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Dynamic Regulation by Polycomb Group Protein Complexes Controls Pattern Formation and the Cell Cycle in *Drosophila*

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SUMMARY

Polycomb group (PcG) proteins form conserved regulatory complexes that modify chromatin to repress transcription. Here, we report genome-wide binding profiles of PhoRC, the *Drosophila* PcG protein complex containing the DNA-binding factor Pho/dYY1 and dSfmbt. PhoRC constitutively occupies short Polycomb response elements (PREs) of a large set of developmental regulator genes in both embryos and larvae. The majority of these PREs are co-occupied by the PcG complexes PRC1 and PRC2. Analysis of PcG mutants shows that the PcG system represses genes required for anteroposterior, dorsoventral, and proximodistal patterning of imaginal discs and that it also represses cell cycle regulator genes. Many of these genes are regulated in a dynamic manner, and our results suggest that the PcG system restricts signaling-mediated activation of target genes to appropriate cells. Analysis of cell cycle regulators indicates that the PcG system also dynamically modulates the expression levels of certain genes, providing a possible explanation for the tumor phenotype of PcG mutants.

INTRODUCTION

Polycomb group (PcG) genes encode an evolutionary conserved set of transcriptional repressors that are essential for the development of animals and plants (reviewed in Schubert et al., 2005; Pien and Grossniklaus, 2007; Schwartz and Pirrotta, 2007; Schuettengruber et al., 2007). PcG genes were originally identified in *Drosophila* as a set of loci that share a common mutant phenotype, the misexpression of multiple HOX genes in cells where they are normally inactive (Duncan, 1982; Jürgens, 1985). To date, HOX genes are the best characterized target genes of the PcG system in *Drosophila* but over the years several additional genes have been found to be misexpressed in PcG mutants (Dura and Ingham, 1988; Busturia and Morata, 1988; Beuchle et al., 2001; Maurange and Paro, 2002; Zirin and Mann, 2004; Janody et al., 2004). In vertebrates, PcG proteins also repress HOX genes, but early studies showed that mammalian PcG proteins also repress the cell cycle regulator p16/INK4a (Jacobs et al., 1999). More recently, genome-wide PcG protein binding profiles in mammalian tissue culture cells (Kirmizis et al., 2004; Bracken et al., 2006; Squazzo et al., 2006) and in embryonic stem cells (Boyer et al., 2006; Lee et al., 2006) showed that these proteins bind to a large number of developmental control genes. These studies led to the proposal that PcG repression of developmental control genes may be required for maintaining stem cell pluripotency (Boyer et al., 2006; Lee et al., 2006), a view that needs to be kept in perspective because embryonic stem cells lacking PcG proteins maintain their pluripotent state for many cell generations (Chamberlain et al., 2008).

Biochemical analyses of PcG proteins revealed that they exist in distinct multimeric protein complexes that contain two or more different PcG proteins (reviewed in Müller and Kassis, 2006; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). Three principal PcG protein complexes have been characterized to date: Polycomb repressive complex 1 (PRC1), containing E3 ligase activity for ubiquitylation of histone H2A at lysine 119 (Shao et al., 1999; Wang et al., 2004a; Buchwald et al., 2006); Polycomb repressive complex 2 (PRC2), a histone methyltransferase that methylates H3 at lysine 27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002); and Pho repressive complex (PhoRC) (Klymenko et al., 2006). Of these three complexes, only PhoRC contains a sequence-specific DNA-binding protein that is essential for PcG repression: Pho, the *Drosophila* homolog of YY1 (Brown et al., 1998; Klymenko et al., 2006). Nevertheless, at least at HOX genes in *Drosophila*, all three complexes cobind in a sharply localized manner to specific DNA sequences that are called Polycomb response elements (PREs) (Papp and Müller, 2006). Targeting of PRC1 and PRC2 to HOX gene PREs requires Pho function, as seen in *pho* mutant animals (Wang et al., 2004b) or upon disruption of Pho DNA-binding sites (Klymenko et al., 2006). In addition to Pho, a variety of other DNA-binding proteins have been proposed to function as PRC1 and/or PRC2 recruitment factors at *Drosophila* PREs (reviewed in Müller and Kassis, 2006). However, mutants lacking those proteins do not show misexpression of HOX genes or other PcG phenotypes (reviewed in Müller and Kassis, 2006). In vertebrates, YY1 has been reported to interact with PRC2 (Satijn et al., 2001) and has been implicated in PRC2 recruitment to certain target genes (Caretti et al., 2004), while

a noncoding RNA has been implicated in PRC2 targeting to HOX genes (Rinn et al., 2007).

Recent studies reported genome-wide binding profiles of *Drosophila* PRC1 and PRC2 components (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006; reviewed in Ringrose and Paro, 2007). Specifically, Schwartz et al. (2006) reported that, in tissue culture cells, PRC1 and PRC2 components tightly colocalize at about 200 genes in the entire *Drosophila* genome. Studies using the DamID technique in tissue culture cells also found a good correlation between regions occupied by PRC1 and PRC2 components (Tolhuis et al., 2006). Negre et al. (2006), finally, analyzed binding of PRC1 components in *Drosophila* embryos, pupae and adults, using an array platform that covered approximately 10% of the genome. This study revealed a strong coincidence between binding sites for two different PRC1 components (Negre et al., 2006). Taken together, these studies thus provided a comprehensive catalog of PRC1 and PRC2 binding sites in tissue culture cells and a first partial map of PRC1 binding sites in developing *Drosophila*.

Although these studies made important contributions toward identifying possible PcG target genes, many central questions remain unresolved. First, the PRC1- and PRC2-bound regions identified in ChIP-chip studies (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006) show only a limited overlap with in silico PRE predictions based on the presence of binding site motifs for Pho and other DNA-binding proteins (Ringrose et al., 2003). This raises the question where the DNA-binding PcG complex PhoRC binds. Second, apart from the few well-characterized PREs that have been analyzed to date, there is only limited information about the DNA sequences that are bound by PcG protein complexes, and the sequence motifs that make up PREs are thus still largely elusive. Finally, and perhaps most importantly, even though PRC1 and PRC2 were found to bind at a large number of genes, it is still largely unknown if and how these genes are regulated by the PcG system. Here, we determined the genome-wide binding profile of PhoRC at different stages of *Drosophila* development. Our study identifies a large set of genomic sites where PhoRC is constitutively bound together with PRC1 and PRC2, establishing PhoRC as a core-PRE-binding complex. Functional analyses in PcG mutants reveal that the PcG system represses many of the identified target genes and, unexpectedly, that PcG repression at several of these genes is dynamic and can be overcome in response to extracellular signaling.

RESULTS

To identify genes that are bound by PhoRC in developing *Drosophila*, we generated genome-wide binding profiles of two PhoRC components: Pho and dSfmbt. Specifically, we analyzed binding of Pho protein in 6- to12-hour-old embryos by performing chromatin immunoprecipitation (ChIP) assays with two independent antisera that had been raised against distinct portions of the Pho protein. Only genomic regions significantly enriched by both anti-Pho antibodies were considered. In parallel, we also analyzed Pho and dSfmbt binding at later developmental stages. To this end, we performed ChIP assays with the two Pho antisera and with an antibody against dSfmbt in imaginal disc cells isolated from third instar larvae. In all cases, the immunoprecipitated material was hybridized to high-density whole-genome tiling arrays and analyzed using TileMap (Ji and Wong, 2005) to identify a high-confidence set of Pho- and dSfmbt-bound regions, using a stringent cutoff (see Experimental Procedures).

PhoRC Binding at Target Genes Is Sharply Localized

These experiments resulted in three high resolution in vivo binding maps of the two PhoRC complex components: 407 genomic regions bound by Pho in embryos (Pho^E; see Table S1 available online), 670 regions bound by Pho in larval imaginal discs (Pho^L; Table S2) and 666 regions bound by dSfmbt in larval imaginal discs ($dSfmbt^L$; Table S3). In all three binding profiles, the genomic intervals with enriched binding were on average 1100–1600 bp in length with few regions bound by either protein across more extended chromosomal regions (Figure 1A). Pho and dSfmbt are thus more frequently bound at discrete short *cis*-regulatory sequences.

Comparison of Pho^E and Pho^L binding profiles identified 236 regions in common (Figure 1B), corresponding to chromosomal sequences where Pho is constitutively bound during embryonic and larval stages. To identify regions specifically bound by PhoRC, as opposed to Pho alone, we next compared the binding profiles in Pho^L versus $dSfmbt^L$, which revealed that 50% of regions are cobound by both Pho and dSfmbt proteins in larvae (Figure 1C). These 338 regions thus represent sequences bound by the PhoRC complex in imaginal discs. Importantly, among those, 196 regions (58%) correspond to regions also bound by Pho in embryos, suggesting that these regions are occupied by PhoRC throughout development. We refer to these 196 regions as the core-PhoRC binding sites (Table S4). Notably, these core-PhoRC sites include previously characterized PREs in the Bithorax complex (BXC), *engrailed* and *polyhomeotic* genes (Figure S1).

Genome-wide Cotargeting of PhoRC, PRC1, and PRC2

To determine the overlap between PhoRC, PRC1, and PRC2 target sites, we compared the 196 core-PhoRC binding sites with PRC1- and PRC2-bound regions identified through genome-wide binding studies in *Drosophila* S2 cells (Schwartz et al., 2006; M. Biggin and V. Pirrotta, personal communication). Considering the differences in experimental conditions (developing embryos and larvae presented here versus tissue culture cell lines), there is a significant degree of overlap: 88 of the 196 core-PhoRC binding sites were also identified as bound by PRC1 and PRC2 (45%) (Figure S2A). A substantial number of core-PhoRC binding sites thus correspond to PRC1 and PRC2 target sites, supporting the view that PhoRC is a central PRE-binding complex.

