

The DNA-binding Polycomb-group protein Pleiohomeotic maintains both active and repressed transcriptional states through a single site

Miki Fujioka, Galina L. Yusibova, Jian Zhou and James B. Jaynes*

Although epigenetic maintenance of either the active or repressed transcriptional state often involves overlapping regulatory elements, the underlying basis of this is not known. Epigenetic and pairing-sensitive silencing are related properties of Polycomb-group proteins, whereas their activities are generally opposed by the trithorax group. Both groups modify chromatin structure, but how their opposing activities are targeted to allow differential maintenance remains a mystery. Here, we identify a strong pairing-sensitive silencing (PSS) element at the 3' border of the *Drosophila even-skipped (eve)* locus. This element can maintain repression during embryonic as well as adult eye development. Transgenic dissection revealed that silencing activity depends on a binding site for the Polycomb-group protein Pleiohomeotic (Pho) and on *pho* gene function. Binding sites for the trithorax-group protein GAGA factor also contribute, whereas sites for the known Polycomb response element binding factors Zeste and Dsp1 are dispensable. Normally, *eve* expression in the nervous system is maintained throughout larval stages. An enhancer that functions fully in embryos does not maintain expression, but the adjacent PSS element confers maintenance. This positive activity also depends on *pho* gene activity and on Pho binding. Thus, a DNA-binding complex requiring Pho is differentially regulated to facilitate epigenetic transcriptional memory of both the active and the repressed state.

KEY WORDS: Polycomb group, Trithorax group, Epigenetics, Pleiohomeotic, Even-skipped, *Drosophila*

INTRODUCTION

Polycomb group (PcG) proteins associate with specific DNA regions, forming complexes that modify chromatin and maintain a transcriptionally silent state (Grimaud et al., 2006; Muller and Kassis, 2006; Ringrose, 2007; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). Among the genetically identified *Drosophila* PcG proteins, only Pleiohomeotic (Pho) and its homolog Pho-like (Phol) have sequence-specific DNA-binding activity (Brown et al., 2003; Brown et al., 1998; Fritsch et al., 1999). They, together with other DNA-binding proteins (Americo et al., 2002; Brown et al., 2005; Muller and Kassis, 2006; Ringrose and Paro, 2007; Schuettengruber et al., 2007), have been implicated in targeting each of the known PcG complexes (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Shao et al., 1999; Wang et al., 2004a) to their target genes. In mammals, PcG proteins are involved in maintenance of the stem cell niche (Buszczak and Spradling, 2006; Sparmann and van Lohuizen, 2006) and in oncogenesis (Sparmann and van Lohuizen, 2006).

Trithorax group (trxG) proteins oppose the action of the PcG (Kennison, 2004), form complexes on target DNA (Badenhorst et al., 2002; Bantignies et al., 2000; Fyodorov et al., 2004; Papoulas et al., 1998; Petruk et al., 2001) and maintain an active state, by modifying histones and remodeling chromatin. PcG and trxG proteins are considered to be distinct from other regulators in that they can mediate heritable maintenance of the transcriptional state from one cellular generation to another (Grimaud et al., 2006; Muller and Kassis, 2006; Ringrose and Paro, 2007; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). Target sequences that

mediate the functions of these proteins are termed PcG- and trxG-responsive elements (PREs and TREs, respectively) or, collectively, maintenance elements (MEs) (Brock and van Lohuizen, 2001), because they often overlap. Dissection of these target elements has proven to be difficult, because of their complexity, and because the associated activities manifest themselves fully only in a developmental context. Consequently, how PcG and trxG complexes are targeted to DNA, and how this targeting varies with the transcriptional state, is just beginning to be elucidated.

Transgenes carrying PREs/TREs in *Drosophila* have been seen to share a property known as pairing-sensitive silencing (PSS) (Gindhart and Kaufman, 1995; Kassis, 2002). Transgenic flies carrying the *mini-white* gene typically have eye colors ranging from yellow to orange in a *white* mutant background. Normally, flies that are homozygous for such a transgene have a darker eye color than heterozygotes, as the genetic dose of *mini-white* is doubled. However, with transgenes carrying PRE/TREs, the eye color is often lighter in homozygotes than in heterozygotes. PSS is also often accompanied by a reduction in *mini-white* activity from heterozygous transgenes, causing a relatively faint eye color, or a variegated pattern of eye coloration. This phenomenon was first reported for a pairing-sensitive element (PSE) near the *engrailed (en)* transcription start site (Kassis, 1994; Kassis et al., 1991). It was also shown that the *en* PSE can function as a PRE in embryos, in that it can restrict expression of a transgenic reporter gene driven by the *Ultrabithorax (Ubx)* regulatory region *bithoraxoid (bxo)* to the posterior part of the embryo, where *Ubx* is normally expressed, in a PcG-dependent manner (Americo et al., 2002). More generally, although PREs usually exhibit PSS activity, PSEs may not be sufficient to function as a PRE (Kassis, 1994; Kassis, 2002).

Genome-wide predictions of possible *Drosophila* PREs using sequences common to known PREs, including binding sites for the PcG and trxG proteins Pho/Phol, GAGA factor (GAF) and Zeste,

Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA.

* Author for correspondence (e-mail: jaynes@jci.tju.edu)

identified 167 candidates, including the *even skipped* (*eve*) promoter region (Ringrose et al., 2003). Other studies using two different techniques to determine *in vivo* protein binding (ChIP on chip and DamID) identified other candidate PREs with some, although not extensive, overlap (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006). These studies suggested that the *eve* locus is bound by the Polycomb (Pc) protein (Schwartz et al., 2006; Tolhuis et al., 2006). Earlier, mapping of binding sites for Pc and Polyhomeotic (Ph) on polytene chromosomes identified the cytological location of *eve* (Sinclair et al., 1998). Consistent with this, *eve* is ectopically expressed in the embryonic nervous system in mutants of the PcG gene *ph* (Smouse et al., 1988).

Here, we show that the *Drosophila eve* gene is regulated by a Pho-dependent maintenance element located 9 kb downstream of the promoter. This element maintains repression in cells where *eve* is turned off during early development, but maintains the active state in other cells. Both negative and positive maintenance depend on Pho binding, and on *pho* gene activity. The element shares properties with other PREs, including binding sites for several factors. Dissection suggests that although GAF is involved, Zeste and Dsp1 may be dispensable, and that Grainyhead/Elf1 (Bray and Kafatos, 1991), as well as unknown factors, may also contribute. Thus a 'core' DNA-binding component, differentially modified or regulated by the existing transcriptional state, mediates epigenetic maintenance of that state.

