Published in final edited form as:
Adv Genet. 2013 ; 81: 83–118. doi:10.1016/B978-0-12-407677-8.00003-8.

Polycomb Group Response Elements in *Drosophila* and Vertebrates

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Abstract

Polycomb group genes (PcG) encode a group of about 16 proteins that were first identified in *Drosophila* as repressors of homeotic genes. PcG proteins are present in all metazoans and are best characterized as transcriptional repressors. In *Drosophila*, these proteins are known as epigenetic regulators because they remember, but do not establish, the patterned expression state of homeotic genes throughout development. PcG proteins, in general, are not DNA binding proteins, but act in protein complexes to repress transcription at specific target genes. How are PcG proteins recruited to the DNA? In *Drosophila*, there are specific regulatory DNA elements called Polycomb group response elements (PREs) that bring PcG protein complexes to the DNA. *Drosophila* PREs are made up of binding sites for a complex array of DNA binding proteins. Functional PRE assays in transgenes have shown that PREs act in the context of other regulatory DNA and PRE activity is highly dependent on genomic context. *Drosophila* PREs tend to regulate genes with a complex array of regulatory DNA in a cell or tissue-specific fashion and it is the interplay between regulatory DNA that dictates PRE function. In mammals, PcG proteins are more diverse and there are multiple ways to recruit PcG complexes, including RNA-mediated recruitment. In this review, we discuss evidence for PREs in vertebrates and explore similarities and differences between *Drosophila* and vertebrate PREs.

1. INTRODUCTION

Polycomb group proteins (PcG) are important regulators of developmental genes in all metazoans (two reviews: Beisel & Paro, 2011; Simon & Kingston, 2009). First discovered in genetic studies as regulators of homeotic genes in *Drosophila*, genome-wide chromatin-immunoprecipitation (ChIP) studies using antibodies against various PcG proteins show that there are likely hundreds of PcG targets in *Drosophila* (Négre et al., 2006; Schwartz et al., 2006, 2010; Tolhuis et al., 2006). In vertebrate embryonic stem (ES) cells, PcG proteins are important regulators of developmental genes and are important in both maintenance of pluripotency and in differentiation. Because they regulate developmental and cell cycle genes, altered expression of *PcG* genes has been associated with many cancers (reviews: Geini & Hendzel, 2009; Sparmann & van Lohuizen, 2006).

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There are at least 16 *PcG* genes in *Drosophila*, identified by mutations that derepress homeotic gene expression. Many *PcG* genes encode proteins that act in protein complexes to regulate transcription via alterations to chromatin structure. The two best studied PcG protein complexes are PRC1 and PRC2 (Polycomb repressive complexes 1 and 2), which are conserved from flies to mammals (for reviews, see Kerppola, 2009; Müller & Verrijzer, 2009; Schuettengruber & Cavalli, 2009; Simon & Kingston, 2009). PRC2 contains the PcG proteins Enhancer of zeste (E(z)), Extra sex combs (Esc), Suppressor of zeste 12 (Su(z)12), as well as the protein p55 (Cao et al., 2002; Czermin et al., 2002; Müller et al., 2002), and in some tissues Polycomb-like (Pcl), which modifies PRC2 function (Nekrasov et al., 2007; Savla, Benes, Zhang, & Jones, 2008). E(z), the catalytic component of PRC2, trimethylates histone H3 lysine 27 creating the H3K27me3 mark characteristic of Polycomb-regulated genes. The PRC1 core complex is comprised of Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc), and Sex combs extra/dRing1 (Sce/dRing1; Fritsch, Beuchle, & Müller, 2003) and acts to inhibit chromatin remodeling and compact chromatin (Saurin, Shao, Erdjument-Bromage, Tempst, & Kingston, 2001; Shao et al., 1999). Psc and Sce/dRing1 are also in another complex called dRAF that contains a histone demethylase, dKDM2 (Lagarou et al., 2008). Another PcG complex, PR-DUB (Polycomb repressive deubiquitinase) consists of two other PcG proteins, Calypso and Additional sex combs (Asx), which deubiquitinates H2A118 (Scheuermann et al., 2010; Schuettengruber & Cavalli, 2010). Additional PcG proteins required for homeotic gene silencing not yet assigned to a protein complex include Sex combs on middle leg (Scm), which loosely associates with PRC1, and Super sex combs (Sxc/Ogt) that encodes O-GlcNAc transferase and modifies Ph (Gambetta, Oktaba, & Müller, 2009). Finally, *Drosophila* has the protein complex Pho-repressive complex (PhoRC), consisting of the DNA binding PcG protein Pleiohomeotic (Pho) and the methyl-lysine-binding protein, dSfmbt (Klymenko et al., 2006). Both Pho and dSfmbt have homologues in vertebrates, but no vertebrate PhoRC complex has been described.

Genome-wide studies have shown that PcG proteins colocalize to many or most PcG target genes in *Drosophila*. Despite this, mutations in various *PcG* genes give very different phenotypes (Breen & Duncan, 1986). This suggests that different target genes have different requirements for specific PcG proteins. For example, at the PcG target gene *engrailed* (*en*), a gene required for segmentation, mutations in *ph* cause a massive *en* derepression in embryos, while mutations in *Pc* cause very little *en* misexpression (Moazed & O'Farrell, 1992). Since Ph and Pc are both components of PRC1, and both are bound to *en* PREs in *Drosophila* embryos (Négre et al., 2006), why *en* is so sensitive to the loss of Ph but not Pc is unclear. In a recent study, Gutiérrez et al. (2012) began to classify different PcG targets based on their response to *Sce* mutations. They studied targets that bind the PcG proteins Pho, Sce, Pc, Ph, and Psc, and found that expression of only a subset of the targets was altered in *Sce* mutants. Clearly, there is still a lot we need to learn about the role of different PcG proteins in transcriptional repression of different target genes.

In this review, we focus on the fundamental question of how PcG proteins are recruited to their target genes. This topic has been comprehensively covered in a number of recent reviews (Beisel & Paro, 2011; Müller & Kassis, 2006; Ringrose & Paro, 2007;

Schuettengruber & Cavalli, 2009); here, we bring our own perspective and discuss unresolved issues. In *Drosophila*, we concentrate on target genes that are bound by both PRC1 and PRC2 and describe what is known about Polycomb group response element (PREs), DNA fragments that can recruit PRC1 and PRC2 to target genes via a complex array of DNA binding proteins. In vertebrates, recruitment of PcG proteins is more complicated, given that there are many more PcG proteins and varieties of PcG protein complexes (Beisel & Paro, 2011; Kerppola, 2009). While it has been demonstrated that RNA is involved in recruitment of PcG protein complexes at some vertebrate PcG targets, a role for RNA in PcG protein binding in *Drosophila* has not been demonstrated. Thus, for the vertebrate system, we limit our discussion to presumptive PREs.

2. PREs IN *DROSOPHILA*

2.1. PRE maintenance assays

PREs were first discovered in transgenes of regulatory DNA from the Bithorax complex (BX-C). In 1991, Müller and Bienz, while studying regulatory DNA from the *Ubx* gene, identified a DNA fragment that they called BXD, which, in combination with another regulatory fragment that initiated correct *Ubx* expression, could maintain β-galactosidase expression throughout embryonic development in a pattern similar to *Ubx*. Notably, in a *Polycomb* mutant, β-galactosidase expression was derepressed late in embryonic development, suggesting that *Polycomb* directly repressed the expression of the transgene (Müller & Bienz, 1991). In 1993, Simon et al. identified three discrete fragments of regulatory DNA from the BX-C that rendered transgenes responsive to PcG repression and coined the term Polycomb group response elements (PREs). Several important principles came out of this and other work on PREs from the BX-C complex. First, PREs act with other regulatory DNA to remember boundaries set up directly by gap and pair-rule regulators. Second, PREs respond to mutations in many different *PcG* genes, suggesting PcG proteins act together to repress gene expression. Third, discrete BX-C PREs have similar activities and can be interchangeable within transgenes (Simon, Chiang, Bender, Shimell, & O'Connor, 1993).

The principles of PREs learned from the early experiments on BX-C PREs apply to most if not all of the PREs studied to date. We study PREs from the *Drosophila engrailed* (*en*) and *invected* (*inv*) genes. *en* and *inv* encode coregulated, highly related homeodomain proteins which function together during development to control embryonic segmentation and formation of the posterior compartment in imaginal disks (Gustavson, Goldsborough, Ali, & Kornberg, 1996). *en* and *in* exist in a 115-kb chromatin domain, marked by the distinctive H3K27me3 modification put on by the PRC2 PcG protein complex (Kharchenko et al., 2011). There are four major PREs present in the *en*/*inv* domain (Cunningham, Brown, & Kassis, 2010; DeVido, Kwon, Brown, & Kassis, 2008) and their activity can be demonstrated in the functional assay shown in Fig. 3.1. A transgene containing 8 kb of upstream *en* regulatory DNA fused to the reporter gene *lacZ* expresses β-galactosidase in *en*-like stripes throughout embryonic development. However, deletion of a 2-kb fragment, that includes two PREs, while still giving *en*-like stripes early in development, gives expression throughout the embryo in late development. Thus, the 2-kb fragment, containing two PREs,

is required for maintenance of *en* stripes. In this construct, the activity of both PREs is required for full maintenance (DeVido et al., 2008). Interestingly, the *en* PREs are also able to act as PREs in an embryonic *Ubx*-reporter construct (Americo et al., 2002) and PRE_D, a PRE from the *Ubx* gene, can act with *en* regulatory DNA to maintain *en*-expression in stripes (Cunningham et al., 2010). These data emphasize the functional similarity between *en* and *Ubx* PREs.