An Extended Pho-Binding Motif as PRE Signature

We next asked whether the chromosomal intervals identified by Pho or dSfmbt ChIP-chip are enriched for particular DNA sequence motifs. To this end, we performed de novo sequence motif discovery on the Pho^E-, Pho^L-, $dSfmbt^L$-bound regions and on the 196 core-PhoRC regions. Several 8-mers, based on a GCCAT core were significantly overrepresented in the Pho datasets and were used as the basis to reconstruct a position sequence-specific matrix (PSSM; Figure 2A). This sequence, $G^C/_A{}^C/_G GCCAT^T/_C TT$, closely matches the motif previously

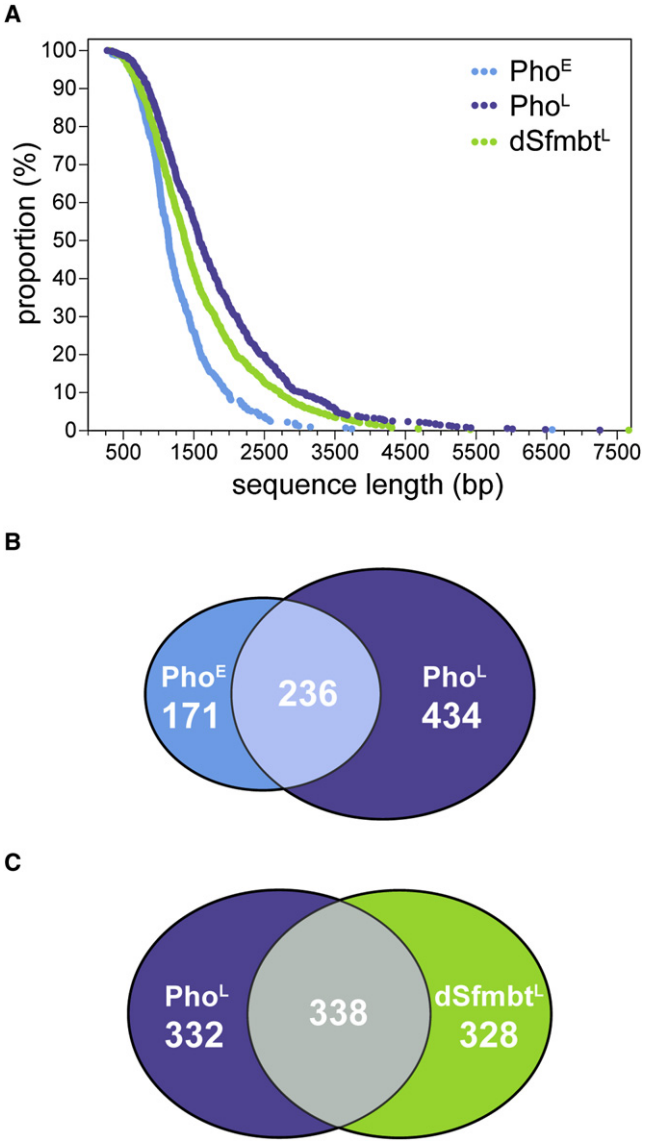


Figure 1. Pho and dSfmbt Cobind Short *cis*-Regulatory Regions in Embryos and Imaginal Discs

(A) Cumulative size distribution of TileMap reported regions bound by Pho or dSfmbt. Pho^E, Pho^L, and $dSfmbt^L$ data sets are indicated in blue, purple, and green, respectively. A position (x,y) on the curve represents the proportion y (in percent) of regions of size x or more. Note that the median size (obtained for y = 50%) of regions enriched by ChIP lies between 1100 and 1600 bp for all three data sets. Not more than 10% of the regions are longer than 3kb.

(B) Venn diagram showing the overlap of regions bound by Pho in embryos (Pho^E) and in imaginal discs (Pho^L).

(C) Venn diagram showing the overlap of regions cobound by Pho (Pho^L) and dSfmbt ($dSfmbt^L$) in imaginal discs.

identified in vitro as an optimal Pho-binding site using electro mobility shift assays (Brown et al., 1998; Fritsch et al., 1999; Shimell et al., 2000; Mishra et al., 2001). However, our in vivo binding data suggest a more extensive Pho-binding motif containing an additional Guanine nucleotide at the −3 position and a Thymine pair at positions +7 and +8 (Figure 2A). This extended Pho-binding motif is enriched in all data sets (Figure 2B). However, it is noteworthy that we failed to identify the Pho-binding site as an overrepresented motif in the subset of $dSfmbt^L$ regions not bound by Pho (i.e., the 328 $dSfmbt^L$ regions shown in Figure 1C) (data not shown). The lack of Pho enrichment by ChIP and the absence of detectable Pho binding sites in this fraction of the $dSfmbt^L$ dataset indicate that dSfmbt is targeted to these regions independently of Pho.

Having generated this refined motif, we next assayed the number of Pho motifs per PRE. While many regions among the 196 core-PhoRC binding sites contain multiple Pho-binding motifs (Figure 2C), several bound regions contain only a single recognizable Pho-binding motif. Previous in vitro studies with recombinant proteins reported that Pho directly recruits PRC1 to the *bxd* PRE in the HOX gene *Ubx*, and it was proposed that cooperative assembly of such a "silenceosome" requires two appropriately juxtaposed and oriented Pho binding sites (Mohd-Sarip et al., 2005, 2006). However, when we measured the distance between Pho-binding motifs in our Pho^E and Pho^L data sets, we were unable to detect any specific constraints for distance or strand orientation between computationally identified GCCAT motifs (data not shown). It is possible that Pho bound at canonical sites cooperates with Pho bound to sites with imperfect matches to this consensus to recruit PRC1 by the mechanism proposed by Mohd-Sarip et al. (2005, 2006). Alternatively, there may be multiple modes of PRC1 recruitment by PhoRC and/or other factors (e.g., Horard et al., 2000) and the mechanism may differ, depending on sequence context.

In addition, we also compared PhoRC datasets with the reported binding profiles for Zeste in *Drosophila* embryos (Moses et al., 2006) and GAGA factor/Trithorax-like (GAF/Trl) in S2 cells (Lee et al., 2008). Previous studies had implicated Zeste and GAF/Trl in PRE binding (reviewed in Müller and Kassis, 2006). We found significant overlap in the PhoRC and GAF/Trl binding profiles (Figure S2B) but only little overlap in the Pho and Zeste binding profiles (Figure S2C). GAF/Trl but not Zeste thus appears to be bound at many PhoRC-bound PREs. In concordance with these binding data, we also identified GAF/Trl-binding motifs (GAGAG) enriched in PhoRC regions using de novo motif discovery (data not shown). Finally, we used a pattern-match approach to examine PhoRC data sets for enrichment of binding motifs for Grainyhead (Grh), Dsp1, and Sp1/KLF, all of which had been reported to bind to PRE DNA in case studies (Brown et al., 2005; Dejardin et al., 2005; Blastyák et al., 2006). Grh binding sites are enriched in all PhoRC bound regions (Figure S2D), but we have not been able to identify this motif by de novo discovery. Dsp1 and Sp1/KLF binding sites have low sequence complexity; they are present in PhoRC-bound regions but not enriched (Figure S2D). In summary, these in silico analyses identified a refined and extended Pho-binding site together with GAF/Trl binding sites as the signature of PhoRC-bound PREs.

Most PREs Are Located Close to Transcription Start Sites

By analyzing the location of the Pho^E-, Pho^L-, and $dSfmbt^L$-bound regions with respect to transcription start sites (TSS), we found that in all three cases the majority of the regions lies within ±1 kb from the nearest TSS and that there is a significant enrichment of binding between ±500 bp compared to a random dataset (Figures S3 and 2D).