MATERIALS AND METHODS

Plasmids construction

All sequence coordinates in the *eve* locus are relative to the transcription start site (+1). The parental P-element plasmid containing Glass-binding sites has been described previously (Fujioka et al., 1999). To construct the PSS assay transgenes of Fig. 1, *eve* DNA from +8.9 to +9.2 kb (PRE300) was cloned upstream of the Glass-binding sites followed by *mini-white*. DNA from -275 bp to +116 bp was similarly used to make PSEpro. The *mini-white* P-element-based GFP-RR construct was made by fusing *eve* DNA from -275 bp to +99 bp with the EGFP-coding region, driven by the RR regulatory DNA (+7.9 to +9.2 kb), which consists of the neuronal RP2+*a*/pCC enhancer and PRE300 (Fujioka et al., 2003). For ΦC31 recombinase-mediated cassette exchange (RMCE) (Bateman et al., 2006; Groth et al., 2000), the piB-GFP construct (Bateman et al., 2006) was first modified to increase the number of unique cloning sites (attBΔ2; details available on request). Then, GFP-RR described above was transferred to this vector. For the PRE assays of Fig. 2, a P-element based *bx-d-Ubx-Z* vector (with a *bx-d* enhancer fragment inserted upstream of the -3.1 *Ubx-βgal* fusion in Uβghz) (Muller and Bienz, 1991) was modified by inserting PRE300 (with or without the Pho site mutated) into the *Xba*I site, oriented such that the PRE300 3' end was closer to the *Ubx* promoter. For RMCE, the PRE300 (with or without the Pho site mutated)-*bx-d-Ubx-Z* region was transferred to attBΔ2. PRE300 was also replaced by a 500 bp λ phage DNA fragment as non-PRE negative control in this context (*λ500-bx-d-Ubx-Z*). Binding site mutations used in this study are shown in Fig. 3.

Transgenic and genetic analysis

P-element-based transgenic lines were established as previously described (Fujioka et al., 2000; Rubin and Spradling, 1982). RMCE was performed as previously described (Bateman et al., 2006). The attP target lines used were chosen at chromosomal locations that were not suggested to have PREs nearby (Negre et al., 2006; Schwartz et al., 2006; Sinclair et al., 1998; Tolhuis et al., 2006). The attP target lines 25C, 52D (Bateman et al., 2006) and 78C4 (obtained in our laboratory) were used for inserting GFP-RR. For RMCE of *bx-d-Ubx-Z* constructs, 25C, 52D, 78C4 and 95E5 (obtained in our laboratory) lines were tested. 52D and 95E5 were used for further analysis. For staining with anti-β-gal (ICN, 1:1000 dilution), embryos were collected for 6 hours at room temperature (RT), then incubated at 17°C for 15 hours. The antibody was visualized using biotin-conjugated anti-rabbit IgG followed by peroxidase-conjugated streptavidin, with DAB as a substrate.

For Fig. 4, each central nervous system (CNS) was dissected out in phosphate-buffered saline, placed on a poly-L-lysine-coated cover slip, and covered with a non-coated cover slip using another cover slip as a spacer to prevent flattening.

For *pho* mutant analysis of PSS, six PRE300-carrying lines (including two GFP-RR lines) that showed PSS were crossed to generate PRE300:*pho*¹/*ci*^D. Larvae from these stocks were grown at room temperature, and pharate adults were dissected to analyze their eye color. For *pho* and *phol* mutant analysis of positive maintenance in the CNS, a *mini-white* P-element-based GFP-RR line was used to produce GFP-RR:*phol*^{81A}/*TM6B,Tb*, GFP-RR:*pho*¹/*ci*^D or GFP-RR:*phol*^{81A}/*TM6B,Tb;pho*¹/*+*, which were then self-crossed. Homozygous *phol* larvae were tentatively identified as non-*Tubby*, and confirmed post hoc by PCR. In all cases, homozygous *pho* and *pho*⁺ larvae and pupae were identified post hoc by PCR analysis of their DNA (Brown et al., 2003). GFP-RR at 52D was crossed into mutant backgrounds to produce GFP-RR(52D);*E(z)*⁶¹/*TM6B,Tb*, GFP-RR(52D);*trx*¹/*TM6B,Tb*, and GFP-RR(52D);*Trl*^{13C}/*TM6B,Tb*. Homozygous mutant larvae from these lines were identified by their non-*Tb* phenotype. For *E(z)*⁶¹, eggs were collected at 18°C for 24 hours, kept at 18°C until near hatching, then moved to 29°C until the third larval instar. For *trx*¹, eggs were collected similarly, then moved to 25°C until the third larval instar. For *Trl*^{13C}, eggs were collected similarly, and kept at 18°C until the third larval instar.

To test effects of PcG mutations on *eve* PRE activity, lines homozygous for *bx-d-Ubx-Z*-derived transgenes (at target site 52D) were each crossed with *ph*⁵⁰³/*FM7,B* or *Pc*⁴/*TM3,Sb*. Then, *ph*⁵⁰³/*+*; *bx-d-Ubx-Z*/*+* (non-*B*) females were crossed with homozygous *bx-d-Ubx-Z* males, while *bx-d-Ubx-Z*/*+*; *Pc*⁴/*+* (non-*Sb*) males and females were intercrossed. Resulting embryos were collected as described above. Homozygous mutant embryos were identified by ectopic *lacZ* expression in anterior regions of embryos, which were not present in a wild-type background. For *ph*⁵⁰³, the ectopic *lacZ* phenotype of hemizygous mutant embryos was confirmed by double-staining for ectopic *eve* expression in the CNS (Smouse et al., 1988).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed as follows. Dechorionated embryos were crosslinked with 2% formaldehyde for 15 minutes at room temperature, washed and stored at -80°C. About 200 μl of embryos were homogenized in 1 ml Buffer A (350 mM sucrose, 15 mM HEPES pH 7.6, 10 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM EGTA). Homogenates were centrifuged at 600 g for 5 minutes to pellet nuclei, which were then washed gently with 1 ml Buffer A and repelleted. Pellets were resuspended in 1 ml Buffer A containing 0.2% Triton-X100, incubated for 5 minutes on ice, then layered on top of 3 ml 800 mM sucrose (in Buffer A) in 15 ml conical-bottom tubes, and centrifuged at 250 g for 10 minutes. The pellets were subjected to ChIP analysis essentially as described previously (Wang et al., 2004b), using Pho-specific antiserum (Brown et al., 1998). PCR primer sequences will be provided on request.