In addition to embryonic reporter constructs, there are at least two imaginal disk *Ubx-lacZ*-reporter constructs for testing PRE activity (Fritsch, Brown, Kassis, & Müller, 1999; Poux, Kostic, & Pirrotta, 1996); both constructs contain combinations of *Ubx*-regulatory DNA, including an imaginal disk enhancer. Without a PRE, β-galactosidase is ubiquitously expressed in the wing disk from these constructs, where *Ubx* is not normally expressed; addition of a PRE represses wing disk expression of the *Ubx-lacZ*-reporter. An *Ubx-lacZ* imaginal disk reporter construct was used to refine the size of the *Ubx bxd* PRE, PRE_D to a 567-bp fragment and to demonstrate the importance of the PcG-DNA-binding protein Pho to PRE_D activity in larvae (Fritsch et al., 1999). Recently, an imaginal *Ubx-lacZ*-reporter was used to test the activity of presumptive PREs from the *Psc–Su*(*z*)*2* gene complex (Park, Schwartz, Kahn, Asker, & Pirrotta, 2012). These results again demonstrated that DNA from different genes can act as PREs with *Ubx*-regulatory DNA.

Only a few of the hundreds of PREs identified in genome-wide studies have been tested for activity in either embryonic or imaginal disk PRE reporter constructs and even fewer have been tested in both (see Table 3.1). It might be that some PREs are stage or tissue specific (see below). The development of a series of PRE vectors, with regulatory DNA from different genes, designed to assess activity at different developmental stages using the phi-C31 system (Groth, Fish, Nusse, & Calos, 2004) to insert transgenes at the same chromosomal location, would be key to assessing the functional equivalency between PREs.

2.2. Mini-*white* silencing

The discovery of silencing of the mini-*white* gene in transgenic *Drosophila* coincided with the discovery of PREs (Kassis, Vansickle, & Sensabaugh, 1991). In 1988, Pirrotta developed *pCaSpeR*, a vector for P-element transformation using the mini-*white* gene as a reporter for transgenic flies (Pirrotta, 1988). *white* encodes a member of the ABC family of transporter superfamily and is necessary for the transport of the precursors of eye color pigment into the eye. Flies with a null mutation in *white* have a white-eye color. To make transgenic flies with the *pCaSpeR* vector, embryos mutant for the *white* gene are injected, mated, and screened for transgenic progeny that have colored eyes. The amount of *white* gene product produced determines the eye color; that is, a little White product gives a light yellow eye, more White product gives an orange eye all the way to the wild-type red eye color. The mini-*white* gene lacks most of the enhancers that cause *white* to be expressed in the eye; eyes from *pCaSpeR*-containing transgenic flies have an eye color ranging from yellow to red. This is because the expression of mini-*white* is highly susceptible to regulation by flanking genomic DNA. The *pCaSpeR* vector soon became the vector of choice because of two properties: (1) it was easier to clone into than other P-based vectors and (2) transgenic flies with independent insertion sites could be recognized by their different eye colors.

In 1991, Kassis et al. reported that a fragment of regulatory DNA from the *Drosophila en* gene could mediate mini-*white* silencing in flies homozygous for the *en*-mini-*white* containing transgenes (*P*[*en-mw*]). Normally, when using a vector with mini-*white*, flies homozygous for the transgene have a darker eye color than flies heterozygous for it (*P*[*mw*] in Fig. 3.2). However, when a 2.4-kb fragment of *en* regulatory DNA was included in the mini-*white* vector, the eye color of homozygous flies was often lighter than that of heterozygous flies, sometimes even white, suggesting that the mini-*white* expression was completely silenced in the eye. This mini-*white* silencing was dependent on the two transgenes being inserted near each other in the genome, either in *cis* (a duplicated *P*[*en-mw*] element would also silence mini-*white*) or in *trans*, that is, on homologous chromosomes (Kassis et al., 1991). Somatic chromosomes are paired in *Drosophila*, and this mini-*white* silencing was dependent on the pairing of the two *P*[*en-mw*] transgenes. This phenomenon has been called pairing-dependent repression or pairing-sensitive silencing (PSS) (Kassis, 1994). The 2.4-kb *en* fragment contained within the *P*[*en-mw*] transgenes contains two PREs (Americo et al., 2002; DeVido et al., 2008; Kassis, 1994).

Mini-*white* silencing is a property of PREs (reviewed in Kassis, 2002). In 1994, Chan, Rastelli, and Pirrotta reported that a 2.2-kb PRE from *Ubx* caused silencing of the mini-*white* gene in the *pCaSpeR* vector. In that case, an *Ubx* PRE repressed mini-*white* expression even in the heterozygous state; many *Ubx* PRE-mini-*white* transformants had variegated eyes, some with the eye color only present in a few ommatidia. Using a different vector that contained both mini-*white* and *hs-neo* genes and selecting flies using G418 resistance, many of the lines obtained showed no detectable eye color and thus would not have been obtained if eye color had been used as the selectable marker. The 2.2-kb *Ubx bxd* PRE fragment they used was subsequently dissected into subfragments, six of which, when multimerized, mediated mini-*white* suppression (Horard, Tatout, Poux, & Pirrotta, 2000) in slightly different ways. Some subfragments gave transgenic flies with variegated eyes; one gave flies with patterned eyes (eye color in only part of the eye, but reproducibly in the same part of each eye). Of three multimerized subfragments tested for PREs in a maintenance assay in embryos, only one acted as a PRE. The data suggest that the 2.2-kb *Ubx bxd* PRE fragment contains multiple elements that function together to regulate *Ubx* expression throughout development.

Use of the FRT-Flp and LoxP-Cre recombinase systems to remove PREs from *pCaSpeR*-containing transgenes has shown that all PREs tested repress eye color to some extent in the heterozygous state. Figure 3.2 shows an example of this. In *P*[*PREmw*], the 181-bp *en* PRE is flanked by LoxP sites. At this chromosomal insertion site, *P*[*PREmw*] flies have a light orange eye color. Removal of the PRE via Cre recombinase yields flies with a slightly darker eye color. The effect of the 181-bp *en* PRE is much more dramatic in the homozygous flies; with the PRE, the eye color is white; without the PRE, the eye color is red (Noyes et al., 2011).

Silencing of mini-*white* expression either in the heterozygous state or via PSS is the most common assay used to test the function of a presumptive PRE (Table 3.1). Is this a legitimate assay for PRE activity? There are a few reported cases where PREs that work in maintenance assays in embryos or larvae do not repress mini-*white* expression and vice

versa (reviewed in Kassis, 2002), but, in general, there is an excellent correlation between PRE activity and PSS. Some minimal PREs may be stage or tissue specific and may not work in both assays. Results from our lab show a perfect correlation between fragments of *en*/*inv* DNA that mediate PcG repression in embryos and PSS (Americo et al., 2002; Brown, Grau, DeVido, & Kassis, 2005; Cunningham et al., 2010; DeVido et al., 2008). Thus, PSS is a good indication of PRE activity.

2.3. Effect of flanking regulatory DNA on PRE activity

In vivo, PREs can be located tens of kilobases away from the promoter they regulate. How are they able to act over such large distances? *In vitro*, PRC1 inhibits chromatin remodeling and transcription (reviewed in Simon & Kingston, 2009). Interestingly, PRC1 bound to a polynucleosome template *in vitro* can recruit a second template and inhibit chromatin remodeling and transcription of that template as well (Lavigne, Francis, King, & Kingston, 2004). Further, the *Drosophila* PRC1 subunit Psc can bind to and compact chromatin, an activity conserved in the M33 Polycomb homolog in mice (Grau et al., 2011).

The PSS assay suggests that PREs act together to facilitate gene silencing. The physical interaction between the PREs and promoters of the BX-C has been well documented by chromatin chromosome capture (3C) and fluorescent *in situ* hybridization (Lanzuolo, Roure, Dekker, Bantignes, & Orlando, 2007). Genome-wide chromatin conformation studies suggest that PcG proteins are a major organizer of the three-dimensional structure of the genome (recently reviewed in Delest, Sexton, & Cavalli, 2012; Pirrotta & Li, 2012). PcG proteins are present in “Polycomb bodies” where concentrations of PcG proteins appear as “dots” in the cell. Interestingly, PcG target genes colocalize with PcG bodies only in those cells where they are repressed (see Delest et al., 2012; Pirrotta & Li, 2012).