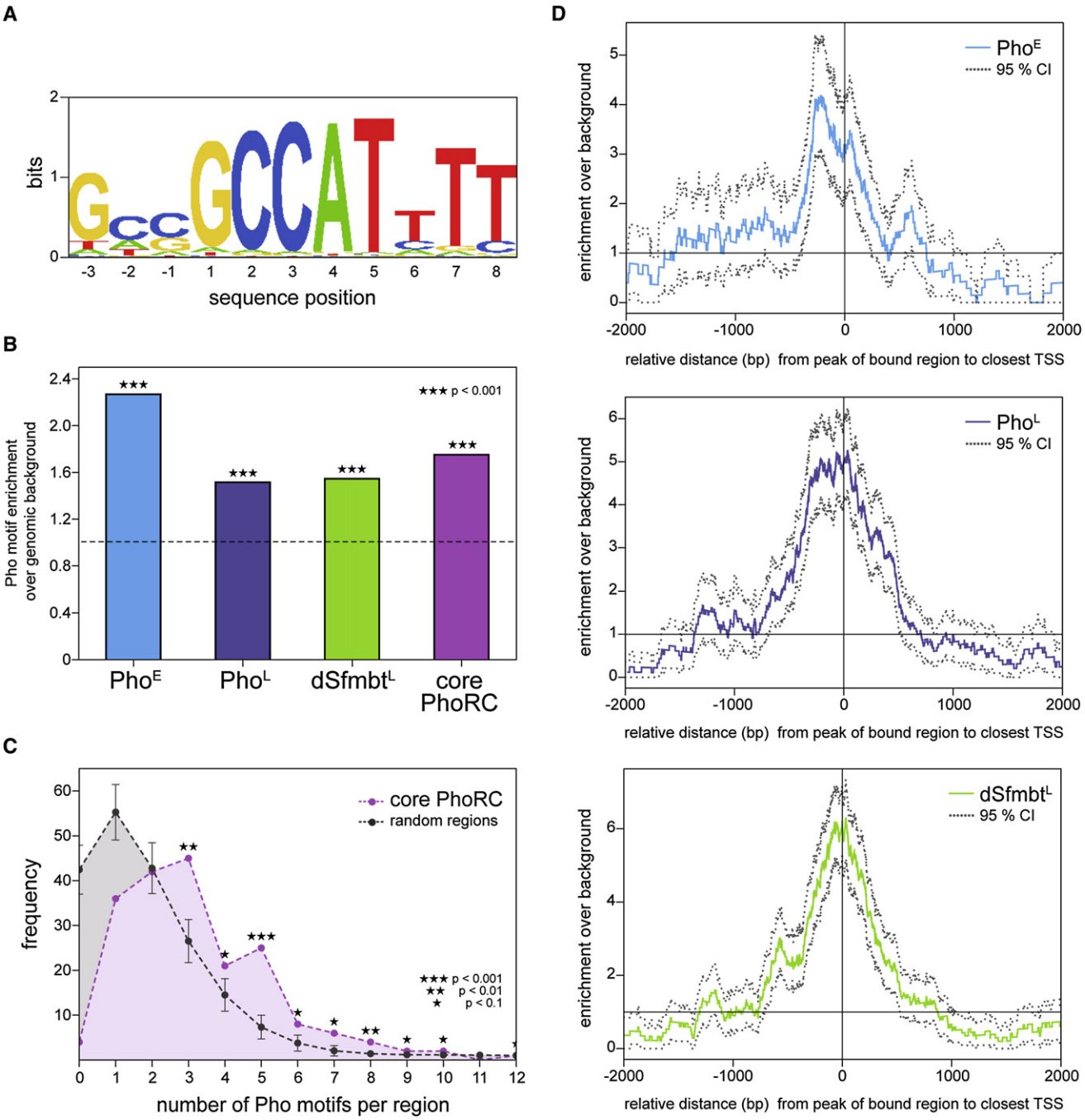


Figure 2. Properties of PhoRC-Bound PREs

(A) Identification of an extended Pho-binding motif in genomic regions bound by Pho in ChIP. Depicted is the sequence logo generated from a position sequence-specific matrix (PSSM). See Supplemental Data for details.

(B) Pho-binding site prediction enrichment in bound regions in the Pho^E (blue), Pho^L (purple), dSfmbt^L (green), and core-PhoRC (magenta) datasets. Enrichment in Pho-binding site prediction is computed as the ratio of the PSSM (presented above) match frequency in the dataset under test and the global genome frequency. Significance is assessed using a binomial test (right test, 95% confidence). PhoRC-bound regions show more often Pho-binding sites than expected by chance.

(C) Number of Pho-binding site predictions per region in the core-PhoRC dataset (magenta) and in simulated random regions (gray). Significance is assessed using Monte Carlo simulation. Reported values for random data correspond to the mean and the standard deviation.

(D) Enrichment of bound region locations in the Pho^E (blue), Pho^L (purple), and dSfmbt^L (green) data sets with respect to the closest transcription start site (TSS). x axis represents distance to the closest TSS, and y axis represents enrichment over random expectation, assuming a uniform distribution of the positions in the genome. Smoothened estimate of the enrichment (moving average) in blue, purple, and green show enrichment over background (values greater than 1). 95% confidence intervals (CIs) in gray show significant enrichment for all three data sets in a zone of about ±500 bp around TSS.

The promoter-proximal location that we uncovered allowed us to readily link the PREs to their potential target genes. Genes encoding developmental regulators are highly overrepresented among PcG targets (Tables S1–S7). Noteworthy, the majority of these target genes contain a single PRE (Figure S4). Table S4 lists core-PhoRC target genes and indicates if they were previously identified as PcG targets in S2 cells (Schwartz et al., 2006).

PcG Control of Body Patterning through Repression of Developmental Regulators

We next explored whether and how the PcG system regulates the identified target genes. In a first set of experiments, we focused our analyses on the patterning genes *engrailed* (*en*), *apterous* (*ap*), *pannier* (*pnr*), *teashirt* (*tsh*), *Distall-less* (*Dll*), *even-skipped* (*eve*), and *Dorsocross* (*Doc*). These genes are bound by Pho in embryos, by PhoRC in imaginal discs, and by PRC1 and PRC2 in tissue culture cells (Tables S1–S4; Schwartz et al., 2006).

To validate and extend our microarray data, we performed ChIP assays followed by real-time quantitative PCR (qPCR) analysis to monitor binding of the PhoRC subunits Pho and dSfmbt, the PRC2 subunit Su(z)12 and the PRC1 subunits Pc and Ph at these genes in imaginal disc cells. At each gene, we analyzed binding of PhoRC, PRC1, and PRC2 at (1) the identified PRE(s) and (2) at a region within the transcribed portion of the gene. Additional euchromatic and heterochromatic sequences served as further controls (control 1 and 2). These assays revealed that not only PhoRC but also PRC1 and PRC2 are localized at the PRE of each of these genes (Figures 3 and 4). Like the PhoRC components Pho and dSfmbt, Ph and Su(z)12 are also specifically localized at PREs of these genes but not at the analyzed intervals of the coding regions (Figures 3 and 4). In contrast, Pc protein shows highest binding at PREs, but there is also significant low-level binding detected at the analyzed coding region in several target genes (Figure 3), consistent with earlier studies (Papp and Müller, 2006; Schwartz et al., 2006). Taken together, these results demonstrate that PhoRC, PRC1, and PRC2 are cobound to the PREs of each of these target genes in larval cells.

We next analyzed if PcG protein complexes repress transcription of these target genes. Previous studies reported misexpression of *en*, *eve*, and *tsh* in certain PcG mutants (Dura and Ingham, 1988; Busturia and Morata, 1988; Moazed and O'Farrell, 1992; Janody et al., 2004; Zirin and Mann, 2004). Here, we systematically removed different PhoRC, PRC1, or PRC2 subunits in larval cells and then tested whether target genes become misexpressed in cells where they are normally not expressed. Specifically, we generated clones of cells homozygous for a given PcG loss-of-function mutation in wing imaginal discs and analyzed mutant clone cells for misexpression of target genes by antibody staining (see Figure 3).

en, *ap*, *pnr*, *tsh*, and *Dll* all encode transcription factors that are expressed in specific compartments of the wing imaginal disc where their presence is essential for the correct patterning and growth of the disc (Figure 3, top row). *en* and *ap* control growth and patterning along the anteroposterior (A/P) and dorsoventral (D/V) axis, respectively, whereas *pnr*, *tsh*, and *Dll* control the subdivision of the wing disc along the proximodistal (P/D) axis to generate primordia for notum, hinge, and wing blade (reviewed in Mann and Morata, 2000). As illustrated in Figure 3, upon removal of PcG proteins, these target genes indeed become misexpressed in territories of the disc where they are normally not expressed. However, similar to HOX genes (Beuchle et al., 2001), the extent to which repression of these target genes is lost and the kinetics of derepression depend on which PcG protein is removed (Figures 3 and S5). Generally, *Psc-Su(z)2* or *ph* mutant clones show the most rapid and widespread loss of target gene repression, whereas removal of the other PRC1 subunits *Pc*, *Sce*, or *Scm* results in a more delayed loss of repression, and clones lacking the PRC2 components *E(z)* or *Su(z)12* show an even further delayed loss of repression (Beuchle et al., 2001; Birve et al., 2001). Nevertheless, we also found examples where target genes are strongly misexpressed in clones lacking certain PcG proteins but show no detectable misexpression in clones lacking other PcG proteins (see Figures 3 and S5 legends for details). This indicates that even though PhoRC, PRC1, and PRC2 are all cotargeted to these genes (Figure 3), certain PcG components appear to be less stringently required for their repression than others, at least in the tissues analyzed here.

This analysis uncovered another important aspect that is relevant for analyzing PcG regulation of target genes. We found that misexpression of certain target genes cannot be straightforwardly monitored in PcG single mutants because it is masked by other regulatory interactions. For example, the HOX proteins Ubx, Abd-A and Abd-B are known to repress *Dll* transcription in embryos (Vachon et al., 1992), and all three HOX proteins are strongly misexpressed in PcG mutant cells in the wing disc (Beuchle et al., 2001). Consequently, we found that PcG single-mutant clones show complete loss of *Dll* expression (Figures 3 and S5) and that misexpression of *Dll* in PcG mutant clones is only revealed in clones lacking both PcG and HOX gene functions (i.e., in clones of *Scm Ubx abd-A Abd-B* quadruple mutant cells, Figure 3). Similarly, ectopic expression of the HOX protein Ubx activates *tsh* expression outside its normal expression domain in embryos (Röder et al., 1992), raising the question whether the reported misexpression of *tsh* in PcG mutant clones in discs (Zirin and Mann, 2004) might be indirectly activated by misexpressed HOX proteins. However, repression of *tsh* is also lost in *Scm* mutant clones lacking the three HOX proteins Ubx, Abd-A, and Abd-B (Figure 3). Taken together with the in vivo binding data, misexpression of *tsh* in *Scm* mutant clones is thus most likely a direct consequence of loss of PcG repression at the *tsh* locus.