RESULTS

The *eve* 3' boundary region contains a strong PSE

In a previous study, we reported that a region between +8.6 and +9.2 kb (relative to the *eve* transcription start site), at the 3' end of the *eve* locus, caused PSS of *mini-white* expression in transgenes (Fujioka et al., 1999). This region also caused repression of *mini-white* expression in heterozygotes, making it difficult to use a standard *mini-white*-containing P-element vector to generate transgenic lines, because complementation of the *white*⁻ phenotype was extremely weak at most chromosomal locations (data not shown). In such a case, it is likely that insertions at many sites are missed, resulting in a biased sampling of potential insertion sites. To overcome this problem, three tandem Glass activator-binding sites, which are active specifically in the eye disc, were introduced just upstream of the *mini-white* promoter to increase expression (Fujioka et al., 1999). This Glass site vector was also used in the analysis of the *en* PRE (Americo et al., 2002), where it was shown that the presence of Glass

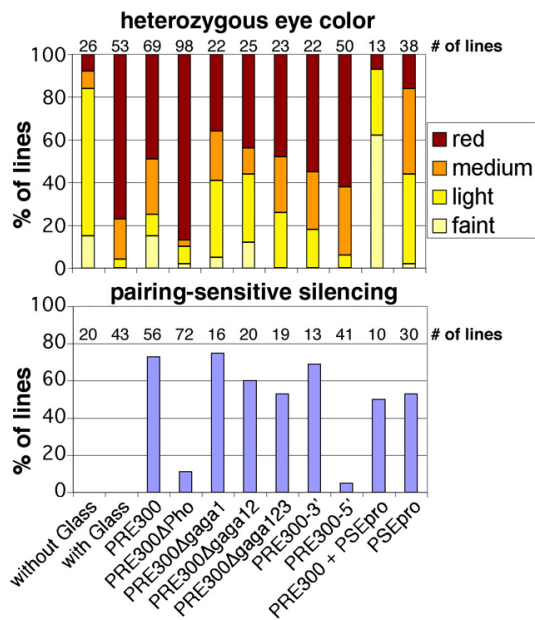


Fig. 1. Silencing activities of sequences from *even skipped*. (Top panel) Range of eye colors exhibited by heterozygous flies of different lines carrying each construct (indicated below both panels) is graphed in colors that approximate the eye color. The extent of each color bar represents the percentage of lines exhibiting that color. (Bottom panel) The percentage of lines showing pairing-sensitive silencing (PSS) is graphed. In both panels, the number of lines analyzed is indicated above each bar. The first two bars in each panel represent transgenes carrying *mini-white* without a PSS element. These showed no PSS activity, either with or without Glass-binding sites, and the presence of Glass activator binding sites strongly shifted the intensity of eye colors, indicative of increased expression of *mini-white*. PSS elements and derivatives were each assayed in the context of transgenes with Glass-binding sites (see Results). In addition, PRE300 caused PSS activity in 73% of lines and decreased the average eye color intensity; mutation of the Pho binding site (PRE300ΔPho) drastically decreased PSS activity (to 11%) and abolished the effect on eye color; mutation of either one, two or all three GAF-binding sites (PRE300Δgaga123) had a relatively weak effect on both PSS activity and eye color. The 3' region of PRE300 (PRE300-3') harbors most of its PSS activity (69% of lines for PRE300-3' compared to 5% for PRE300-5'), even though binding sites for several known PRE-binding proteins are removed (see Fig. 3). The *eve* promoter region (PSEpro) also confers PSS activity (53% of lines); combining it with PRE300 (PRE300 + PSEpro) did not significantly change this activity (50%), although it did increase the repression of heterozygous *mini-white* activity. Mutations in Pho- and GAF-binding sites, and deletions of PRE300, are as indicated in Fig. 3.

sites does not affect the percentage of PRE-carrying transgenic insertions that exhibit PSS. As expected, with the Glass-binding sites, the range of eye colors of heterozygous transgenic flies shifted toward the dark end of the spectrum (Fig. 1, compare 'with Glass' with 'without Glass'; note that none of these lines carry a PSE). Among 53 independent lines carrying the Glass vector without the PSE, 43 were homozygous viable and none showed PSS (Fig. 1, 'with Glass').

We dissected the original 600 bp PSE (+8.6 to +9.2 kb, data not shown) down to an active region of 300 bp (+8.9 to +9.2 kb). The resulting element caused repression of Glass-augmented *mini-white* expression in heterozygous transgenic flies, as evidenced by the resulting shift toward lighter eye colors ('PRE300' in Fig. 1;

compare with 'with Glass'). Among homozygous viable lines, 73% of them showed PSS activity (Fig. 1, Fig. 2A,B). We refer to this element as a PRE because of these results together with further results described below.

The *eve* promoter region (from about -700 to -100 bp), but not the *eve* 3' region, was previously identified in a genome-wide prediction of PRE/TREs based on a clustering of Pho-, GAF- and Zeste-binding sites (Ringrose et al., 2003). We discovered that the *eve* promoter region from -275 to +166 bp also has PSS activity. Fifty-three percent of transgenic lines carrying this region showed PSS (PSEpro, Fig. 1). Although the combination of PRE300 with the *eve* promoter caused more repression of heterozygous *mini-white* expression than did either one alone, PSS activity itself was not increased relative to that of PRE300 (PRE300 + PSEpro, Fig. 1).

eve* PSS activity depends on a Pho binding site, and requires *pho

As the *eve* 3' PRE showed stronger activity than the promoter, we focused on the 3' PRE in further experiments. Previous dissections of PSS regions from the *en* gene (Americo et al., 2002), the MCP silencer (Busturia et al., 2001) and the *iab-7* PRE (Hagstrom et al., 1997; Mishra et al., 2001) (both from the *bithorax* complex) showed that activity depended on both Pho- and GAF-binding sites. PRE300 contains one perfect match to the Pho-binding site consensus and three close matches to the GAF consensus. Mutating the single Pho site (Fig. 3) strongly reduced PSS activity from 73% to 11% (PRE300ΔPho, Fig. 1). This was accompanied by a shift in the spectrum of heterozygous eye colors back to that obtained without the PSE, suggesting that the cis-repressive activity of PRE300 is entirely dependent on the Pho site. Mutating two or all three of the GAF sites (Fig. 3) caused a progressive decrease of PSS activity, but only to 53% (Fig. 1: PRE300Δgaga1, Δgaga12 and Δgaga123). Mutating GAF sites also did not have as much of an effect on heterozygous eye color as did mutating the Pho site. These data suggest that the PSS activity of PRE300 depends only weakly on GAF binding sites, whereas, like other PSEs examined in detail, it is strongly dependent on the Pho-binding site.