In a PRE-transgene assay, a PRE is removed from its normal chromatin context, often combined with regulatory DNA from a gene it normally does not regulate, and then inserted into a region of the genome where it normally does not reside. Given that PREs are designed to interact with other regulatory DNA, it is not surprising that the activity of a PRE in a transgene is dependent on where it is inserted in the genome. For example, PSS mediated by *en* PREs occurs in only about 50% of transgenic lines, and the extent of silencing also depends on the chromosomal insertion site (Americo et al., 2002; Noyes et al., 2011). Various models can be proposed to explain this. First, since PREs often work together, perhaps PSS only occurs in regions of the genome where there is another PRE nearby; there is some evidence to suggest that PRE-containing transgenes have a tendency to insert in the genome near PcG-regulated genes (reviewed in Kassis, 2002 and see below). Second, if the transgene inserts downstream of a promoter, it might be transcribed; transcription through a PRE has been shown to inactivate it (Schmitt, Prestel, & Paro, 2005). Finally, genomic enhancers flanking the PRE-transgene insertion site might activate mini-*white* expression via a mechanism not subject to PcG repression. A mammalian enhancer that can evict PcG proteins has recently been described (Vernimmen et al., 2011).

Recent work from our lab shows the effect of chromatin context (or flanking DNA) on PRE function. We carried out a genetic screen for dominant suppressors of PSS of an *en* PRE-mini-*white* transgene (*P*[*enPRE-mw*]), hoping to recover mutations in PcG genes or other

genes necessary for the PSS activity of the *en* PRE (Noyes et al., 2011). We screened for suppressors of mini-*white* silencing of a *P*[*enPRE-mw*] transgene inserted on chromosome 2R between the genes *CG30456* and *GstS1*. We reasoned that if the mutations we recovered affected the activity of the *en* PRE directly, then they should suppress mini-*white* silencing by *P*[*enPRE-mw*] regardless of where it was inserted in the genome. Mutations that suppressed the mini-*white* silencing of a specific *P*[*enPRE-mw*] insertion must act on flanking regulatory DNA. Interestingly, most of the mutations we recovered suppressed the mini-*white* silencing at only one or a subset of *P*[*enPRE-mw*] insertion sites, again showing the profound effect of genomic location on PRE activity.

We have characterized two of the dominant suppressors of PSS by *P*[*enPRE-mw*] (Cunningham et al., 2012; Noyes et al., 2011). One was a dominant mutation in the gene encoding the transcriptional activator Without children (Woc^D) (Noyes et al., 2011). Woc^D is caused by a single amino acid change that we hypothesize increases the ability of Woc to activate transcription. Woc^D suppresses *P*[*enPRE-mw*]-mediated PSS at only two chromosomal insertion sites (out of 16 tested). This suggests that Woc^D does not act at the PRE directly but rather acts on flanking genomic DNA. Woc^D may change the local chromatin conformation, perhaps by increasing the transcription of genes flanking the *P*[*enPRE-mw*] insertion site, inactivating PRE function at that chromosomal location.

The other PSS suppressor mutation we characterized was a dominant mutation in the cohesin-associated regulatory protein *wings apart-like* (*wapl*) (Cunningham et al., 2012), $wapl^{AG}$. Interestingly, $wapl^{AG}$ mutants have a classic PcG phenotype, sex comb teeth on the second and third legs (Kennison, 1995), suggesting that Wapl is also a regulator of the homeotic gene *Scr*. Wapl acts in a protein complex with Pds5 to remove cohesin from chromosomes (Shintomi & Hirano, 2009). Our results suggest that increasing cohesin-binding stability antagonizes PcG silencing (Cunningham et al., 2012). The relationship between cohesin binding and PcG activity has been recently reviewed (Dorsett, 2011).

2.4. PREs can facilitate transcriptional activation or repression

The patterned expression of the *HOX* genes in *Drosophila* is initially set up by the gap and pair-rule transcription factors that directly bind HOX-regulatory DNA. Soon after, these direct regulators disappear, and the PcG and trithorax-group (trxG) proteins take over to maintain the repressed or active state, respectively. It is well established that PcG proteins act through PREs, and there is evidence that trxG proteins act through the same or closely linked DNA elements (trxG response elements, TREs, reviewed in Ringrose & Paro, 2004). Interestingly, in genome-wide studies, the C-terminus of Trithorax localizes to the same locations as PcG proteins, the presumptive PREs (Schuettengruber et al., 2009; Schwartz et al., 2010).

The first indication that PREs could maintain either the repressed or active chromatin state was for a DNA fragment from the BX-C, *Fab-7* (Cavalli & Paro, 1998). Remarkably, *Fab-7* was able to maintain either transcriptional repression or activation of two reporter genes throughout both mitosis and meiosis depending on the transcriptional state of the transgenes in the embryo. The authors designed a vector with a GAL4-inducible *lacZ* gene followed by the mini-*white* gene. Inclusion of the *Fab-7* fragment (that contains a PRE) repressed the

expression of both *lacZ* and mini-*white*. After transient activation of the *lacZ* gene by GAL4 in embryos, both *lacZ* and mini-*white* expression were maintained throughout development. This fragment of DNA was subsequently called a "cellular memory module" or CMM. Three PRE-containing fragments from the BX-C have been shown to act as CMMs in this assay: *Fab7*, *Mcp*, *bxd*, and also a PRE from the *hedgehog* (*hh*) gene (Maurange & Paro, 2002; Rank et al., 2002). The *en* PRE did not act as a CMM in this assay (DeVido et al., 2008). Further experiments indicated that transcription through a PRE could turn it from a PRE to a TRE (Schmitt et al., 2005). One model states that it is the transcriptional state of PREs that determines whether they will maintain the active or repressed chromatin state. This may be true for some genes, but it is unlikely that this simple model will explain how the activity state is set for all PREs.

The ability of two closely linked *engrailed* PREs to mediate either transcriptional activation or repression was revealed in an enhancer-trap assay. An *en-lacZ* transgene containing 2.4 kb of upstream *en* upstream sequences (that includes the two PREs) driving *lacZ* expression had no intrinsic patterning activity on its own, but *lacZ* was expressed in patterns driven by genomic enhancers near the transgene insertion site. By flanking the *en* PREs with loxP or FRT sites, the expression of the *en-lacZ* transgene with and without the PREs in the same location could be examined. Remarkably, each PRE could act with flanking regulatory DNA as either a positive or negative regulatory element depending on the genomic context and the tissue examined. These data suggest that PREs, or closely associated sequences, mediate long-range interactions with enhancers or silencers around the PRE-transgene insertion site.

An excellent example of a fragment of DNA that acts as both a PRE and a presumptive TRE is provided by studies on a PRE from the *eve* locus (Fujioka et al., 2008). A 300-bp *eve* DNA fragment was shown to (1) mediate PSS of mini-*white*; (2) act as a PRE in an *Ubx*-reporter construct in embryos; and (3) maintain the continued expression of an *eve-lacZ* reporter construct in cells of the larval CNS; that is, without the PRE, the *eve-lacZ* gene was not expressed in the larval CNS. Remarkably, all three activities were dependent on a single binding site for the PcG-DNA binding protein Pho (Fujioka et al., 2008). Pho has been shown to interact with both PcG proteins and the trxG proteins Brahma, Moira, and Osa in *Drosophila* embryo extracts (Mohd-Sarip, Venturini, Chalkley, & Verrijzer, 2002). It would be interesting to know whether PREs from other genes could maintain the expression of the *eve-lacZ* reporter in the larval CNS, or whether this activity is specific for the *eve* PRE.

2.5. DNA binding proteins and sequence motifs associated with PREs

PREs are made up of multiple binding sites for many different factors. Despite the overwhelming amount of literature investigating the nature of *Drosophila* PREs, we still do not have a clear understanding of what precise combination of DNA binding sites/proteins are required for PRE activity. Studies of a number of different *Drosophila* PREs from *en* (Americo et al., 2002; DeVido et al., 2008; Kassis, 1994), *inv* (Cunningham et al., 2010), *eve* (Fujioka et al., 2008), *Ph* (Bloyer, Cavalli, Brock, & Dura, 2003), *hh* (Chanas & Machat, 2005; Maurange & Paro, 2002), *vestigal* (*vg*) (Okulski et al., 2011), and PREs of the BX-C: *Fab7*, *Mcp*, *bxd* (also called PRE_D), and *iab-2* (Busturia et al., 2001, Hagstrom, Müller, & Schedl, 1997, Mishra et al., 2001, Strutt, Cavalli, & Paro, 1997) have identified several

different factors/binding sites as being important for PRE function. These include Pho/Phol, Sp1/KLF factors, GAGA/Psq, Zeste/Fs(1)h, Dsp1, Grainyhead (Grh), and other as yet unidentified factors (reviewed in Müller & Kassis, 2006; Ringrose & Paro, 2007) that we will discuss below.