In summary, these experiments provide strong evidence that the PcG system not only controls the subdivision of segments into A/P compartments via *en* repression but also controls the D/V subdivision by repressing *ap*, and the P/D subdivision by repressing the *pnr*, *tsh* and *Dll* genes. Finally, we note that the transcription factor Eve, which is normally expressed in a subset of cells in the genital disc (Gorfinkiel et al., 1999), becomes misexpressed in *ph* and *Psc-Su(z)2* mutant clones in the wing disc (Figure 3).

Global, Tissue-Specific, and Cell-Type-Specific Repression by the PcG System

The misexpression of *tsh*, *eve*, and *Doc* in *ph* or *Psc-Su(z)2* mutant embryos shown in Figure 4 illustrates how the PcG system is

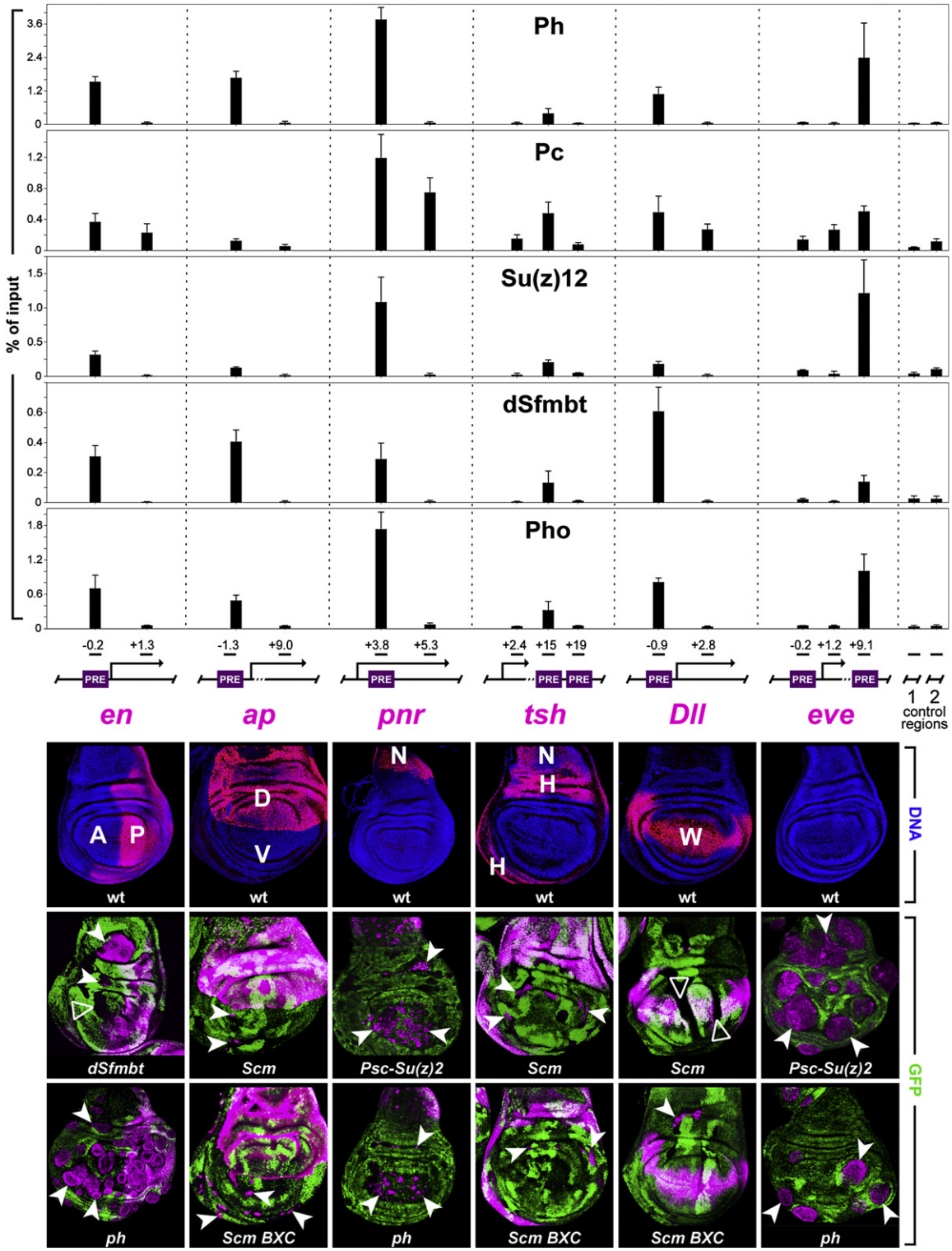


Figure 3. Control of *Drosophila* Limb Patterning by the PcG System

(Top) ChIP analysis at the *en*, *ap*, *pnr*, *tsh*, *Dll*, and *eve* loci in wild-type imaginal discs. Each bar shows the result from at least three independent immunoprecipitation reactions on independently prepared batches of chromatin, performed with the indicated antibodies against Ph, Pc, Su(z)12, dSfmbt, or Pho. ChIP signals at PREs (dark purple boxes) and other regions (distances from transcription start sites indicated in kb) are presented as percentage of input chromatin precipitated for each region, error bars correspond to standard deviation. Note that at *tsh*, PhoRC, PRC1, and PRC2 components are bound at the +15 kb PRE but not at the +19 kb PRE where these complexes are bound in embryos (compare to Figure 4). Similarly, at *eve*, PcG protein complexes are bound at the +9.1 kb PRE but not at the promoter proximal −0.2 kb PRE where these complexes are bound in embryos (compare to Figure 4).

used in different ways to restrict target gene expression. *tsh* is misexpressed in many different tissues in every segment in *ph* or *Psc-Su(z)2* mutants (Figure 4). This misexpression is probably activated by different tissue-specific enhancer-binding proteins that are present in every segment along the anteroposterior body axis but normally only activate *tsh* transcription within its appropriate expression domain. Consequently, *tsh* becomes globally expressed if PcG function is removed (Figure 4). At the *eve* locus, the situation is different. In *ph* or *Psc-Su(z)2* mutant embryos, *eve* is misexpressed in most if not all cells of the nervous system but there is only subtle misexpression in the epidermis and mesoderm (Figure 4, cf. Dura and Ingham, 1988). At *eve*, the primary function of the PcG system therefore seems to be to restrict expression to a specific subset of cells within a particular tissue, i.e., by preventing global neuronal-specific transcription factors from activating *eve* transcription throughout the nervous system. *Doc*, finally, is initially expressed in ectodermal and mesodermal cells on the dorsolateral side of wild-type embryos and expression subsequently becomes restricted to two pairs of cardioblast cells in the mesoderm of every segment (Figure 4; Reim et al., 2003). In *ph* or *Psc-Su(z)2* mutants, this restriction fails to occur, and *Doc* expression is maintained at high levels in the dorsolateral epidermis (Figure 4). At *Doc*, the PcG system is thus required to efficiently turn off transcription in a subset of cells in which it was initially active.

A final point worth noting in this context comes from the analysis of PRE occupancy at different developmental stages. At the *tsh* and *eve* loci, PcG protein complexes assemble at different PREs within these genes during embryonic and larval stages (Figures 3 and 4). This suggests that PcG complexes repress *tsh* and *eve* transcription through different PREs during these two distinct stages of the life-cycle (Figures 3 and 4).

PcG Control of Cell Division and Cell Growth

Clones of *Psc-Su(z)2* or *ph* mutant cells in imaginal discs show tumor-like phenotypes that are characterized by unrestricted cell proliferation and a failure to exit the cell cycle at the end of larval development (Beuchle et al., 2001). We wondered whether any of the identified PcG target genes might give us a better understanding of the molecular basis of this phenotype. To better characterize the tumor phenotype of *Psc-Su(z)2* or *ph* mutant

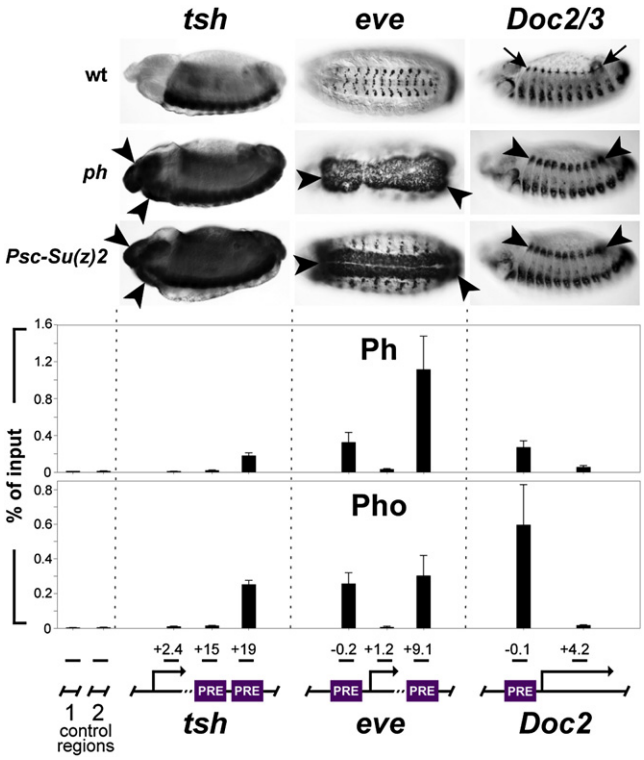


Figure 4. Global, Tissue- and Cell Type-Specific Regulation of Target Genes by the PcG System

(Top) Wild-type (wt, top row), *ph* mutant (middle row) and *Psc-Su(z)2* mutant (bottom row) embryos stained with antibodies against Tsh, Eve, or Doc2/3 protein as indicated. *ph* and *Psc-Su(z)2* mutants show global activation of *tsh* in all segments (left column, arrowheads mark misexpression in head), general activation of *eve* in the nervous system (arrowheads) but, apart from a few cells, not in the epidermis and mesoderm (middle column), and failure to restrict Doc expression (arrowheads) to the two pairs of cardioblast cells in the mesoderm of every segment (small arrows).

