Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins

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Polycomb group (PcG) and trithorax group (trxG) proteins act as antagonistic regulators to maintain transcriptional OFF and ON states of HOX and other target genes. To study the molecular basis of PcG/trxG control, we analyzed the chromatin of the HOX gene *Ultrabithorax* (*Ubx*) in *Ubx*^{OFF} and *Ubx*^{ON} cells purified from developing *Drosophila*. We find that PcG protein complexes PhoRC, PRC1, and PRC2 and the Trx protein are all constitutively bound to Polycomb response elements (PREs) in the OFF and ON state. In contrast, the trxG protein Ash1 is only bound in the ON state; not at PREs but downstream of the transcription start site. In the OFF state, we find extensive trimethylation at H3-K27, H3-K9, and H4-K20 across the entire *Ubx* gene; i.e., throughout the upstream control, promoter, and coding region. In the ON state, the upstream control region is also trimethylated at H3-K27, H3-K9, and H4-K20, but all three modifications are absent in the promoter and 5' coding region. Our analyses of mutants that lack the PcG histone methyltransferase (HMTase) E(z) or the trxG HMTase Ash1 provide strong evidence that differential histone lysine trimethylation at the promoter and in the coding region confers transcriptional ON and OFF states of *Ubx*. In particular, our results suggest that PRE-tethered PcG protein complexes act over long distances to generate Pc-repressed chromatin that is trimethylated at H3-K27, H3-K9, and H4-K20, but that the trxG HMTase Ash1 selectively prevents this trimethylation in the promoter and coding region in the ON state.

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Polycomb group (PcG) and trithorax group (trxG) proteins are evolutionary conserved proteins that regulate expression of developmental control genes in animals and plants. In both animals and plants, PcG proteins are required for maintaining long-term repression of particular target genes that were switched off by transient signals and need to be kept inactive after these signals are no longer present (for review, see Ringrose and Paro 2004). The classic example for this kind of regulation is the maintenance of HOX gene expression in *Drosophila*, where PcG and trxG regulators were first identified through genetic studies (for review, see Kennison 1995). PcG proteins are defined as regulators required for keeping *Drosophila* HOX genes repressed in cells in which they must stay inactive, whereas trxG proteins are defined as proteins required to keep HOX genes active in cells in which their products are needed.

Biochemical studies revealed that PcG and trxG proteins exist in distinct multimeric protein complexes that

bind to and modify chromatin in in vitro assays (for review, see Ringrose and Paro 2004). To date, three different *Drosophila* PcG protein complexes have been purified: PhoRC, PRC1, and PRC2. PhoRC contains the DNA-binding PcG protein Pho that targets the complex to PREs, and dSfmbt, a novel PcG protein that selectively binds to histone H3 and H4 tail peptides that are mono- or dimethylated at H3-K9 or H4-K20 (H3-K9me1/2 and H4-K20me1/2, respectively) (Klymenko et al. 2006). PRC1 contains the PcG proteins Ph, Psc, Sce/Ring, and Pc (Shao et al. 1999). PRC1 inhibits nucleosome remodeling and transcription in in-vitro assays (Francis et al. 2001; King et al. 2005) and its subunit Pc specifically binds to trimethylated K27 in histone H3 (H3-K27me3) (Fischle et al. 2003; Min et al. 2003). PRC2, finally, contains the PcG proteins E(z), Su(z)12, and Esc as well as Nurf55, and this complex functions as a histone methyltransferase (HMTase) that specifically methylates K27 in histone H3 (H3-K27) in nucleosomes (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Müller et al. 2002).

trxG proteins also exist in several different multiprotein complexes and, intriguingly, two of those also pos-

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sess HMTase activity. Notably, TAC1 contains Trithorax (Trx), an HMTase with specificity for H3-K4 (Petruk et al. 2001; Smith et al. 2004), whereas Ash1, an HMTase reported to methylate H3-K4, H3-K9, and H4-K20 (Beisel et al. 2002; Byrd and Shearn 2003), exists in a different, currently uncharacterized protein assembly (Papoulas et al. 1998). Thus, HMTases that target distinct histone lysine residues and proteins that bind methylated histone lysine residues appear to play a central role in transcriptional control by the PcG/trxG system.