Genome-wide ChIP studies are yielding specific information about the distribution of these proteins at PREs in different cell types and at different developmental stages (Kwong et al., 2008; Négre et al., 2006; Schuettengruber et al., 2009; Schwartz et al., 2006, 2010; Tolhuis et al., 2006). These studies show that the identified PRE-associated DNA binding proteins are not present at all PREs and bind to many sites that are not within PREs. The phenotypes of mutants of some of these DNA binding factors argue for a role of some of these proteins in *trxG* gene activation rather than PcG repression. This may reflect the close association of PREs and TREs.

In addition, genome-wide studies have shown that PcG repression is not the all or nothing system that was originally thought and is in fact much more dynamic. A study of PcG distribution in different tissue culture cells reflecting different activity states of some genes shows that PcG proteins can be bound to PREs in both the on and the off transcriptional state raising interesting mechanistic questions (Schwartz et al., 2010). Interestingly, Papp and Müller (2006) found binding of PcG proteins in both the on and off transcriptional states at *Ubx* PREs in imaginal disks. Comparison of embryo versus imaginal tissue PcG protein binding suggests the presence of stage-specific PREs (Kwong et al., 2008; Schuettengruber et al., 2009). The general consensus seems to be that no one factor alone is responsible for PRE activity and that PcG recruitment to PREs involves the cooperative contribution of a number of different DNA binding factors. The exact combination of factors that constitute a PRE may be different at different genes.

2.5.1 Pho and Phol—The only known bonafide member of the *PcG* genes that encodes a DNA binding protein is *pho. pho* zygotic mutants die as pharate adults with sex combs on the second and third legs (the “classic” PcG phenotype), while embryos derived from germ line clones that lack both maternal and zygotic *pho* die at the embryonic stage with segmentation defects (Breen & Duncan, 1986). The *Drosophila* genome encodes a protein highly related to Pho in the DNA binding domain, Pho-like (Phol) (Brown, Fritsch, Müller, & Kassis, 2003). *phol* mutants are homozygous viable with no homeotic phenotypes. Genetic experiments suggest that Pho and Phol act together to repress homeotic gene expression since *pho; phol* double mutants have more derepression of the homeotic gene *Ubx* in wing disks than do *pho* mutants. Phol also works independently of Pho; eggs derived from Phol females are fertilized but do not develop, a phenotype not observed for eggs that lack Pho (Breen & Duncan, 1986).

Pho and Phol are the *Drosophila* homologs of the mammalian proteins YY1 and YY2, respectively (Brown et al., 1998, 2003 Drews, Klar, Dame, & Bräuer, 2009; Nguyen, Zhang, Olashaw, & Seto, 2004). YY1 is a very dynamic protein that can be involved with repression, activation, and transcriptional initiation in a context-dependent way (Gordon, Akopyan, Garban, & Bonavida, 2006; Wang, Chen, & Yang, 2006). Pho has 96% amino acid identity with YY1 over four zinc fingers that are used for DNA binding and protein–

protein interactions. There is also conservation of another region of the protein (18/22 amino acid identity between YY1 and Pho), a small spacer region implicated in protein–protein interactions. Phol has 80% amino acid identity to Pho over the four zinc fingers, and 10/22 and 11/22 amino acid identity to YY1 and Pho, respectively, in the spacer region. YY1 has been postulated to be involved in PcG repression in vertebrates (discussed in more detail below), and mammalian YY1 can partially rescue a *pho* zygotic mutant (Atchison, Ghias, Wilkinson, Bonini, & Atchinson, 2003).

Although Pho and Phol both bind to *Ubx* PRE_D and Phol can silence *Ubx* transcription in the absence of Pho (Wang et al., 2004), genome-wide studies show that, in wild-type embryos, the distribution of Pho and Phol is different (Schuettengruber et al., 2009). Consistent with the PcG phenotype of zygotic *pho* mutants, Pho binding is highly correlated with the binding of Ph and Pc. Of genomic positions that share Ph and Pc binding, 96% correspond to Pho peaks but only 21% correspond to Phol peaks (Schuettengruber et al., 2009). The data suggest that Phol preferentially binds to a slightly different sequence than Pho and may be involved in transcriptional activation rather than repression (Schuettengruber et al., 2009). The 96% overlap between regions that bind Pc/Ph and Pho suggest that Pho could be absolutely required for PRC1 recruitment. However, in another study, also in embryos, there was only a 50% overlap of Pc binding sites with Pho (Kwong et al., 2008). It should also be pointed out that there are many Pho sites that do not overlap with either Pc or Ph binding.

What does Pho do at the PRE? In embryos, Pho was found in two protein complexes, Pho-dINO80, a nucleosome-remodeling complex, and PhoRC where it is in a complex with dSfmbt, a protein that has methyl-lysine-binding activity (Grimm et al., 2009; Klymenko et al., 2006). Both Pho and Phol form stable complexes with dSfmbt in Sf9 cells. In contrast, Phol is not found associated with dINO80. dSfmbt is also bound to PREs, and mutations in the gene encoding *dSfmbt* cause homeotic derepression (Klymenko et al., 2006; Oktaba et al., 2008) showing it is also important for PcG repression. Therefore, one function of Pho and Phol may be to bring dSfmbt to the PRE. Biochemically, Pho has been reported to interact with components of both PRC1 and PRC2 in coimmunoprecipitation and GST pulldown assays (Mohd-Sarip, Cleard, Mishra, Karch, & Verrijzer, 2005; Wang et al., 2004). Further, both Pho and dSfmbt copurified with a Pc protein labeled *in vivo* by biotinylation (Strübbe et al., 2011). Pho and Phol are thought to play a key role in the binding of both PRC1 and PRC2 to chromatin (Wang et al., 2004). Interestingly, the PcG protein Scm can bind to an *Ubx* PRE in the absence of Pho/Phol (Wang et al., 2010).

There is no doubt that Pho plays a key role at PREs. To our knowledge, all PREs tested in either the mini-*white* silencing or the embryonic or larval maintenance assays bind Pho. Mutation of Pho binding sites within PREs abrogated the function of all PREs where it has been tested (reviewed in Kassis, 2002; Fujioka et al., 2008). There is some data that other PRE-binding proteins may be necessary to facilitate the binding of Pho to the PRE. For example, Grh, GAF, and Dsp1 have all been postulated to cooperatively assist Pho binding to chromatinized substrates (Blastyák, Mishra, Karch, & Gyurkovics, 2006; Dejardin et al., 2005; Mahmoudi, Zuijderduijn, Mohd-Sarip, & Verrijzer, 2003). Pho is generally thought to have a repressive role but, as discussed above, Fujioka et al. (2008) have shown that Pho can act as an activator in a specific subset of cells in the *Drosophila* nervous system. It remains

to be seen whether this activating role of Pho is specific for this case or can be generalized to many PRE/TREs.

2.5.2 Sp/KLF proteins—An analysis of a PRE from the *en* gene identified a Sp1/KLF binding site as being required for PSS and PRE activity of a 181-bp PRE (Americo et al., 2002; Brown et al., 2005). There are nine members of the Sp1/KLF family of proteins in *Drosophila* and *in vitro* transcription/translation of the DNA binding domains showed that 8/9 of the proteins were capable of binding to the site in the *en* PRE (Brown et al., 2005). As seen in the extensive family of mammalian Sp1/KLF proteins (reviewed in Kaczynski, Cook, & Urrutia, 2003), there were differences in the binding site preferences of the *Drosophila* family members. Further analysis identified a member of this group, Spps (Sp1 factor for PSS) as being involved in PRE activity of the *en* PRE (Brown & Kassis, 2010). Flies mutant for *Spps* die as late pharate adults and *Spps* enhances the *pho* mutant phenotype. This indicates that Pho and Spps either work together or in parallel parts of the same pathway to silence PcG target genes. PSS of mini-*white* in constructs carrying either an *en* PRE or a *bxd* PRE is lost in *Spps* mutants, showing that Spps is required for PSS. ChIP-qPCR showed that Spps binds to both the *en* and *bxd* PREs in larvae. ChIP experiments showing the genome-wide distribution of Spps have not yet been published. However, a general role for Spps in PRE activity is predicted given that Spps colocalizes almost perfectly with Psc in polytene chromosomes (Brown & Kassis, 2010). Mammalian YY1 and Sp1 have been shown to be able to interact (Lee, Calvin, & Shi, 1993; Seto, Lewis, & Shenk, 1993) which raises the possibility for interaction here. The involvement of other members of the *Drosophila* Sp1/KLF factors in PcG repression has yet to be determined. The mammalian Sp1/KLF proteins are a dynamic set of proteins involved in many aspects of gene expression, differentiation, and cancer (Kaczynski et al., 2003; McConnell & Yang, 2010).