(Below) ChIP analysis to monitor Ph and Pho binding in wild-type embryos. Analysis was performed and results are presented as in Figure 3. Note, at *tsh*, Ph, and Pho are bound at the +19 kb PRE and not at the +15 kb PRE where PcG protein complexes are bound in discs (compare with Figure 3). At *eve*, PcG protein complexes are bound at the promoter proximal −0.2 kb PRE and at the +9.1 kb PRE (compare with Figure 3).

(Below) Misexpression of patterning genes in PcG mutant cell clones in wing imaginal discs. Row 1: discs from wild-type (wt) third instar larvae stained with antibodies to detect expression of *en*, *ap*, *pnr*, *tsh*, *Dll*, and *eve* as indicated (magenta in each case) and costained with Hoechst (blue) to visualize nuclei. Rows 2 and 3: discs with clones of cells that are homozygous for the indicated PcG mutations; mutant cells are marked by absence of GFP. *en*: in wt animals, expression is confined to the posterior compartment (P) and not detected in the anterior compartment (A). *en* is misexpressed in most *ph* and many *dSfmbt* mutant clones in the anterior compartment (arrowheads) but consistently remains repressed in *dSfmbt* clones in the presumptive wing blade (empty arrowhead). *ap*: in wt animals, *ap* expression is restricted to the dorsal compartment (D) and is absent in the ventral compartment (V). *ap* is misexpressed in *Scm* mutant clones in the ventral compartment (arrowheads); misexpression is more widespread *Scm BXC* mutant clones also lacking the products of the three Bithorax complex genes *Ubx*, *abd-A* and *Abd-B*. *pnr*: in wt animals, *pnr* expression is confined to the notum (N), and it is not expressed in the hinge and wing blade. *pnr* is misexpressed in *Psc-Su(z)2* and *ph* mutant clones in hinge and wing blade cells (arrowheads). *tsh*: in wt animals, *tsh* expression is confined to the notum (N) and hinge (H), and it is not expressed in the wing blade. *tsh* is misexpressed in *Scm* mutant clones in the wing blade (arrowheads); slightly less extensive misexpression is observed in *Scm BXC* mutant clones, suggesting that misexpressed HOX proteins contribute to *tsh* activation in Scm mutant clones. *Dll*: in wt animals, *Dll* expression is confined to wing blade (W) cells and it is not expressed in the notum and hinge. *Dll* expression is lost in *Scm* single mutant clones (empty arrowheads) but is restored in *Scm BXC* mutant clones, and *Dll* is misexpressed in a subset of clone cells in a specific region of the hinge (arrowhead). *eve*: in wt animals, *eve* is not expressed in the wing disc. *eve* is misexpressed in all *Psc-Su(z)2* and *ph* mutant clones throughout the disc (arrowheads). Images show representative examples to illustrate extent of misexpression of target genes. *en*, *tsh*, *Dll*, and *eve* expression was visualized with antibodies against their protein products; *ap* and *pnr* expression was visualized using *Gal4*-tagged alleles in *UAS-nLacZ* transgenic animals and staining with β-galactosidase antibodies. Clones were analyzed at different time points after clone induction (row 2 from left to right) *dSfmbt*/96 hr, *Scm*/68 hr, *Psc-Su(z)2*/48 hr, *Scm*/72 hr, *Scm*/96 hr, *Psc-Su(z)2*/96 hr (row 3 from left to right), *ph*/96 hr, *Scm BXC*/72 hr, *ph*/72 hr, *Scm BXC*/72 hr, *Scm BXC*/96 hr, *ph*/96 hr.

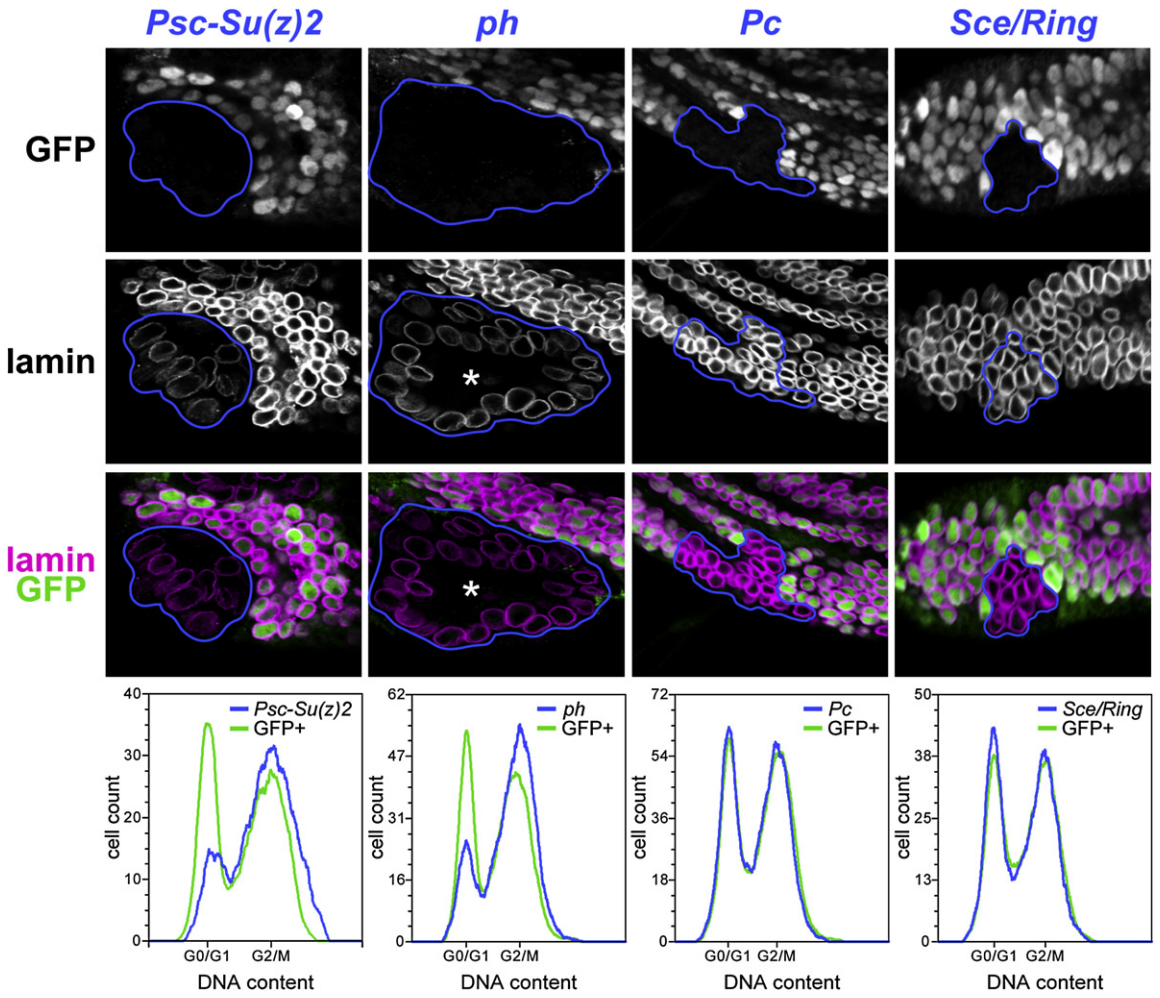
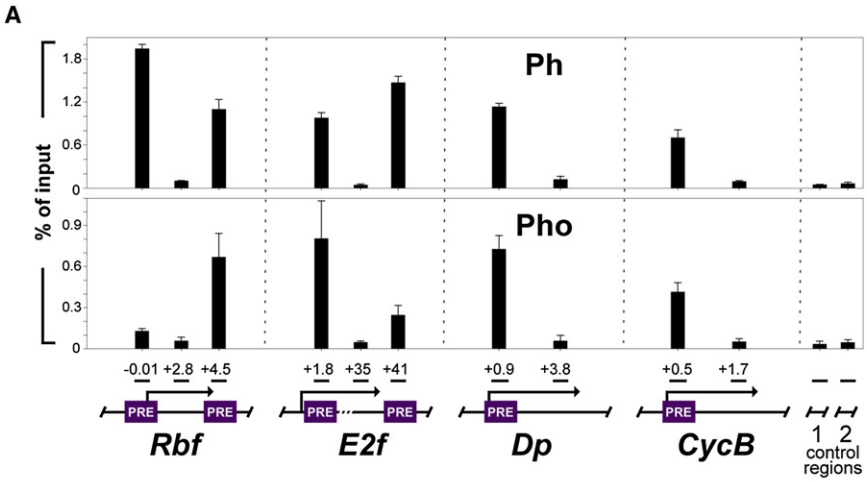


Figure 5. PcG Control of Cell Growth and Proliferation

(Top) Wing imaginal discs from third-instar larvae with clones of cells homozygous for the indicated PcG mutations stained with an antibody against nuclear lamin (magenta). Clones were induced 48 hr prior to analysis and are marked by absence of GFP; a single clone is marked with a blue line in each image. Note the increased size and altered morphology of *Psc-Su(z)2* and *ph* mutant nuclei compared to GFP-positive wild-type nuclei. *Psc-Su(z)2* and *ph* mutant clone tissue forms vesicles, often with cavities (asterisk), that sort out from surrounding wild-type tissue. *Pc* or *Sce/Ring* mutant clones do not show these phenotypes. (Below) DNA content and cell cycle profile of *Psc-Su(z)2*, *ph*, *Pc* and *Sce/Ring* mutant cells and GFP-positive wild-type control cells in imaginal discs from third-instar larvae. Clones were induced 96 hr prior to analysis. Note the change in cell cycle phasing in *Psc-Su(z)2* and in *ph* mutant cells (see text for details).