In order to test whether this Pho-binding-site dependence reflects an actual dependence on Pho binding, or whether it might be due to some overlapping DNA-binding activity, we placed several PRE300-carrying lines in a *pho*¹ mutant background. As *pho*¹ mutants die as pharate adults, it was necessary to dissect pupae in order to examine their eye color. Compared with a wild-type background, the homozygous eye color was darker in *pho*¹ mutants in all six lines examined. This effect was very strong in one line (Fig. 2E,F), whereas the other lines showed less complete, albeit still clear, derepression (Fig. 2C,D; data not shown). Incomplete derepression is expected in the *pho* mutants, because the *pho-like* gene (*phol*) provides a partially redundant function with that of *pho*. It is not possible to carry out this analysis in *phol*^{81A}; *pho*¹ double mutants, because they fail to pupariate (Brown et al., 2003). The convergence of these genetic data with the Pho-binding-site dependence of PSS strongly suggests that Pho is directly involved in silencing through PRE300.

The involvement of known factors in *eve* PSS activity

Among known PREs/TREs, binding sites for Pho/Phol, GAF/Pipsqueak, Sp1/KLF, Zeste and Dsp1 were found to be clustered (Brown et al., 2005; Muller and Kassis, 2006; Ringrose, 2007; Schuettengruber et al., 2007). PRE300 contains all of these

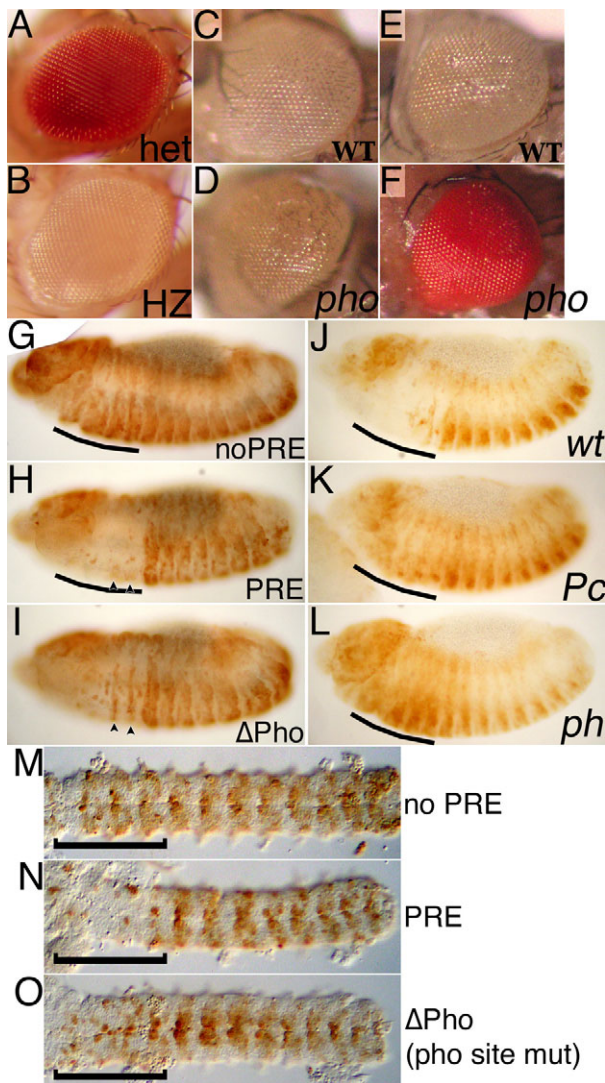


Fig. 2. The *eve* locus contains a Pho-dependent PRE. (A,B) Eyes of adult flies closely matched in age (based on body color) and within 14 hours of eclosion (during which time no discernable changes in eye color occurred), either heterozygous (A) or homozygous (B) for transgenic PRE300 in a *white* mutant background. Note the dramatically lighter color in B, owing to PSS of transgenic *mini-white*. (C,D) Eyes of pharate adults carrying a homozygous PRE300 transgene in a heterozygote (C; indistinguishable from wild-type eyes in this line) or homozygous (D) *pho*¹ mutant background. Note the slightly darker eye color in D compared with C. (E,F) Eyes of pharate adults carrying a homozygous GFP-RR transgene in a heterozygote (E; indistinguishable from wild-type eyes in this line) or homozygous (F) *pho*¹ mutant background. GFP-RR (described in the Materials and methods) carries the neuronal RP2+*a/pCC* enhancer and PRE300 (+7.9 to +9.2 kb) (Fujioka et al., 2003). As with PRE300 alone (Fig. 1), GFP-RR transgenic lines showed PSS (64%: 7 out of 11 lines). Note the darker eye color in F. (G-L) Anti-β-gal-stained stage 13 embryos carrying a *bx-d-Ubx-Z*-derived transgene. (G-I) Transgenes (indicated below) inserted at attP-target site 95E5. (G) λ500-*bx-d-Ubx-Z*, a non-PRE-containing negative control. (H) PRE300-*bx-d-Ubx-Z*. Note the lack of β-gal expression in the anterior region indicated by the black line, relative to G. (I) PRE300ΔPho-*bx-d-Ubx-Z*. Note that mutation of the Pho-binding site caused de-repression of β-gal in many cells in the anterior regions, indicated by arrowheads (compare with H). (J-L) PRE300-*bx-d-Ubx-Z* inserted at attP-target site 52D (Bateman et al., 2006). (J) Wild-type background. (K) *Pc*⁴ background. (L) *ph*⁵⁰³ background. Note de-repression of β-gal in the anterior region indicated by the black line in K and L, relative to J. (M-O) Anti-β-gal-stained CNS from stage 15 embryos carrying transgenes (indicated below) inserted at attP-target site 95E5. (M) λ500-*bx-d-Ubx-Z*. (N) PRE300-*bx-d-Ubx-Z*. Note the lack of β-gal expression in the anterior region indicated by the black bracket, relative to M. (O) PRE300ΔPho-*bx-d-Ubx-Z*. Note that mutation of the Pho-binding site caused de-repression of β-gal in many cells in the anterior region indicated by the black bracket, relative to N.