2.5.3 GAGAG sites—The sequence GAGAG is required for the activity of many PREs (reviewed in Kassis, 2002; Fujioka et al., 2008). GAGAG elements are binding sites for two proteins, GAGA factor (GAF) and Pipsqueak (Psq) (Lehmann, Siegmund, Lintermann, & Korge, 1998). GAF recruits a chromatin-remodeling complex and the role of GAF in PRE activity may be to remove nucleosomes at the PRE, allowing the binding of other transcription factors (Mahmoudi et al., 2003). Genome-wide studies show that GAF is present at about 50% of sites bound by both Pc and Ph (Négre et al., 2006; Schuettengruber et al., 2009). Psq also binds to the GAGAG sequence although it is reported to have a preference for longer GAGA sequences in keeping with the fact that it has a different DNA binding domain (HLH; Siegmund & Lehmann, 2002) from GAF (a single zinc finger; Lehmann et al., 1998). Psq has been isolated from S2 cells in a protein complex (CRASCH; Huang & Chang, 2004; Huang, Chang, Yang, Pan, & King, 2002) with PcG proteins. The genome-wide distribution of Psq binding is not known but GAF and Psq proteins colocalize on polytene chromosomes. GAF and Psq both have the same type of protein–protein interaction domains, BTB domain, and can interact with each other (Schwendemann & Lehman, 2002) and with some other members of this class of proteins, for example, Tramtrack (Pagans, Ortiz-Lombardia, Esinas, Bernues, & Azorin, 2002). GAF is not a stable component of any of the PcG complexes isolated to date. Recently, a vertebrate homolog of

GAF was identified that also binds to GAGAG sequences and shows extensive homology with GAF (Matharu, Hussain, Sankaranarayanan, & Mishra, 2010). It will be interesting to know whether this protein plays a role in PcG silencing in mammals.

GAF is encoded by the gene *trithorax-like* (*trl*; Farkas et al., 1994). As suggested by the name, *trl* mutants have a trxG phenotype. GAF is required for many aspects of gene expression and also for nuclear division in *Drosophila* embryos (Bhat et al., 1996). GAF is bound to GAGA satellite sequences. Because of its multifunctional role in controlling gene expression and cell division, it is impossible to directly test the role of GAF at PREs in embryos. Psq acts during oogenesis in the establishment of the anterior/posterior axis of the oocyte (Siegel, Jongens, Jan, & Jan, 1993). A role for Psq in PcG silencing is suggested by a genetic interaction between a *psq* and *Pc*. A mutation in *psq* strongly enhances the derepression of *Ubx* in wing and leg disks of larvae heterozygous for a *Pc* allele, strongly suggesting Psq plays a role in PcG silencing (Huang et al., 2002). The exact role of GAF versus Psq at the PREs needs further exploration.

2.5.4 GTGT sequence—This sequence has shown up in a number of studies to be enriched in PRE sequences (Ringrose et al., 2003; Schuettengruber et al., 2009). Deletion of the GTGT sequences in the *vg* PRE led to reduction in silencing activity of this fragment suggesting that this sequence may play a role in PcG repression (Okulski et al., 2011). Interestingly, in mammals, the Sp1/KLF factor Sp2 was shown to bind to a GTGT box (GGTGTGGGG) implicated in regulating T-cell receptor gene expression (Kaczynski et al., 2003; Kingsley & Winoto, 1992; Suske, Bruford, & Philipsen, 2005). It would be interesting to know whether the GTGT sequences deleted in the *vg* PRE bind to Spps or other *Drosophila* Sp1/KLF family members.

2.5.5 Dsp1—Dsp1 was identified as a PRE DNA binding protein important for the recruitment of PcG complexes to polytene chromosomes (Dejardin et al., 2005). These authors reported that Dsp1 binds to the consensus sequence GAAAA and that mutation of GAAAA within the *en* and *Fab7* PREs not only abrogated PSS of mini-*white* but also changed the PREs into constitute TREs. These data suggest that Dsp1 is required for PRE repression activity.

Genome-wide studies with 4–12 h embryo extracts have shown that Dsp1 binds to a subset of PREs (about 50% of the Ph/Pc sites identified (225/439); Schuettengruber et al., 2009). Curiously, the GAAAA consensus sequence is not enriched within these PREs. Clearly, the DNA binding site of Dsp1 requires further definition; however, Dsp1 might recognize DNA structure instead of sequence. Dsp1 is a member of the class of HMG proteins that have two HMG domains; these proteins bind to the minor groove of DNA without sequence specificity, instead recognizing DNA structural features. This type of HMG protein has been reported to be able to introduce a significant distortion or bend into the DNA (for reviews, see Agresti & Bianchi, 2003; Stros, 2010). Dsp1 could facilitate PcG protein binding or long-range interactions by changing the structure of the DNA.

Mutants of Dsp1 exhibit features more typical of a *trxG* gene than a *PcG* gene (Decoville, Giacomello, Leng, & Locker, 2001; Rappailles, Decoville, & Locker, 2005). Dsp1 has been

shown to coimmunoprecipitate with the chromo domain containing protein, Corto (Salvaing et al., 2006). These authors suggest that Dsp1 binds to the *Scr* PRE only when *Scr* is being transcribed, thus Dsp1 plays a critical role in TRE activity. Clearly, more work needs to be done to understand the role of Dsp1 in PcG and trxG activity.

2.5.6 Grh—Grh was found to bind to the *iab-7* PRE and to cooperatively interact with Pho both *in vitro* and genetically (Blastyák et al., 2006). The binding site for Grh identified in the BX-C *iab-7* PRE was reported to be TGTTTTTT. However, other groups report a Grh consensus sequence as WCHGGTT (where W is A or T and H is not G) (Almeida & Bray, 2005; Venkatesan, McManus, Mello, Smith, & Hansen, 2003). To date, a genome-wide study of Grh binding in relationship to PcG protein binding has not been reported. A presumptive Grh binding site is present in an *eve* PRE (Fujioka et al., 2008) but not in the 181-bp *en* PRE (J. Lesley Brown and Judith A. Kassis, unpublished observation), suggesting it may be important for only a subset of PREs.

Other evidence that Grh may be involved in PcG repression comes from work on the mammalian Grh-family member CP2. CP2 was shown to interact with a mammalian Ring protein, DinG. The CP2–DinG interaction was necessary for transcriptional repression (Tuckfield et al., 2002). The authors also showed that *Drosophila* Grh could interact with dRing in Gst-pull down experiments *in vitro*.

Drosophila Grh regulates the expression of many genes and can act as either a transcriptional repressor or activator dependent on the context (see references cited in McQuilton, Pierre, Thurmon, & The FlyBase Consortium, 2012). Of particular interest for this review, Grh, Zeste, and GAGA binding sites activate transcription of an *Ubx* promoter reporter construct through intermingled clusters of binding sites (Hur, Laney, Jeon, Ali, & Biggin, 2002 and references therein). Interestingly, Hur et al. (2002) found that Zeste and GAGA promoter-proximal binding sites were able to maintain PcG-mediated repression of a 22-kb *Ubx-lacZ* reporter construct in embryos, whereas Grh binding sites did not have this activity. The role of Grh in PcG repression requires further study.

2.5.7 Zeste binding sites—The role of Zeste in PcG repression is also unclear. Zeste has been reported to be an activator or repressor of transcription dependent on the context and has been reported to be a stoichiometric component of PRC1 (Saurin et al., 2001). Schuettengruber et al. (2009) found only a 25% overlap of Ph with Zeste in genome-wide binding data. Oktaba et al. (2008) report very little overlap between Zeste and Pho in genome-wide binding data. Zeste mutants are homozygous viable and fertile and do not show PcG phenotypes (Goldberg, Colvin, & Mellin, 1989). *female sterile* (*1*) *homeotic* (*fs*(*1*)*h*) is a member of the *trxG* genes. *fs*(*1*)*h* encodes a double bromodomain-containing protein that binds to Zeste sites within the *Ubx* promoter and activates transcription of the *Ubx* gene (Chang, King, Lin, Kennison, & Huang, 2007). It is not known if Fs(1)h binds to Zeste sites in PREs.

2.6. Are all PREs alike?

Algorithms based on clustered consensus binding sites for PRE DNA binding proteins have been marginally successful in identifying PREs, giving both false positive and false negative

results (Fiedler & Rehmsmeier, 2006; Ringrose et al., 2003; Zeng, Kirk, Gou, Wang, & Ma, 2012). In one study, of 167 potential PREs that Ringrose et al. (2003) identified, only 16% of them were PcG binding sites in PcG ChIP genome-wide studies of *Drosophila* embryos (Schuettengruber et al., 2009). A weakness of this approach is the unreliability of the consensus binding sequences that are used in the search. A consensus sequence is our best guess but it does not take into account cooperativity between weak sites, the influence of nearby binding proteins, or the fact that a protein may be able to bind to sites that deviate from the consensus. For example, in a study on PREs at the *invected* locus (Cunningham et al., 2010), a fragment that had both PSS and PRE activity did not contain any matches to the core consensus binding sequence for Pho (GCCAT); thus this fragment was not predicted to be a PRE by the jPREdictor algorithm; however, this fragment clearly bound Pc and Ph in ChIP studies, suggesting it was a PRE. There were several CCAT sequences in this PRE, and 5/6 bound Pho *in vitro*. In addition, some PREs require Pho binding sites that contain sequences beyond the minimal Pho consensus. Two extended Pho binding sites were required to interact and recruit Pc complexes to chromatin in the *iab-7* PRE (Mohd-Sarip et al., 2002; reported to be a PcG target Mohd-Sarip et al., 2005, 2006). In studies examining the genome-wide distribution of Pho, Oktaba et al. (2008) noted that an extended Pho consensus sequence was enriched in regions that bound both Pho and dSfmbt. They also examined the number of Pho consensus sites within a Pho-bound region and found that, while often there was more than one Pho site, the spacing of these sites was variable, and many fragments had only one Pho binding site (Oktaba et al., 2008). Further, the number and order of Pho and other motifs varies greatly between orthologous PREs in different *Drosophila* species (Hauenschild, Ringrose, Altmutter, Paro, & Rehmsmeier, 2008); clearly there is great flexibility in PRE-binding site configuration.