clones, we first analyzed the morphology and DNA content of the mutant cells. Staining of imaginal discs with *Psc-Su(z)2* or *ph* mutant clones with antibodies against nuclear lamin revealed that the mutant cells show a substantial increase in nuclear and cellular volume (Figure 5 and data not shown). Interestingly, this phenotype is not observed in clones of cells that are homozygous for a protein null mutation in *Pc* or in clones of cells homozygous for Sce^{1}, the strongest available *Sce/Ring* allele (Figure 5). To test whether the increased nuclear size of *Psc-Su(z)2* or *ph* mutant cells may be due to a change in DNA content, we dissociated imaginal discs with PcG mutant clones into individual cells and measured the DNA content of mutant (i.e., GFP-negative) and wild-type (i.e., GFP-positive) cells from the same disc by FACS analysis. In each case, the mutant cells have a normal 2C/4C DNA content (Figure 5). However, we found that *Psc-Su(z)2* and *ph* mutant cells show a drastic change in cell cycle phasing, with the fraction of G0/G1 cells being strongly reduced and the fraction of G2/M cells being substantially increased compared to wild-type control cells in the same disc (Figure 5). In contrast, the cell cycle phasing of *Pc* or *Sce/Ring* mutant cells is comparable to wild-type cells (Figure 5). These observations using loss-of-function mutations in developing *Drosophila* larvae are in contrast with previous studies using RNAi knockdown of *Pc* in proliferating S2 cells where an altered cell cycle profile with a lengthened G2/M phase was reported (Martinez et al., 2006). Taken together, our analyses demonstrate that the PRC1 components Ph, Psc and Su(z)2 are critically required for restricting growth and proliferation of imaginal disc cells.

Interestingly, our ChIP-chip analyses identified the cell cycle regulator genes *Rbf*, *E2F*, *Dp*, and *CycB* as PhoRC targets (Tables S1–S4). This is intriguing because Rbf, E2F, and Dp were shown to control cell growth and proliferation in imaginal disc



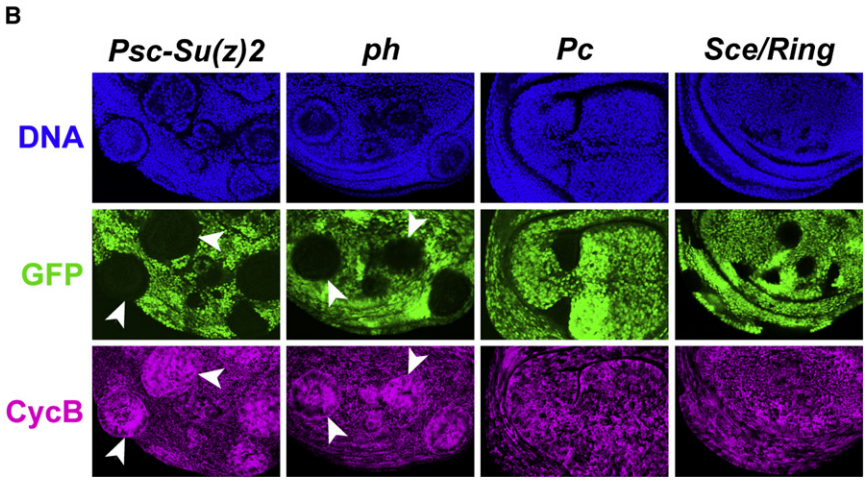


Figure 6. PcG Complexes Target Cell Cycle Regulator Genes

(A) ChIP analysis to monitor Ph and Pho binding in wild-type embryos. Analysis was performed and results are presented as in Figure 3.

(B) Wing imaginal discs from third instar larvae with clones of cells homozygous for the indicated PcG mutations stained with an antibody to detect CycB (magenta) and costained with Hoechst (blue) to visualize nuclei; mutant cells are marked by absence of GFP. *Psc-Su(z)2* and *ph* mutant clones were induced 72 hr prior to analysis, and *Pc* and *Sce/Ring* mutant clones 96 hr prior to analysis. CycB protein levels are increased in *Psc-Su(z)2* and *ph* mutant clones (arrowheads) but not in *Pc* or *Sce/Ring* mutant clones.

cells (Neufeld et al., 1998). ChIP assays in imaginal discs further showed that not only Pho but also the PRC1 component Ph is bound at the identified PREs in the *Rbf*, *E2F*, *Dp*, and *CycB* genes (Figure 6A). Interestingly, none of these genes had been described as PRC1 or PRC2 targets in tissue culture cells (Schwartz et al., 2006).

To determine whether the tumor phenotypes of *Psc-Su(z)2* or *ph* mutant clones might be associated with altered expression levels of these cell cycle regulators, we stained PcG mutant clones with antibodies against Rbf, E2F, or CycB. As expected, all three proteins are ubiquitously expressed in proliferating imaginal disc cells. We have been unable to detect altered Rbf or E2F protein levels in *Psc-Su(z)2* or *ph* mutant cell clones (data not shown), suggesting that regulation of Rbf or E2F by the PcG system may be subtle or does not occur in the tissues analyzed here. In contrast, we found that CycB protein levels were elevated in both *Psc-Su(z)2* and *ph* mutant clones (Figure 6B). Together with the observation that PhoRC and PRC1 components are bound at the *CycB* PRE (Figure 6A), this result suggests that PcG protein complexes directly regulate the levels of *CycB* transcription. Understanding the relationship between the tumor phenotype of *Psc-Su(z)2* and *ph* mutant clones and transcriptional control of cell cycle regulators such as *CycB* by the PcG system will need further investigation. However, the finding that the PcG system controls the expression levels of *CycB* and possibly other cell cycle regulators suggests that the system is not only a binary ON/OFF switch to confine expression of genes to subsets of cells within an organism, but that it is also used to dynamically modulate the levels of transcription of certain target genes within cells where these genes are expressed.

DISCUSSION

Global identification of genomic locations to which PcG protein complexes bind and unraveling how expression of target genes is regulated by these complexes is important to understand how the PcG system controls transcription of the genome. The following main conclusions can be drawn from the work presented here: (1) PhoRC is sharply localized at discrete PRE sequences, many of which are co-occupied by PRC1 and PRC2. (2) 196 PREs were identified in the *Drosophila* genome where PhoRC is constitutively bound in embryos and larvae. In general, these PREs are located within ±1 kb from the closest gene transcription start site and the majority of target genes contain only one PRE. (3) Sequence analyses of identified PREs allowed us to define an extended Pho-binding motif that is part of the signature of PhoRC-bound PREs. (4) Functional analyses in *Drosophila* reveal that PcG proteins repress transcription of several key developmental regulators. In particular, the PcG system is required for maintaining the subdivision of segment primordia into anteroposterior, dorsoventral and proximodistal compartments by repressing the genes *en*, *ap*, *pnr*, *tsh*, and *Dll*. (5) As discussed in detail below, these analyses suggest that extracellular signaling can selectively induce transcription of previously silent PcG target genes, even though PcG protein complexes are bound at their PREs. (6) Among the PcG target genes are also cell cycle regulators such as *Rbf*, *E2F*, *Dp*, and *CycB*, and we provide evidence that the PcG system regulates expression levels of the *CycB* gene in *Drosophila*.

PcG Complex Binding at PREs

Genome-wide binding profiling and ChIP analyses at selected target genes revealed an extensive overlap between PhoRC-, PRC1-, and PRC2-bound regions. These results, together with previous reports that Pho interacts with and is required for targeting of PRC1 and/or PRC2 at HOX gene PREs (Poux et al., 2001; Mohd-Sarip et al., 2002, 2005; Wang et al., 2004b; Klymenko et al., 2006), suggest that PhoRC is needed for PRC1 and PRC2 binding at many PcG target genes. It should also be recalled that in larvae, PhoRC, PRC1, and PRC2 are all constitutively bound to PREs of the HOX gene *Ubx*, both in cells where *Ubx* is repressed and in cells where it is active (Papp and Müller, 2006). The observation that the same PRE sites are occupied in both tissue culture cells and in developing *Drosophila* thus further implies that PhoRC, PRC1, and PRC2 may be constitutively bound to a large fraction if not most of their target genes.

What are the sequences that make up a PRE? Using an algorithm based on binding site motifs for Pho, GAF/Trl, and Zeste proteins, Ringrose et al. (2003) predicted 167 PREs across the *Drosophila* genome. We detected PhoRC binding at 26 of these predicted PREs (15%; Ringrose et al., 2003). Intriguingly, we found a significant overlap between PhoRC-bound regions and regions bound by GAF/Trl but only a limited overlap with Zeste-bound regions. *GAF/Trl* mutants do not show HOX misexpression phenotypes, making its role at HOX gene PREs somewhat enigmatic (reviewed in Müller and Kassis, 2006). However, it is possible that GAF/Trl is required for PRC1 and/or PRC2 targeting to PREs of non-HOX genes. Finally, it is important to keep in mind that PRC1 and PRC2 are also bound at genomic regions where we have been unable to detect PhoRC, suggesting that they are targeted there by other factors.