binding sites, including two Dsp1 sites, three GAF sites, two Sp1/KLF sites, one Zeste site and one Pho site (Fig. 3). It has also been shown that the *grainyhead* (*grh*) gene product (Bray and Kafatos, 1991), which was originally identified as a transcriptional activator termed Elf-1 (Bray et al., 1989), physically interacts with RING, a PcG gene product (Tuckfield et al., 2002). Importantly, Grh/Elf-1 was shown to cooperatively interact with Pho on the *iab-7* PRE in vitro, and to genetically interact with *pho* (Blastyak et al., 2006). PRE300 also contains a reasonable match to the Grh consensus site (Fig. 3). In order to test whether these binding sites are required for its PSS activity, PRE300 was split into two overlapping parts (PRE300-5' and PRE300-3'). As the Pho site is necessary for the activity, this site was included in both parts (Fig. 3). Surprisingly, PRE300-5', which includes all of these sites except for one Sp1/KLF, one GAF and the Grh site, showed PSS activity in only 5% of transgenic lines (Fig. 1). By contrast, PRE300-3', which contains only the Pho site, one Sp1/KLF site, the Grh site and one of the GAF sites, showed almost full PSS activity (69% versus 73% for PRE300, Fig. 1). These data suggest that only a subset of known binding sites are required for the PSS activity of PRE300, and further suggest that other, unidentified factors may well be involved.

Is the *eve* PSE a PRE?

Although PSS activity is often seen with transgenes carrying a PRE, not all PSEs can act as a PRE (Kassis, 2002). We directly tested for PRE activity of *eve* PRE300 by combining it in a transgene with a *bx-d-Ubx-Z* reporter gene. This reporter is expressed throughout the ectoderm and nervous system in late-stage embryos (Muller and Bienz, 1991). However, when a PRE is inserted into this reporter, it can prevent activation of *lacZ* expression in parasegments 1-5, similar to the endogenous *Ubx* PRE from the *bx-d* region (Americo et al., 2002; Brown et al., 2005; Busturia and Bienz, 1993; Hagstrom et al., 1997; Horard et al., 2000; Muller and Bienz, 1991). In two out of four P-element-based transgenic lines carrying PRE300 in this reporter, we observed an anteriorly restricted *lacZ* pattern (similar to Fig. 2H, data not shown). Similarly, studies of other PREs in this reporter found that not all transgenic lines showed repression (Americo et al., 2002; Brown et al., 2005; Busturia and Bienz, 1993; Hagstrom et al., 1997; Horard et al., 2000; Muller and Bienz, 1991), presumably owing to the effects of different chromosomal contexts. In order to further analyze the PRE activity of PRE300, we incorporated the *bx-d-Ubx-Z* reporter into a vector for RMCE (Bateman et al., 2006; Groth et al., 2004), which allows the analysis of multiple

independently inserted transgenes tested, eight gave normal expression in the embryonic CNS. Of these eight, three completely failed to maintain expression to the third larval instar (data not shown, similar to Fig. 4F), and the other five showed relatively weak and variable expression of GFP in third instar larvae (Fig. 4B and data not shown). As the Pho dependence of positive maintenance seemed to be affected somewhat by the chromosomal context, we confirmed these results by examining expression in a pair of lines, one with the Pho site mutated, inserted at the same chromosomal location using RMCE. At this target site, although GFP expression in third instar larvae was somewhat weaker than in the random insertion line shown in Fig. 4A, expression in third instar larvae was nonetheless abolished when the Pho site was mutated (Fig. 4E,F). Thus, the maintenance of CNS expression by PRE300 requires the Pho-binding site.

GAF has been shown to affect the activity of other PREs (Busturia et al., 2001; Hagstrom et al., 1997; Hodgson et al., 2001; Horard et al., 2000; Mishra et al., 2001), and to facilitate binding of Pho to a chromatinized PRE (Mahmoudi et al., 2003). Moreover, mutations in GAF sites affect the PSS activity of PRE300 (Fig. 1). Therefore, we tested the effects of GAF site mutations using both conventional P-element transformation and RMCE. When all three GAF sites were mutated, GFP expression depended strongly on the insertion site. Although two out of 11 random insertion lines showed similar levels of expression in third instar larvae as transgenes carrying wild-type PRE300, four out of 11 lines showed weak expression, and five out of 11 lines showed ectopic expression throughout the CNS, such that we could not discern the intensity in RP2 and a/pCC neurons. When RMCE was used to compare directly the activity of wild-type and triply GAF-mutated PRE300, there was no apparent change in GFP expression (data not shown). Thus, mutating sites for GAF can have either a positive or a negative effect on CNS maintenance by PRE300. The ectopic expression observed with the GAF-mutated element also suggests that wild-type PRE300 may have a negative maintenance activity in cells of the CNS where *eve* is not normally expressed, in addition to its role in positive maintenance.

As stated above in relation to PSS, it is important to determine whether the Pho-binding site dependence reflects an actual dependence on Pho binding, or whether it might be due to some overlapping DNA-binding activity. In order to test this, the GFP-RR transgene was placed in a *phol*^{81A}, *pho*¹ double mutant background. Indeed, relative to a wild-type background, GFP expression was clearly reduced (Fig. 4C, compare with 4A). In *pho*¹ single mutants, there was a reduction in most larvae (Fig. 4D), but the difference versus wild type was not as clear-cut as in the double mutant. In *phol*^{81A} single mutants, there was no apparent effect (not shown). This is consistent with the fact that *pho*¹ mutants die as pharate adults, whereas *phol*^{81A} mutants survive to adulthood (Brown et al., 2003). These data indicate that Pho and Phol are indeed involved in the maintenance not only of repression, but also of activation. Consistent with PRE300 having a negative maintenance activity in the CNS in addition to its role in positive maintenance, as suggested above, we observed widespread ectopic expression (seen as a higher background of GFP fluorescence) in some PRE300-carrying lines both in *pho/phol* double mutants and when the Pho site was mutated (e.g. Fig. 4C and Fig. 4F, respectively).