PRE prediction algorithms make the assumption that all PREs have the same requirements for activity. This seems unlikely given the complexity of the different PcG target loci and the indication that different PREs function at different times in development. There is also a difference in the arrangement of PREs within a gene that may reflect underlying differences in the way the PRE works. A subset of PREs are located immediately upstream of the promoter, while others are many kilobases away. The available data suggest that some DNA binding proteins are present at most PREs (Pho), for example; however, it is likely that locus specific factors influence the activity of at least a subset of PREs.

A limited number of PREs have been shown to work with regulatory DNA from other genes (discussed above). For example, PREs from the genes *en*, *eve*, and Abd-B maintain silenced expression of an *Ubx-lacZ* reporter construct in the correct pattern in embryos. Further, the *iab-7* and *iab-8* core PREs completely replaced the activity of a core *bxd* PRE in the endogenous *Ubx* gene (Kozma, Bender, & Sipos, 2008). In general, these studies used minimal PRE fragments of only a few hundred base pairs or less that contained binding sites for Pho and GAF. Larger PRE fragments are more likely to contain other regulatory DNA and may not work in these assays. For example, while a 181-bp minimal *en* PRE maintained an *Ubx*-like expression pattern of an *Ubx-lacZ* transgene in embryos, a larger fragment (2.6 kb) that contained the 181-bp PRE as well as an additional PRE and flanking DNA totally silenced the *Ubx-lacZ* reporter gene in a *Pc*-dependent way (Americo et al., 2002). Recently,

Okulski et al. (2011) used the phi-C31 system to integrate two different PRE-mini-*white* transgenes (*vg* and *Fab7*) at four different chromosomal insertion sites and quantitated the amount of mini-*white* repression by the PREs both in flies heterozygous and homozygous for the transgene. They noted that the *vg* and *Fab7* PREs gave quantitatively different amounts of mini-*white* silencing and behaved differently at the different insertions sites. Although this was a very careful study, one problem with it was that the PRE fragments they used were 1.6 kb and were likely to have other regulatory DNA for their respective loci. In this regard, the 181-bp *en* PRE also has promoter-tethering activity (Kwon et al., 2009). It is important to keep in mind that PREs act in the context of other regulatory DNA and that other regulatory elements may be closely linked or even intertwined.

Strübbe et al. (2011) recently purified Pc under physiological conditions designed to maintain the weakest associations and then analyzed copurifying products by mass spectrometry. This identified all of the PRC1 core components Pc, Psc, Sce/dRing, Ph and Su(z)2 and PhoRC (dSfmbt and Pho) but components of PRC2 were not found. Fs(1)h and Grh also copurified. In addition to known PcG complexes, the data also showed unexpected interactions with the cohesin complex and with proteins related to the Moz/Morf histone acetyltransferase complex. This type of approach, combined with a rigorous analysis of individual PREs will be necessary to understand how PcG proteins are recruited.

3. CHARACTERISTICS OF PcG TARGET GENES IN *DROSOPHILA*

3.1. Many PcG targets have multiple PREs

PcG target genes tend to have a large amount of regulatory DNA reflecting their complex and often dynamic expression patterns throughout development (Kharchenko et al., 2011; Négre et al., 2006). PcG target genes often occur in gene clusters. The best known of these are the *HOX* gene clusters including the BX-C that contains the homeotic genes *Ubx*, *Abd-B*, and *Abd-A*. The arrangement and function of PREs within the BX-C have been extensively studied (reviewed in Maeda & Karch, 2006, 2011); in fact, much of what we know about PREs comes from studies on BX-C PREs. Here, we describe the PREs of three PcG target loci, *eve*, an example of a single gene PcG target and two PcG targets with two coregulated genes, *en*/*inv* and *Psc*/*Su*(*z*)*2*.

3.1.1 eve—The *eve* gene is a pair-rule gene required for segmentation and the development of specific lineages in the mesoderm and nervous system. A single *eve* 1.5-kb transcription unit is flanked by about 9 kb of regulatory DNA downstream and 6-kb upstream, containing many *eve* enhancers (Fujioka et al., 1999). *PcG* genes regulate *eve* expression in the nervous system (Fujioka et al., 2008; Smouse, Goodman, Mahowald, & Perrimon, 1988). In S2 cells, a 20-kb "*eve* domain" is covered with the characteristic H3K27me3 PcG mark generated by PRC2 (Kharchenko et al., 2011). There are two fragments of *eve* DNA that mediate PSS of mini-*white*, a 300-bp fragment located 8.9-kb downstream of the *eve* transcription unit (*eve* PRE300), and another 441-bp fragment that includes the *eve* promoter. *eve* PRE300 acts as a PRE in a *Ubx-lacZ* reporter gene and also as a positive element to maintain expression of an *eve*-reporter gene in specific cells of the larval nervous system (discussed above). The *eve* proximal-promoter PRE has not been tested in these assays. Both *eve* PREs contain Pho, GAF, and Zeste binding sites (Fujioka et al., 2008). Interestingly, *eve* PRE300 is not

required for correct *eve* expression in an *eve* rescue transgene (Fujioka et al., 1999). This suggests that the promoter-proximal *eve* PRE is sufficient for maintaining *eve* expression.

3.1.2 en/inv—The *en* and *inv* genes are coexpressed genes that share regulatory DNA (Gustavson et al., 1996) and form a 115-kb H3K27me3 domain in S2 cells, embryos, and larvae (Kharchenko et al., 2011). There are four well-characterized PREs in the *inv*/*en* locus; two closely linked ones located between −400 bp and −2 kb upstream of the *en* transcription start site (DeVido et al., 2008) and two upstream of *inv*, one coincident with the major *inv* transcription start site and another about 6-kb upstream of that (Cunningham et al., 2010). In S2 cells and adults, PcG proteins are bound to a single 2-kb peak just upstream of the *en* transcription start site (encompassing the two PREs) and to the two *inv* PREs, however, in embryos and larvae, PcG proteins bind to additional regions upstream of the *en* gene (Négre et al., 2006; Oktaba et al., 2008). It is not known whether these other PcG binding regions represent additional stage-specific PREs or are the result of the three-dimensional structure of the locus causing cross-linking to fragments because of chromatin compaction. PRE activity assays will need to be done on these other fragments to distinguish between these two possibilities. Interestingly, deletion of a 1.5-kb fragment ($en^{\Delta 1.5}$, that includes the two PREs upstream of the *en* transcription start site) from the endogenous *en*/*inv* locus does not alter the pattern of *en* or *inv* expression (Cheng, Kwon, Arai, Mucci, & Kassis, 2012). These data suggest that PREs might act redundantly in the *en*/*inv* domain; however, it is not know whether deletion of the PREs in $en^{\Delta 1.5}$ causes quantitative difference in *en*/*inv* expression.

3.1.3 Psc/Su(z)2—The PcG group genes *Psc* and *Su*(*z*)*2* exist in an H3K27me3 domain that extends over about 91 kb in S2 cells; insulator elements may limit the spread of H3K27me3 into flanking genes (Park et al., 2012). *Psc* and *Su*(*z*)*2* are ubiquitously expressed genes, so, obviously, PcG proteins do not silence gene expression in this case. It is thought that PcG proteins regulate the level of *Psc* and *Su*(*z*)*2* expression. ChIP experiments in S2 cells show four large discrete Psc binding sites as well as additional smaller peaks (Schwartz et al., 2006). Park et al. (2012) showed that two of these peaks had PRE activity in both a mini-*white* silencing assay and in a larval *Ubx*-reporter gene functional assay. Another Psc binding fragment behaved as a downregulatory module rather than a typical PRE. Deletion of one of the *Psc*/*Su*(*z*)*2* PREs from the endogenous locus caused a twofold increase in the level of *Psc* and *Su*(*z*)*2* transcription suggesting that this element downregulates the level of transcription. Why do PREs from the *Psc*/*Su*(*z*)*2* gene act as silencers in transgenes, but not silence expression of the endogenous gene? One hypothesis is that the type of enhancers associated with this gene are not subject to PcG silencing. More work needs to be done on this interesting locus.