PcG Regulation of Developmental Regulator Genes

The most obvious phenotype of PcG mutants in *Drosophila* are homeotic transformations, caused by the global misexpression of multiple HOX genes. In this study, we monitored loss of repression of several non-HOX target genes and we show that these genes are indeed also misexpressed in PcG mutants. In particular, we found that the PcG system represses key regulator genes required for the subdivision of appendages into anteroposterior, dorsoventral, and proximodistal compartments. This suggests that the PcG system is responsible for maintaining many more cell fate decisions than may be immediately evident from the phenotype. In this context, it is important to note that at some target genes, control by PcG proteins is masked by other regulatory interactions and can only be revealed in the absence of those regulatory inputs. The downregulation of *Dll* in PcG mutant clones by HOX proteins represents a prime example for such a masking effect; regulation of *Dll* by the PcG system could only be revealed in cells lacking both PcG and HOX gene functions.

Finally, we found that some target genes are only strongly misexpressed in certain PcG mutants but not in others, even though PhoRC, PRC1, and PRC2 are all cobound at these genes. This implies that in those cases not all PcG protein complexes or complex components are required for repression. In the most extreme scenario, recruitment of all three complexes reflects the default state that even occurs at genes that require i.e., only H2A ubiquitylation by PRC1 but not H3-K27 methylation by PRC2 for repression.

Signaling-Induced Activation at PcG-Repressed Target Genes

The finding that PcG protein complexes are constitutively bound at PREs of target genes in both embryonic and imaginal disc cells has implications for understanding regulation by the PcG system. In particular, target genes such as *ap*, *Dll*, or *pnr* are not active in the wing disc primordium in embryos, remain silent as the primordium grows during the early larval stages and only become transcriptionally active at later larval stages (Vachon et al., 1992; Cohen et al., 1992; Williams et al., 1993; Gorfinkiel et al., 1997; Tomoyasu et al., 2000). The factors responsible for activating *ap* transcription during the second larval instar are not known (Williams et al., 1993), but induction of *Dll* expression in the wing blade primordium during the third larval instar occurs in response to *wg* signaling (Gorfinkiel et al., 1997; Neumann and Cohen, 1997), and expression of *pnr* in the notum primordium during the second larval instar is activated by *dpp* signaling (Sato and Saigo, 2000; Tomoyasu et al., 2000). The most straightforward explanation of these observations is that these signaling pathways are able to switch on expression of these target genes even though PcG complexes are bound at their PREs and even though their chromatin bears the Polycomb-repressive H3-K27me3 mark in embryos (Nekrasov et al., 2007). In wild-type wing discs, Wg-signaling would thus be able to overcome PcG repression at the *Dll* gene in wing pouch cells but not in the notum and hinge region where Wg protein is also present (Neumann and Cohen, 1997). Similarly, in wild-type animals, Dpp-signaling only activates *pnr* expression in the notum and not along the whole length of the anteroposterior compartment boundary where Dpp protein is expressed (Posakony et al., 1990). One possible explanation for this selective activation in discrete parts of the disc would be a requirement for signaling pathways to act in a combinatorial manner with other (unknown) factors to relieve PcG repression. Consistent with this, removal of PcG function often results in misexpression of target genes only in specific regions of the disc and that these regions receive the same signals that are also responsible for activation of these target genes in their normal wild-type expression domain. For example, misexpression of *pnr* mainly occurs in PcG mutant clones in the wing blade and thus in cells that receive Dpp, the signal that also activates *pnr* expression in its wild-type expression domain in the notum. This raises the intriguing possibility that an important function of the PcG system may be to spatially restrict activation of target genes in response to more widely distributed extracellular signals.

Concluding Remarks

Previous ChIP-chip studies showed that PcG protein complexes are bound to a large number of developmental regulators in mammalian embryonic stem cells (Boyer et al., 2006; Lee et al., 2006). The majority of the genes bound by PcG proteins in stem cells are orthologs of PcG target genes identified in flies, including the family orthologs of *en*, *ap*, *pnr*, *Dll*, *eve*, and *Doc* whose regulation we analyzed here. The finding that these genes are regulated by the PcG system during *Drosophila* development implies that the mammalian PcG system may also regulate the orthologous genes in differentiating cells and tissues, beyond the known regulation in stem cells.

Our study identified cell cycle regulator genes as PcG targets, and we provided evidence that the PcG system directly regulates *CycB* expression. Control of cell cycle regulators by the PcG system may provide a molecular explanation for the tumor phenotype observed in proliferating imaginal disc cells lacking the PRC1 components *Psc-Su(z)2* or *ph*. The observation that the PcG system controls transcription of genes whose expression is modulated during the cell cycle suggests that the PcG system is also used to regulate target genes more dynamically than previously thought. In mammalian cells, knockout of the Psc homolog *bmi-1* results in cellular senescence via loss of p16/INK4A transcriptional repression, a cyclin D regulator without any obvious ortholog in the *Drosophila* genome (Jacobs et al., 1999). It therefore appears that the PcG system has a conserved role in regulating expression of genes involved in body patterning but that it evolved in different ways to control cell growth and proliferation in mammals and flies.

EXPERIMENTAL PROCEDURES

Antibodies and *Drosophila* Strains

All antibodies and fly strains used in this study are listed in Supplemental Data.

ChIP-chip in *Drosophila* Using Affymetrix Whole-Genome Microarrays

ChIP from *Drosophila* embryos and larval imaginal discs was performed essentially as described in Klymenko et al. (2006) and in Papp and Müller (2006), respectively. The detailed protocols, including description of the quantitative analysis of ChIP material, a list with the PCR primers used for gene-specific amplification and the ChIP-chip procedure can be found in Supplemental Data. Data analysis is described in detail in Supplemental Data. In brief, for each of the three datasets (Pho^E, Pho^L, $dSfmbt^L$) a quantile normalization (Bolstad et al., 2003) was applied to normalize together the ChIP hybridizations and three genomic DNA hybridizations. Significantly bound regions were identified using TileMap (Ji and Wong, 2005) with the hidden Markov model (HMM). The relative distance of the midpoint of each region with respect to the closest transcription start site (TSS) was computed. Target genes were assigned based on TSS-proximal location. Assigned genes to each dataset were tested for enriched GO slim term annotations. For Pho-binding motif analysis, enrichment for transcription factor binding signatures was performed on each of the three datasets (Pho^E, Pho^L, and $dSfmbt^L$) using RSAT (van Helden, 2003). GCCAT enriched patterns were used for position-specific scoring matrix (PSSM) reconstruction. Matrices obtained for each dataset were averaged yielding to the PSSM available in Supplemental Data. Predictions of Pho-binding sites were made using Patser (Hertz and Stromo, 1999). Frequency of the Pho-binding site in each dataset was compared to the frequency of the motif in the genome. Frequency of the motif in the core-PhoRC bound regions was compared to the frequency observed in 1000 randomly generated region sets.

Immunostaining of *Drosophila* Embryos and Larval Imaginal Discs

Antibody staining of embryos was done following standard protocols. Staining of imaginal discs was performed as described in Beuchle et al. (2001).

FACS Analysis of *Drosophila* Larval Wing Disc Cells

Ninety-six hours after mutant clone induction, wing discs of third-instar larvae were dissected in ice-cold PBS. For each measurement, five to eight discs were incubated in Trypsin-EDTA solution at 28°C for 2–4 hr. Twenty minutes prior to FACS analysis, Hoechst 33342 was added to the sample. FACS data were collected (DAKO MoFlo High Speed Sorter) and analyzed using Summit software. See Supplemental Data for details.

ACCESSION NUMBERS

The raw and processed microarray data have been deposited in the ArrayExpress database under accession number E-TABM-525.

SUPPLEMENTAL DATA

The Supplemental Data include five figures, seven tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.developmentalcell.com/supplemental/S1534-5807(08)00432-2.

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REFERENCES

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

Birve, A., Sengupta, A.K., Beuchle, D., Larsson, J., Kennison, J.A., Rasmuson-Lestander, A., and Muller, J. (2001). Su(z)12, a novel *Drosophila* Polycomb group gene that is conserved in vertebrates and plants. Development *128*, 3371–3379.

Blastyák, A., Mishra, R.K., Karch, F., and Gyurkovics, H. (2006). Efficient and specific targeting of Polycomb group proteins requires cooperative interaction between Grainyhead and Pleiohomeotic. Mol. Cell. Biol. *26*, 1434–1444.

Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics *19*, 185–193.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonat, A., Ray, M.K., et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349–353.

Bracken, A.P., Dietrich, N., Pasini, D., Hansen, K.H., and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev. *20*, 1123–1136.

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

Brown, J.L., Grau, D.J., DeVido, S.K., and Kassis, J.A. (2005). An Sp1/KLF binding site is important for the activity of a Polycomb group response element from the Drosophila engrailed gene. Nucleic Acids Res. *33*, 5181–5189.

Buchwald, G., van der Stoop, P., Weichenrieder, O., Perrakis, A., van Lohuizen, M., and Sixma, T.K. (2006). Structure and E3-ligase activity of the Ring-Ring complex of polycomb proteins Bmi1 and Ring1b. EMBO J. *25*, 2465–2474.

Busturia, A., and Morata, G. (1988). Ectopic expression of homeotic genes caused by the elimination of the Polycomb gene in *Drosophila* imaginal epidermis. Development *104*, 713–720.

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.

Caretti, G., Di Padova, M., Micales, B., Lyons, G.E., and Sartorelli, V. (2004). The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. Genes Dev. *18*, 2627–2638.

Chamberlain, S.J., Yee, D., and Magnuson, T. (2008). Polycomb Repressive Complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells *26*, 1496–1505.

Cohen, B., McGuffin, M.E., Pfeifle, C., Segal, D., and Cohen, S.M. (1992). apterous, a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. Genes Dev. *6*, 715–729.