As a preliminary test of the involvement of other PcG genes in positive maintenance by PRE300, we tested the allele *E(z)*⁶¹, as homozygous larvae survive to the third larval instar. However, we did not see any effects on positive maintenance (data not shown).

We also tested some *trxG* alleles that survive to the third larval instar. Larvae homozygous for *trx*¹ and *Trl*^{13C} did not show any effects on CNS expression of our GFP reporter (data not shown). Larvae heterozygous for *trx*^{B11}, *Trl*^{R65} and *Trl*^{R85} were also tested, and, again, no clear effects were observed (data not shown). However, because these alleles have significant maternal and zygotic contributions that allow them to survive long enough to be assayed, further analysis will be required to determine definitively whether these genes are involved along with *pho* and *phol* in positive maintenance by PRE300.

Pho binds to the *eve* PRE in vivo

The convergence of genetic and biochemical evidence presented above strongly suggests that Pho acts directly through the binding site in PRE300. To confirm that Pho interacts with this region, and with this site, in vivo, we performed ChIP assays. First, we surveyed the *eve* locus for Pho binding using a series of primer sets for detection of immunoprecipitated fragments by PCR. As shown in Fig. 5 (B and E, primer set 13), Pho was detected specifically in the region of PRE300, both in early and later-stage embryos. Pho was also detected in the promoter region where binding sites were previously predicted, primarily in early embryos (Fig. 5B, primer set 6), as well as in the region of enhancers driving mesodermal and anal plate expression (Fig. 5B,E, primer set 8). This confirms that Pho interacts with the endogenous *eve* locus. Furthermore, it does so

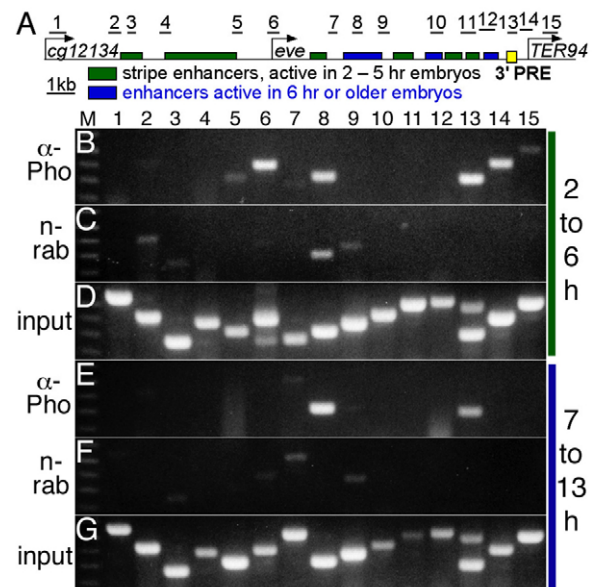


Fig. 5. Pho binds to the *eve* locus. (A) Map of the *eve* locus showing the location of primer sets (numbered black bars) used to analyze Pho binding. The location of the 3' PRE, PRE300, is shown as a yellow box. (B-G) Analysis of Pho binding in wild type (*yw*) 2-6 hours AED (after egg deposition, B-D) and 7-13 hours AED (E-G) embryos. For 2-6 hours AED embryos, eggs were collected for 4 hours and developed for 2 hours at room temperature; these embryos spanned stages 4-11. For 7-13 hours AED embryos, eggs were collected for 6 hours at room temperature and developed overnight at 17°C; these embryos spanned stages late 11 to early 16. (B,E) ChIP with Pho-specific antiserum (raised in rabbit). (C,F) Negative control: parallel analysis using normal rabbit serum. (D,G) Positive control: parallel PCR reactions using 1/300 of input material. Note that, in addition to positive signals from elsewhere in the locus, some of which change with time, Pho binding is specifically detected at the 3' PRE in both early and later-stage embryos.

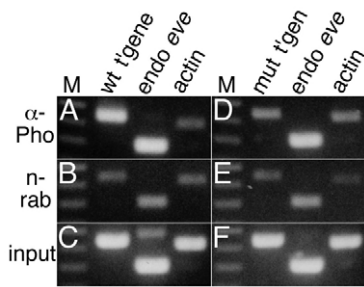


Fig. 6. The Pho consensus site is required for Pho binding in vivo.

ChIP analysis of Pho binding (as in Fig. 5) to transgenes carrying PRE300 (GFP-RR, Fig. 2), either with a normal (A-C) or a mutated (D-F) Pho site, in 0-15 hours AED embryos (eggs were collected for 15 hours at temperature; embryos spanned stages 1 to late 16). The signal from the transgene ('wt t'gene' or 'mut t'gene', using a transgene-specific primer set) is compared in each case with that from the endogenous *eve* 3' PRE ('endo *eve*', primer set 13 of Fig. 5), as well as to a gene (*actin*) that Pho is not expected to bind. Note that the Pho signal is reduced when the Pho site is mutated (transgene signal in D versus that in A), relative both to the endogenous *eve* signal and to the (presumably background) signal from the *actin* gene.

specifically with the region that our transgenic analysis implicated in maintenance of both the repressed and activated state. To test the involvement of the functionally important Pho-binding site, we tested transgenes carrying PRE300, either with the normal sequence, or with the Pho site mutated. Again, ChIP analysis indicated that Pho interacts with the transgenic copy of PRE300 (Fig. 6A). Furthermore, the signal is reduced with the mutant Pho site, relative to both the endogenous *eve* signal and to the background controls (Fig. 6D, compare to Fig. 6A). This indicates that the site is important for Pho binding in vivo, and the loss of binding correlates with the loss of both repression and activation that occurs when the site is mutated.

DISCUSSION

In this study, we dissected a strong pairing sensitive silencing element from the 3' boundary of the *eve* locus. We found that silencing activity depends on a single Pho-binding site, whereas sites for a number of other proteins found in such elements are less important. The element is genetically responsive to PcG-group activity, as it depends on *pho* gene function. This *eve* 3' PRE has bona fide PRE activity, which can maintain a silenced state established in embryos.

Previous studies have suggested that PRE-containing P-element-based transgenes have a tendency to insert near endogenous PREs, and that this can bias reporter gene expression. Here, we applied both P-element analysis and the RMCE system to compare the effects of mutating binding sites. Our data reveal that there is also variation in PRE effects using RMCE into different target sites. Therefore, it would seem important to test several target sites when using RMCE, to ensure that results are not specific to one chromosomal location. Furthermore, where sensitivity to position effects is high, such as with our GAF site-mutated PRE, it remains valuable to use the standard methodology to probe a variety of insertion sites.