3.2. Homing and PcG target genes

P-element based vectors have been used to make transgenic *Drosophila* for about 30 years (Rubin & Spradling, 1982). Aside from some hotspots and a preference to insert near the 5′ end of transcription units, P-based vectors insert in the genome in a relatively nonselective manner and as such, have been used as a tool to generate thousands of transposon-induced mutations in *Drosophila* (Bellen et al., 2011). In 1990, Hama, Ali, and Kornberg reported that P-based constructs that contain *en* regulatory DNA (*P*[*en*]) insert near or into the *en*/*inv*

domain at a very high frequency. They called this phenomenon “homing” (Hama, Ali, & Kornberg, 1990). Further studies on *P*[*en*] homing by *en* regulatory DNA showed that a 2-kb fragment, extending from −2.4- to −0.4-kb upstream of the *en* transcription start site was sufficient for homing, and that the *en* PREs contributed to the homing activity of this fragment (Cheng et al., 2012). Further, *P*[*en*] inserted near PcG targets at an increased frequency compared with a P-based vector used to generate insertions for the *Drosophila* gene disruption project (Cheng et al., 2012). These data suggest that either the chromatin structure or PcG proteins themselves bring *P*[*en*] to the region of PcG-regulated loci. This is consistent with the view that PcG targets colocalize in the nucleus in PcG bodies (Delest et al., 2012; Pirrotta & Li, 2012) and suggest that these bodies also occur in germ cells where homing occurs. Transgenes containing *polyhomeotic* PREs and BX-C PREs have also been reported to insert at a high frequency in or near PcG target genes (reviewed in Kassis, 2002).

Fragments of DNA from the BX-C and *eve* PcG targets have been shown to mediate homing (Bender & Hudson, 2000; Fujioka, Wu, & Jaynes, 2009). Like *P*[*en*], insertions mediated by the BX-C and *eve* homing fragments occurred over a large region, causing insertions throughout and near the parent locus. For these two cases, the fragments that mediate homing are most likely insulators, not PREs (Bender & Hudson, 2000; Fujioka et al., 2009). Anecdotally, 1/5 insertions of a P-based vector that contained one of the *Psc*/*Su*(*z*)*2* PREs was inserted near the promoter of the *Su*(*z*)*12* gene, strongly suggesting that this fragment mediates homing. Note that one reported case of homing, that of the *linotte* gene (now called *drl*; Taillenbourg & Dura, 1999), is not reported to be a PcG target gene in embryos, larvae, or S2 cells; however, it is possible it could be a PcG-regulated gene in germ cells where P-element homing occurs.

4. PREs IN VERTEBRATES

PcG proteins in vertebrates are much more diverse and play a role in gene regulation in many different tissues and stages of development (two recent reviews: Beisel & Paro, 2011; Kerppola, 2009), including X-inactivation (one recent review: Jeon, Sarma, & Lee, 2012). The mechanism of PcG protein recruitment varies dependent on the target and can be either RNA or protein mediated. In vertebrates, there are gene-specific DNA binding proteins that recruit specific PcG proteins to specific genes (reviewed in Beisel & Paro, 2011; Kerppola, 2009; Schuettengruber & Cavalli, 2009). Here, we focus on vertebrate DNA fragments that have been shown to have properties similar to *Drosophila* PREs.

In ES cells, CpG islands have been shown to be required for PRC2 recruitment (for review, see Deaton & Bird, 2011). In one elegant study, Mendenhall et al. (2010) showed that a CpG island was sufficient for recruitment of PRC2 to a BAC transgene integrated in ES cells. Binding sites for transcriptional activators in or next to the GC-rich sequence abrogated its ability to recruit PRC2 pointing to a competition between transcriptional activation and repression. Remarkably, even a GC-rich region from *E. coli* could recruit PRC2 when integrated into the transgene. Interestingly, PRC1 was not recruited to the DNA in their experiments. Lynch et al. (2012) provide a definitive PRE assay in mouse ES cells at the α-globin gene locus. They used recombination-mediated cassette exchange to study PcG protein binding and histone modifications to a single-copy human α-globin locus integrated

into a mouse ES cell line. They also showed the importance of CpG islands in PcG recruitment and their experiments also pointed to a competition between transcriptional activation versus PcG repression. In addition, a key role for unmethylated CpG dinucleotides in PcG recruitment was established as they found *de novo* sites of PcG recruitment at CpG-rich sequences in methyltransferase-deficient ES cells. It is not known if PRC2 recruitment to CpG-rich sequences is protein or RNA mediated or occurs by default in the absence of transcriptional activators.

Are there any PREs in mammals that have binding sites for proteins important for PRE activity in *Drosophila*? Pho/Phol (YY1-homologues), Spps (a Sp1/KLF family member), Dsp1 (a HMG protein), and GAF all have mammalian homologues. In embryonic and neural stem cells, YY1 and PRC2 show almost no colocalization but, instead, YY1 binding is correlated with transcribed genes (Mendenhall et al., 2010). However, there is some genetic and biochemical evidence that YY1 might recruit PcG proteins at some loci (Garcia, Marcos-Gutierrez, del Mar Lorente, Moreno, & Vidal, 1999; Lorente et al., 2006). Below, we describe three examples of vertebrate PREs, studied in tissues other than ES cells that bear some similarities to *Drosophila* PREs.

Sing et al. (2009) identified a putative PRE (PRE-*kr*) that regulates expression of the *Maf*/*Kreisler* gene in rhomdomeres in mice. Endogenous PRE-*kr* binds PRC1 and PRC2 components. A reporter construct containing PRE-*kr* recruits PcG proteins. Like *Drosophila* PREs, PRE-*kr* contains binding sites for YY1 and GAF. The role of the YY1 and GAF binding sites in PRE-*kr* function has not been tested. Interestingly, PRE-*kr* mediates mini-*white* silencing and recruits PcG proteins to polytene chromosomes in *Drosophila* showing that this 3-kb fragment also acts as a PRE in *Drosophila*. Further studies would be necessary to determine if the same sequence motifs are required for PRE activity in the mouse and fly.

A vertebrate PRE from a *HOX* gene also shows characteristics of a *Drosophila* PRE. Woo, Kharchenko, Daheron, Park, and Kingston (2010) identified a 1.8-kb region between the human *HOXD11* and *HOXD12* genes (D11.12) that is bound by PcG proteins at the endogenous locus and functions as a PcG-dependent repressor in reporter constructs. Analysis of this element showed that there is a cluster of conserved YY1 sites important for the recruitment of BMI1 (a PRC 1 component) but less so for the recruitment of the PRC2 component SUZ12. A 237-bp region that is highly conserved across vertebrates was essential for the recruitment of members of both PRC1 and PRC2. The D11.12 region also contains a CpG island but the functional importance of this has not been tested. These results imply that PRC1 and PRC2 have different methods of recruitment.

A potential human PRE was identified in resting T cells downstream of the *SLCA17* gene (Cuddapah et al., 2012). In resting T cells, the *SLCA17* gene is not transcribed and PcG proteins are bound to the presumptive PRE. Treatment of HeLa cells with RNAi to SUZ12 caused a decrease in SUZ12 levels and to the levels of Bmi1 and Ring1B binding to the presumptive *SLCA17* PRE. A 3-kb putative *SLCA17* PRE contains YY1 and GAF binding sites and acted as a pairing-sensitive silencer in *Drosophila*, again suggesting that PcG proteins in *Drosophila* and vertebrates can be recruited by similar mechanisms (Cuddapah et al., 2012).

5. OUTLOOK

Much has been learned about *Drosophila* PREs since their discovery almost 20 years ago. First discovered as silencers of homeotic gene expression, genome-wide studies have revealed hundreds (or, in some studies, thousands) of potential PREs in *Drosophila*. Available data suggest that not all PREs are alike, but the differences between PREs are not well defined. The situation in mammals is even more complex with the increased diversity of mammalian PcG proteins and the involvement of PcG proteins in so many diverse tissues, stages of development, and processes. It is still too early to conclude whether there is a class of mammalian PREs that closely resemble *Drosophila* PREs. There are many fundamental questions about *Drosophila* PREs that need to be addressed to understand PRE composition and function; some of the questions we have discussed in this review are summarized below.

a. Are all PREs alike? Functional assays on hundreds of potential PREs using the phi-C31 integration system and reporter assays for different developmental stages will be necessary to assess whether there are different classes of PREs. In these assays, it will be important to use minimal PRE fragments (hundreds of base pairs) as larger DNA fragments will most likely have gene-specific activities including enhancers and silencers that could interfere with PRE assays.

b. Mutations in different *PcG* genes in *Drosophila* (and mammals) lead to different phenotypes suggesting that not all the PcG proteins act together all the time or at all targets. Do different PREs recruit different PcG complexes or does the nature of the gene activators determine which PcG proteins are required at a particular target gene?

c. At some targets, PcG proteins are bound to transcribed genes. What do PcG proteins do at actively transcribed genes? How can PcG proteins repress transcription at some targets and silence it at others?

d. Many PcG target genes are expressed in many different tissues at many different times in development; only a subset of the expression patterns is subject to PcG regulation. Are there some types of enhancers (or promoters for that matter) that cannot be regulated by PcG proteins?

e. How do PRE-binding proteins recruit or anchor PcG protein complexes? The mechanisms are far from known.

