O'Connor, M. (1993). Elements of the *Drosophila* bithorax complex that mediate repression by Polycomb group products. Dev. Biol. *158*, 131–144.

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

Smith, S.T., Petruk, S., Sedkov, Y., Cho, E., Tillib, S., Canaani, E., and Mazo, A. (2004). Modulation of heat shock gene expression by the TAC1 chromatin modifying complex. Nat. Cell Biol. *6*, 162–167.

Struhl, G., and White, R.A.H. (1985). Regulation of the *Ultrabithorax* gene of *Drosophila* by other *bithorax* complex genes. Cell *43*, 507–519.

Tie, F., Furuyama, T., Prasad-Sinha, J., Jane, E., and Harte, P.J. (2001). 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. Development *128*, 275–286.

Vazquez, M., Moore, L., and Kennison, J.A. (1999). The trithorax group gene *osa* encodes an ARID-domain protein that genetically interacts with the brahma chromatin-remodeling factor to regulate transcription. Development *126*, 733–742.

Wang, L., Ding, L., Jones, C.A., and Jones, R.S. (2002). *Drosophila* Enhancer of zeste protein interacts with dSAP18. Gene *285*, 119–125.