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.

Dejardin, J., Rappailles, A., Cuvier, O., Grimaud, C., Decoville, M., Locker, D., and Cavalli, G. (2005). Recruitment of Drosophila Polycomb group proteins to chromatin by DSP1. Nature *434*, 533–538.

Duncan, I.M. (1982). Polycomblike: a gene that appears to be required for the normal expression of the bithorax and antennapedia gene complexes of *Drosophila* melanogaster. Genetics *102*, 49–70.

Dura, J.M., and Ingham, P. (1988). Tissue- and stage-specific control of homeotic and segmentation gene expression in *Drosophila* embryos by the polyhomeotic gene. Development *103*, 733–741.

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

Gorfinkiel, N., Morata, G., and Guerrero, I. (1997). The homeobox gene Distalless induces ventral appendage development in *Drosophila*. Genes Dev. *11*, 2259–2271.

Gorfinkiel, N., Sanchez, L., and Guerrero, I. (1999). *Drosophila* terminalia as an appendage-like structure. Mech. Dev. *86*, 113–123.

Hertz, G.Z., and Stromo, G.D. (1999). Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. Bioinformatics *15*, 563–577.

Horard, B., Tatout, C., Poux, S., and Pirrotta, V. (2000). Structure of a polycomb response element and in vitro binding of polycomb group complexes containing GAGA factor. Mol. Cell. Biol. *20*, 3187–3197.

Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A., and van Lohuizen, M. (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature *397*, 164–168.

Janody, F., Lee, J.D., Jahren, N., Hazelett, D.J., Benlali, A., Miura, G.I., Draskovic, I., and Treisman, J.E. (2004). A mosaic genetic screen reveals distinct roles for trithorax and polycomb group genes in *Drosophila* eye development. Genetics *166*, 187–200.

Ji, H., and Wong, W.H. (2005). TileMap: create chromosomal map of tiling array hybridizations. Bioinformatics *21*, 3629–3636.

Jürgens, G. (1985). A group of genes controlling the spatial expression of the bithorax complex in *Drosophila*. Nature *316*, 153–155.

Kirmizis, A., Bartley, S.M., Kuzmichev, A., Margueron, R., Reinberg, D., Green, R., and Farnham, P.J. (2004). Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev. *18*, 1592–1605.

Klymenko, T., Papp, B., Fischle, W., Kocher, T., Schelder, M., Fritsch, C., Wild, B., Wilm, M., and Muller, J. (2006). A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. Genes Dev. *20*, 1110–1122.

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.

Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K., et al. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. Cell *125*, 301–313.

Lee, C., Li, X., Hechmer, A., Eisen, M., Biggin, M.D., Venters, B.J., Jiang, C., Li, J., Pugh, B.F., and Gilmour, D.S. (2008). NELF and GAGA factor are linked to promoter-proximal pausing at many genes in Drosophila. Mol. Cell. Biol. *28*, 3290–3300.

Mann, R.S., and Morata, G. (2000). The developmental and molecular biology of genes that subdivide the body of *Drosophila*. Annu. Rev. Cell Dev. Biol. *16*, 243–271.

Martinez, A.M., Colomb, S., Dejardin, J., Bantignies, F., and Cavalli, G. (2006). Polycomb group-dependent Cyclin A repression in *Drosophila*. Genes Dev. *20*, 501–513.

Maurange, C., and Paro, R. (2002). A cellular memory module conveys epigenetic inheritance of hedgehog expression during *Drosophila* wing imaginal disc development. Genes Dev. *16*, 2672–2683.

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.

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

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.

Mohd-Sarip, A., Cleard, F., Mishra, R.K., Karch, F., and Verrijzer, C.P. (2005). Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb core complex. Genes Dev. *19*, 1755–1760.

Mohd-Sarip, A., van der Knaap, J.A., Wyman, C., Kanaar, R., Schedl, P., and Verrijzer, C.P. (2006). Architecture of a polycomb nucleoprotein complex. Mol. Cell *24*, 91–100.

Moses, A.M., Pollard, D.A., Nix, D.A., Iyer, V.N., Li, X.Y., Biggin, M.D., and Eisen, M.B. (2006). Large-scale turnover of functional transcription factor binding sites in *Drosophila*. PLoS Comput. Biol. *2*, e130. 10.1371/journal.pcbi.0020130.

Müller, J., and Kassis, J.A. (2006). Polycomb response elements and targeting of Polycomb group proteins in *Drosophila*. Curr. Opin. Genet. Dev. *16*, 476–484.

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.

Negre, N., Hennetin, J., Sun, L.V., Lavrov, S., Bellis, M., White, K.P., and Cavalli, G. (2006). Chromosomal distribution of PcG proteins during *Drosophila* development. PLoS Biol. *4*, e170. 10.1371/journal.pbio.0040170.

Nekrasov, M., Klymenko, T., Fraterman, S., Papp, B., Oktaba, K., Köcher, T., Cohen, A., Stunnenberg, H.G., Wilm, M., and Müller, J. (2007). Pcl-PRC2 is needed to generate high levels of H3-K27 trimethylation at Polycomb target genes. EMBO J. *26*, 4078–4088.

Neufeld, T.P., de la Cruz, A.F., Johnston, L.A., and Edgar, B.A. (1998). Coordination of growth and cell division in the *Drosophila* wing. Cell *93*, 1183–1193.

Neumann, C.J., and Cohen, S.M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the *Drosophila* wing. Development *124*, 871–880.

Papp, B., and Müller, J. (2006). Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev. *20*, 2041–2054.

Pien, S., and Grossniklaus, U. (2007). Polycomb group and trithorax group proteins in Arabidopsis. Biochim. Biophys. Acta *1769*, 375–382.

Posakony, L.G., Raftery, L.A., and Gelbart, W.M. (1990). Wing formation in *Drosophila* melanogaster requires decapentaplegic gene function along the anterior-posterior compartment boundary. Mech. Dev. *33*, 69–82.

Poux, S., McCabe, D., and Pirrotta, V. (2001). Recruitment of components of Polycomb Group chromatin complexes in *Drosophila*. Development *128*, 75–85.

Reim, I., Lee, H.H., and Frasch, M. (2003). The T-box-encoding Dorsocross genes function in amnioserosa development and the patterning of the dorsolateral germ band downstream of Dpp. Development *130*, 3187–3204.

Ringrose, L., and Paro, R. (2007). Polycomb/Trithorax response elements and epigenetic memory of cell identity. Development *134*, 223–232.

Ringrose, L., Rehmsmeier, M., Dura, J.M., and Paro, R. (2003). Genome-wide prediction of Polycomb/Trithorax response elements in *Drosophila* melanogaster. Dev. Cell *5*, 759–771.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., and Chang, H.Y. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311–1323.

Röder, L., Vola, C., and Kerridge, S. (1992). The role of the teashirt gene in trunk segmental identity in *Drosophila*. Development *115*, 1017–1033.

Satijn, D.P., 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.

Sato, M., and Saigo, K. (2000). Involvement of pannier and u-shaped in regulation of decapentaplegic-dependent wingless expression in developing *Drosophila* notum. Mech. Dev. *93*, 127–138.

Schubert, D., Clarenz, O., and Goodrich, J. (2005). Epigenetic control of plant development by Polycomb-group proteins. Curr. Opin. Plant Biol. *8*, 553–561.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. Cell *128*, 735–745.

Schwartz, Y.B., and Pirrotta, V. (2007). Polycomb silencing mechanisms and the management of genomic programmes. Nat. Rev. Genet. *8*, 9–22.

Schwartz, Y.B., Kahn, T.G., Nix, D.A., Li, X.Y., Bourgon, R., Biggin, M., and Pirrotta, V. (2006). Genome-wide analysis of Polycomb targets in *Drosophila* melanogaster. Nat. Genet. *38*, 700–705.

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.

Shimell, M.J., Peterson, A.J., Burr, J., Simon, J.A., and O'Connor, M.B. (2000). Functional analysis of repressor binding sites in the iab-2 regulatory region of the abdominal-A homeotic gene. Dev. Biol. *218*, 38–52.

Squazzo, S.L., O'Geen, H., Komashko, V.M., Krig, S.R., Jin, V.X., Jang, S.W., Margueron, R., Reinberg, D., Green, R., and Farnham, P.J. (2006). Suz12 binds to silenced regions of the genome in a cell-type-specific manner. Genome Res. *16*, 890–900.

Tolhuis, B., de Wit, E., Muijrers, I., Teunissen, H., Talhout, W., van Steensel, B., and van Lohuizen, M. (2006). Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in *Drosophila* melanogaster. Nat. Genet. *38*, 694–699.

Tomoyasu, Y., Ueno, N., and Nakamura, M. (2000). The decapentaplegic morphogen gradient regulates the notal wingless expression through induction of pannier and u-shaped in *Drosophila*. Mech. Dev. *96*, 37–49.

Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M.E., Botas, J., and Cohen, S.M. (1992). Homeotic genes of the Bithorax complex repress limb development in the abdomen of the *Drosophila* embryo through the target gene Distal-less. Cell *71*, 437–450.

van Helden, J. (2003). Regulatory sequence analysis tools. Nucleic Acids Res. *31*, 3593–3596.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004a). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873–878.

Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004b). Hierarchical recruitment of polycomb group silencing complexes. Mol. Cell *14*, 637–646.

Williams, J.A., Paddock, S.W., and Carroll, S.B. (1993). Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions. Development *117*, 571–584.

Zirin, J.D., and Mann, R.S. (2004). Differing strategies for the establishment and maintenance of teashirt and homothorax repression in the *Drosophila* wing. Development *131*, 5683–5693.