Despite all these questions, it is quite clear that PREs play a major role in regulating the expression of many genes in the *Drosophila* genome. It is incumbent on us to figure out how they work.

Acknowledgments

We thank Yuzhong Cheng and Payal Ray for comments on this chapter. We thank the authors of many recent reviews on this topic; their comprehensive reviews made it easier for us to highlight only a subset of the literature on this important topic. We apologize for any omissions or misstatements of data. J. A. K. and J. L. B. are supported by the intramural research program of the National Institutes of Health and *Eunice Kennedy Shriver* National Institute of Child Health and Human Development.

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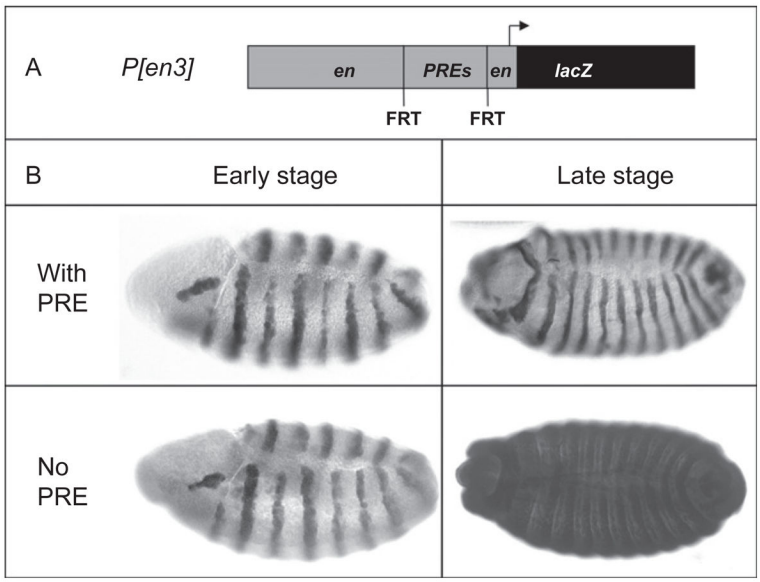

Figure 3.1.

Embryonic maintenance PRE assay. (A) Structure of a vector used to test for PRE activity of the *en* PREs. Eight kilobases of *en* sequence is fused to the reporter gene, *lacZ*. This leads to the expression of β-galactosidase in *en*-like stripes throughout embryonic development. This 8-kb of *en* includes a 2-kb region that encompasses the *en* PREs. Here, the 2-kb fragment containing the PREs is flanked by FRT sites allowing for removal of the PRE with FLP recombinase (Cunningham et al., 2010; DeVido et al., 2008). (B) Early and late stage embryos stained for β-galactosidase with a line that carries the 2-kb *en* PRE and a line that has the 2 kb PRE excised. With the PRE fragment, *lacZ* expression is maintained in *en*-like stripes throughout embryonic development. When the *en* PRE is excised, *lacZ* is expressed throughout the embryo. Early stage embryos: lateral view, anterior left, dorsal up, stage 11. Late stage embryos: dorsal view, anterior left, stage 16.

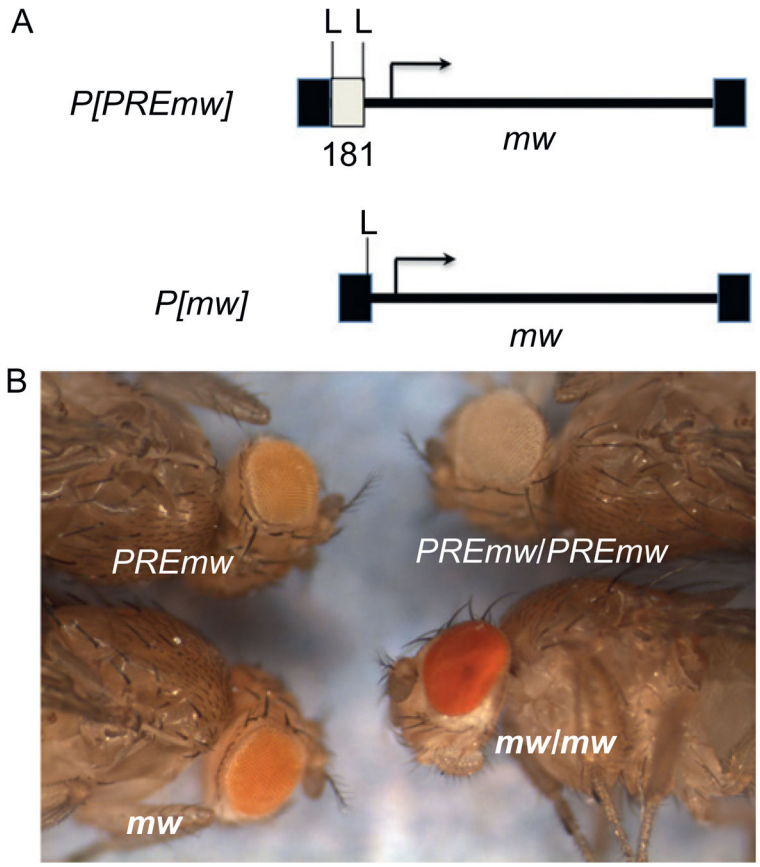

Figure 3.2.

PSS of mini-*white* expression in transgenic flies. (A) Diagrammatic representation of the two reporter constructs used to test for PSS of an *en* PRE. *P*[*mw*] is the P-element vector control without any PRE insert. *P*[*PREmw*] is the same transformation vector carrying a 181-bp PRE from *en* cloned upstream of the mini-*white* promoter. The filled boxes represent the P-element ends. The open box represents the PRE. L is *loxP* sites that allow excision of the PRE. (B) Shows the eye color of transformants carrying the above constructs. *mw* and *PREmw* are heterozygous for the construct. *mw*/*mw* and *PREmw*/*PREmw* are homozygous for the constructs. PSS can be seen by comparing heterozygous versus homozygous eye colors for the two different constructs. *Figure adapted from* Noyes, Stefaniuk, Cheng, Kennison, and Kassis (2011).

Table 3.1

Summary of *Drosophila* genes with PREs that have been characterized by the PSS assay or a maintenance assay in embryos (E) or larvae (L)

PRE	Maintenance assay	PSS mini-*white*
en	Yes, E	Yes
inv	Yes, E	Yes
eve	Yes, E	Yes
Psc/*Su*(*z*)*2*	Yes, L	Yes
Scr	Yes, E	Yes
pb	Yes, E	Yes
vg	ND	Yes
ph	ND	Yes
esg	ND	Yes
hh	ND	Yes
cad	ND	Yes
aPKC	ND	Yes
prod	ND	Yes
BX-C		
Mcp	Yes, E	Yes
bxd(PRE_D)	Yes, E, L	Yes
iab-2	Yes, E*	Yes
iab-6	ND	Yes
iab7 (Fab7)	Yes E	Yes
iab-8	Yes E	Yes

* iab-2 fragment used required additional DNA to work in the embryonic maintenance assay.

ND is for not done. Some of these genes have more than one PRE that have been tested in these assays (see text for discussion of this). *Scr*, Sex combs reduced; *aPKC*, atypical protein kinase C; *prod*, proliferation disruptor; *hh*, hedgehog; *vg*, vestigal; *cad*, caudal; *esg*, escargot; *pb*, probosipedia; *BX-C*, Bithorax complex. Mcp, bxd (PRE_D), iab fragments are regulatory regions within the BX-C.

References: *en*: Kassis et al. (1991), Kassis (1994), Brown, Mucci, Whiteley, Dirksen, and Kassis (1998), Americo et al. (2002), and DeVido et al. (2008). *inv*: Cunningham et al. (2010). *eve*: Fujioka, Emi-Sarker, Yusibova, Goto, and Jaynes (1999) and Fujioka, Yusibova, Zhou, and Jaynes (2008). *Psc*/*Su*(*z*)*2*: Park et al. (2012). *Scr*: Gindhart and Kaufman (1995). *pb*: Kapoun and Kaufman (1995). *vg*: Okulski, Druck, Bhalerao, and Ringrose (2011). *ph*: Bloyer et al. (2003). *esg*: Kassis (1994). *hh*: Rank, Prestel, and Paro (2002), Maurange and Paro (2002) and Chanas and Machat (2005). *cad*, *aPKC*, and *prod:* Ringrose, Rehmsmeier, Dura, and Paro (2003). *BX-C*: Chan, Rastelli, and Pirrotta (1994), Gruzdeva, Kyrchanova, Parshikov, Kullyev, and Georgiev (2005), Vazquez, Muller, Pirrotta, and Sedat (2006), Pérez-Lluch, Cuartero, Azorin, and Espinås (2008), Mishra et al. (2001), Shimell, Peterson, Burr, Simon, and O'Connor (2000), and Barges et al. (2000).