Regulation of HOX genes by PcG proteins requires specific *cis*-regulatory sequences that are typically several hundred base pairs in length and are called Polycomb response elements (PREs) (Simon et al. 1993; Chan et al. 1994). In reporter gene assays in *Drosophila* embryos and larvae, PREs act as potent general silencers that prevent transcriptional activation by a variety of different transcription factors (Chan et al. 1994; Zink and Paro 1995; Dellino et al. 2004; Sengupta et al. 2004). Consistent with the finding that silencing by PREs requires PcG gene function, chromatin immunoprecipitation (X-ChIP) analyses showed that PhoRC, PRC1, and PRC2 are all specifically associated with PREs in *Drosophila* embryos and larvae (e.g., Orlando et al. 1998; Cao et al. 2002; Klymenko et al. 2006). Recent X-ChIP studies reported that H3-K27 methylation is specifically enriched at PREs (Cao et al. 2002; Ringrose et al. 2004; Wang et al. 2004). This led to a model where the role of H3-K27 methylation is to recruit PRC1 to PREs (Cao et al. 2002; Ringrose et al. 2004; Wang et al. 2004). Finally, although PREs act as silencers in all reporter gene assays described to date, several studies provided evidence that the Trx protein binds to and acts through DNA sequences that are either identical or closely linked to PRE sequences (Orlando et al. 1998; Tillib et al. 1999).

But how do the different chromatin-modifying and chromatin-binding activities encoded by PcG and trxG regulators generate transcriptional ON and OFF states at HOX promoters? Genetic studies suggest that there is a tightly balanced antagonism between PcG and trxG regulators. Specifically, in *ash1* or *trx* mutants, expression of HOX genes is lost in their normal expression domains, but HOX expression is restored in *ash1* and *trx* mutants that also lack PcG gene function (Klymenko and Müller 2004). Moreover, such *trxG*/PcG double mutants also show severe misexpression of HOX genes, comparable to PcG single mutants (Klymenko and Müller 2004). These observations suggest that Ash1 and Trx HMTases are not needed for transcriptional activation but are required to prevent “default” PcG silencing of HOX genes in cells in which these genes must stay active (Klymenko and Müller 2004). However, the molecular mechanisms by which these two *trxG* HMTases prevent PcG silencing have remained elusive.

To gain insight into PcG/trxG transcriptional control, we compared the chromatin of the HOX gene *Ultrathorax* (*Ubx*) in its ON and OFF state in imaginal disc cells in developing *Drosophila* larvae. Our quantitative analysis of X-ChIP reveals that most PcG and *trxG* proteins are constitutively bound at PREs, independently of the transcriptional state. However, we find that differential histone trimethylation at the *Ubx* promoter and coding region, generated by PcG and *trxG* HMTases, is critical for maintaining transcriptional ON and OFF states.

Results

In wild-type animals, *Ubx* is expressed in all cells of the haltere and third-leg imaginal discs, but it is stably repressed in cells of the wing imaginal disc (Fig. 1A). Hal-