Surprisingly, we found that the *eve* PRE is also required for positive maintenance of expression in the larval CNS, and that this activity requires both the Pho-binding site and *pho* gene

function. Together, these data strongly suggest that Pho is directly involved in positive maintenance of gene activity. This is surprising because Pho has heretofore been associated only with direct repression of target genes, by recruiting the PRC2 complex and other PcG proteins (Wang et al., 2004b). However, recent studies have blurred the distinction between PcG genes and trxG genes, as some members of each class appear to have dual functions (Grimaud et al., 2006). Furthermore, PREs usually reside in close proximity to TREs (Ringrose and Paro, 2007), and an element from the promoter region of *engrailed* that mediates PSS and can act as a PRE was recently shown to have an activating role in its natural context (Devido et al., 2008). Recent studies of the *Ubx* locus have indicated that PcG proteins are present at PREs in both the off and the on state, and that binding of Ash1 prevents silencing by the PRC complex in cells where *Ubx* is expressed, suggesting that silencing is actively prevented (Papp and Muller, 2006). A similar situation may pertain to Pho function in the *eve* locus.

Because trxG proteins are known to be involved in positive regulation by other maintenance elements, we were interested in whether they are involved in *pho*-dependent positive maintenance by the *eve* PRE. We were also interested in whether other PcG proteins are involved. Because our positive maintenance assay requires survival to the third larval instar, we have so far been able to test only weak alleles of *trx*, *Trl* and *E(z)*, neither of which showed discernable effects in our assays. At this point, we can not definitively say whether other trxG or PcG proteins are involved in the positive maintenance function of the *eve* PRE. However, our observation that a consensus Grh binding site is present in the more active half of PRE300 suggests the involvement of Grh. Indeed, Grh has been shown to interact genetically with Pho, and to facilitate cooperative interaction with Pho in vitro (Blastyak et al., 2006).

Consistent with the broad overexpression of *eve* seen in the CNS of *ph* mutants (Smouse et al., 1988), the *eve* PRE may silence expression in many cells by forming a silencing complex. In wild-type embryos, in the subset of CNS cells where *eve* is expressed, the same Pho-dependent DNA binding platform may recruit a distinct complex that maintains the active state. Consistent with this model, we have found that expression driven by the *eve* RP2+a/pCC enhancer fades prematurely in late stage embryos in *ph* mutants, at the same time that endogenous *eve* is broadly overexpressed (M.F. and J.B.J., unpublished). It will be interesting to determine the composition of Pho-dependent complexes in cells where *eve* is on, and in those where *eve* is off.

How can a region 9 kb away from the basal promoter affect the state of gene expression? There are accumulating data suggesting that locus-wide regulation occurs through direct interactions of the promoter with enhancers and locus control regions. For example, a recent study showed that silencing by the *bxd* PRE directly affects the activity of the transcriptional machinery at the promoter (Dellino et al., 2004). In the *eve* locus, there are PSEs both at the 3' end of the locus and at the promoter. Both contain clusters of binding sites typical of a PRE/TRE. It has been suggested that PRE-containing transgenes have a tendency to insert near endogenous PREs (Chiang et al., 1995; Fauvarque et al., 1995), which might be expected if they mediate long-range interactions. Putting these ideas together, the *eve* 3' PRE may physically interact with the promoter region in a Pho-dependent manner. This may serve to keep *eve* on in some cells and to keep it off in others, depending on whether activating or repressive complexes mediate the association.

We thank Xian Wu for excellent technical assistance, Judy A. Kassis for plasmids and Pho antiserum; Michele P. Calos, C.-Ting Wu and Jack R. Bateman for reagents for the Φ C31 system; and Hugh W. Brock, Paul Schedl, Judy A. Kassis and the Bloomington Stock Center for mutant strains. We also thank Judy A. Kassis, Alex Mazo and Hugh W. Brock for comments on the manuscript. This work was supported by NSF-I0B0416760, NSF-MCB0818118 and NIH-R01GM050231 awards to J.B.J. and M.F.

References

- Americo, J., Whiteley, M., Brown, J. L., Fujioka, M., Jaynes, J. B. and Kassis, J. A.** (2002). A complex array of DNA-binding proteins required for pairing-sensitive silencing by a polycomb group response element from the *Drosophila engrailed* gene. *Genetics* **160**, 1561-1571.
- Badenhorst, P., Voas, M., Rebay, I. and Wu, C.** (2002). Biological functions of the ISWI chromatin remodeling complex NURF. *Genes Dev.* **16**, 3186-3198.
- Bantignies, F., Goodman, R. H. and Smolik, S. M.** (2000). Functional interaction between the coactivator *Drosophila* CREB-binding protein and ASH1, a member of the trithorax group of chromatin modifiers. *Mol. Cell. Biol.* **20**, 9317-9330.
- Bateman, J. R., Lee, A. M. and Wu, C. T.** (2006). Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange. *Genetics* **173**, 769-777.
- Blastyak, 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.
- Bray, S. J. and Kafatos, F. C.** (1991). Developmental function of Elf-1: an essential transcription factor during embryogenesis in *Drosophila*. *Genes Dev.* **5**, 1672-1683.
- Bray, S. J., Burke, B., Brown, N. H. and Hirsh, J.** (1989). Embryonic expression pattern of a family of *Drosophila* proteins that interact with a central nervous system regulatory element. *Genes Dev.* **3**, 1130-1145.
- Brock, H. W. and van Lohuizen, M.** (2001). The Polycomb group-no longer an exclusive club? *Curr. Opin. Genet. Dev.* **11**, 175-181.
- 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., Fritsch, C., Mueller, 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.
- 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.
- Busturia, A. and Bienz, M.** (1993). Silencers in abdominal-B, a homeotic *Drosophila* gene. *EMBO J.* **12**, 1415-1425.
- 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.
- Buszczak, M. and Spradling, A. C.** (2006). Searching chromatin for stem cell identity. *Cell* **125**, 233-236.
- 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.
- Chiang, A., O'Connor, M. B., Paro, R., Simon, J. and Bender, W.** (1995). Discrete Polycomb-binding sites in each parasegmental domain of the *bithorax* complex. *Development* **121**, 1681-1689.
- 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.
- Dellino, G. I., Schwartz, Y. B., Farkas, G., McCabe, D., Elgin, S. C. and Pirrotta, V.** (2004). Polycomb silencing blocks transcription initiation. *Mol. Cell* **13**, 887-893.
- Devido, S. K., Kwon, D., Brown, J. L. and Kassis, J. A.** (2008). The role of Polycomb-group response elements in regulation of *engrailed* transcription in *Drosophila*. *Development* **135**, 669-676.
- Fauvarque, M. O., Zuber, V. and Dura, J. M.** (1995). Regulation of *polyhomeotic* transcription may involve local changes in chromatin activity in *Drosophila*. *Mech. Dev.* **52**, 343-355.
- 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.
- Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T. and Jaynes, J. B.** (1999). Analysis of an *even-skipped* rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development* **126**, 2527-2538.
- Fujioka, M., Jaynes, J. B., Bejsovec, A. and Weir, M.** (2000). Production of transgenic *Drosophila*. *Methods Mol. Biol.* **136**, 353-363.
- Fujioka, M., Lear, B. C., Landgraf, M., Yusibova, G. L., Zhou, J., Riley, K. M., Patel, N. H. and Jaynes, J. B.** (2003). Even-skipped, acting as a repressor, regulates axonal projections in *Drosophila*. *Development* **130**, 5385-5400.
- Fyodorov, D. V., Blower, M. D., Karpén, G. H. and Kadonaga, J. T.** (2004). Acf1 confers unique activities to ACF/CHRAC and promotes the formation rather than disruption of chromatin in vivo. *Genes Dev.* **18**, 170-83.
- Gindhart, J. G., Jr and Kaufman, T. C.** (1995). Identification of Polycomb and trithorax group responsive elements in the regulatory region of the *Drosophila* homeotic gene *Sex combs reduced*. *Genetics* **139**, 797-814.
- Gordon, S., Akopyan, G., Garban, H. and Bonavida, B.** (2006). Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. *Oncogene* **25**, 1125-1142.
- Grimaud, C., Negre, N. and Cavalli, G.** (2006). From genetics to epigenetics: the tale of Polycomb group and trithorax group genes. *Chromosome Res.* **14**, 363-375.
- Groth, A. C., Olivares, E. C., Thyagarajan, B. and Calos, M. P.** (2000). A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* **97**, 5995-6000.
- Groth, A. C., Fish, M., Nusse, R. and Calos, M. P.** (2004). Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics* **166**, 1775-1782.
- Hagstrom, K., Muller, M. and Schedl, P.** (1997). A Polycomb and GAGA dependent silencer adjoins the Fab-7 boundary in the *Drosophila bithorax* complex. *Genetics* **146**, 1365-1380.
- Hodgson, J. W., Argiropoulos, B. and Brock, H. W.** (2001). Site-specific recognition of a 70-base-pair element containing d(GA)(n) repeats mediates *bithoraxoid*-polycomb group response element-dependent silencing. *Mol. Cell. Biol.* **21**, 4528-4543.
- 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.
- Kassis, J. A.** (1994). Unusual properties of regulatory DNA from the *Drosophila engrailed* gene: three 'pairing-sensitive' sites within a 1.6-kb region. *Genetics* **136**, 1025-1038.
- Kassis, J. A.** (2002). Pairing-sensitive silencing, polycomb group response elements, and transposon homing in *Drosophila*. *Adv. Genet.* **46**, 421-438.
- Kassis, J. A., VanSickle, E. P. and Sensabaugh, S. M.** (1991). A fragment of *engrailed* regulatory DNA can mediate transvection of the *white* gene in *Drosophila*. *Genetics* **128**, 751-761.
- Kennin, J. A.** (2004). Introduction to Trx-G and Pc-G genes. *Methods Enzymol.* **377**, 61-70.
- 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.
- Mahmoudi, T., Zuijderduijn, L. M., Mohd-Sarip, A. and Verrijzer, C. P.** (2003). GAGA facilitates binding of Pleiohomeotic to a chromatinized Polycomb response element. *Nucleic Acids Res.* **31**, 4147-4156.
- 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.
- Muller, J. and Bienz, M.** (1991). Long range repression conferring boundaries of *Ultrabithorax* expression in the *Drosophila* embryo. *EMBO J.* **10**, 3147-3155.
- Muller, J. and Kassis, J. A.** (2006). Polycomb response elements and targeting of Polycomb group proteins in *Drosophila*. *Curr. Opin. Genet. Dev.* **16**, 476-484.
- 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.
- 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.
- Papp, B. and Muller, J.** (2006). Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. *Genes Dev.* **20**, 2041-2054.
- Petruk, S., Sedkov, Y., Smith, S., Tillib, S., Kraevski, V., Nakamura, T., Canani, E., Croce, C. M. and Mazo, A.** (2001). Trithorax and dCBP acting in a complex to maintain expression of a homeotic gene. *Science* **294**, 1331-1334.
- Ringrose, L.** (2007). Polycomb comes of age: genome-wide profiling of target sites. *Curr. Opin. Cell Biol.* **19**, 290-297.
- 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.

- Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
- 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.
- Sinclair, D. A., Milne, T. A., Hodgson, J. W., Shellard, J., Salinas, C. A., Kyba, M., Randazzo, F. and Brock, H. W. (1998). The *Additional sex combs* gene of *Drosophila* encodes a chromatin protein that binds to shared and unique Polycomb group sites on polytene chromosomes. *Development* **125**, 1207-1216.
- Smouse, D., Goodman, C., Mahowald, A. and Perrimon, N. (1988). *polyhomeotic*: a gene required for the embryonic development of axon pathways in the central nervous system of *Drosophila*. *Genes Dev.* **2**, 830-842.
- Sparmann, A. and van Lohuizen, M. (2006). Polycomb silencers control cell fate, development and cancer. *Nat. Rev. Cancer* **6**, 846-856.
- Tolhuis, B., de Wit, E., Muijers, 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.
- Tuckfield, A., Clouston, D. R., Wilanowski, T. M., Zhao, L. L., Cunningham, J. M. and Jane, S. M. (2002). Binding of the RING polycomb proteins to specific target genes in complex with the grainyhead-like family of developmental transcription factors. *Mol. Cell. Biol.* **22**, 1936-1946.
- 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.