tere/third-leg discs and wing discs thus represent uniform populations of *Ubx^{ON}* and *Ubx^{OFF}* cells, respectively. We prepared batches of sonicated *Ubx^{ON}* and *Ubx^{OFF}* chromatin from pools that typically contained 1500 fixed and hand-dissected imaginal discs. This procedure allowed us to perform multiple immunoprecipitation reactions with different antibodies from the same chromatin preparation (see Materials and Methods for details). Furthermore, with each antibody we performed immunoprecipitations from at least three independently prepared batches of chromatin. We then measured the abundance of specific DNA sequences in the immunoprecipitates by real-time quantitative PCR (qPCR) at the following genomic locations. In the *Ubx* gene, we analyzed 17 different regions; the exact location of the fragments (F1–F17) amplified by PCR is shown in Figure 1B. Some of these fragments map to well-characterized *Ubx* cis-regulatory sequences. In particular, F1 is located at the imaginal disc enhancer PBX that activates expression in the haltere and third-leg discs (White and Wilcox 1985; Christen and Bienz 1994), F4 and F5 are located within the core of the *bx*d PRE (Chan et al. 1994; Fritsch et al. 1999), F7 is located within the *BXD* enhancer that activates expression in the embryo but is inactive in discs (Müller and Bienz 1991; Castelli-Gair et al. 1992), F9 encompasses the *Ubx* transcription start site (Saari and Bienz 1987), F13 corresponds to the *bx* PRE (Chiang et al. 1995; Orlando et al. 1998), and F17 is located in the *Ubx* 3' untranslated region. The following regions located outside of the *Ubx* locus served as controls. A first control, F18, is located within the *iab-7* PRE of the HOX gene *Abd-B* (Fig. 1B; Mihaly et al. 1997). Since *Abd-B* is repressed in both wing and haltere/third-leg imaginal discs, X-ChIP signals at the *iab-7* PRE are expected to be comparable, if not identical, in wing and haltere/third-leg disc cells. Three additional control sequences (F19–F21) are located in intergenic regions in euchromatin, whereas F22 is located in heterochromatin (Fig. 1B).

Constitutive binding of PhoRC, PRC1, and PRC2 in the OFF and ON state

We first analyzed the binding of PhoRC, PRC1, and PRC2 in *Ubx^{ON}* and *Ubx^{OFF}* chromatin, using antibodies against different subunits of each complex. For PhoRC, we used antibodies against Pho and dSfmbt (Klymenko et al. 2006). For PRC1, we used antibodies against Psc, Sce/Ring, Pc, and Ph (Francis et al. 2001). For PRC2, we used antibodies against Su(z)12 (Czermin et al. 2002; Müller et al. 2002) as well as an antibody against Pcl, a protein that has been reported to be associated with a fraction of PRC2 in *Drosophila* embryos (O'Connell et al. 2001; Tie et al. 2003; M. Nekrasov and J. Müller, unpubl.). We observe high-level binding of PhoRC, PRC1, and PRC2 at the *bx*d, *bx*, and *iab-7* PREs (Fig. 2). Surprisingly, however, we measured comparable X-ChIP signals in *Ubx^{OFF}* and *Ubx^{ON}* chromatin, not only at the *iab-7* PRE where this is expected, but also at the *bx*d and *bx* PREs (Fig. 2). Thus, all three PcG protein complexes are bound at *Ubx* PREs in both the OFF and ON state.

Nevertheless, there are subtle, but significant differences with respect to the level of binding in the OFF and ON state. First, PhoRC, PRC1, and PRC2 are all bound at comparable levels at the *bx*d PRE (Fig. 2, F4/F5), but binding of PRC1 and PRC2 at the *bx* PRE is about two-fold lower in *Ubx^{ON}* chromatin compared with *Ubx^{OFF}* chromatin (Fig. 2, F13). Note, however, that PhoRC binding at the *bx* PRE is identical in *Ubx^{ON}* and *Ubx^{OFF}* chromatin (Fig. 2, F13). Second, in addition to the peak-level binding of Pc and Su(z)12 at PREs, Pc and Su(z)12 are also detected at regions adjacent of the *bx*d PRE and at low levels across the whole *Ubx* gene (Fig. 2). Pc binding is at least two- to threefold higher than in the control regions F19–F21, but it is reduced to background levels in the 5' coding region in *Ubx^{ON}* chromatin (F9–F12) (Fig. 2). Together, these results suggest PhoRC, PRC1, and PRC2 are constitutively recruited to *Ubx* in the ON and OFF state, but that there are subtle differences in the level of binding at the promoter and in the coding region. These results are schematically summarized in Figure 7, below.

Constitutive binding of Trx, but differential binding of Ash1 in the ON and OFF state

We next analyzed binding of the trxG proteins Trx and Ash1. Trx is specifically bound at the *bx*d, *bx*, and *iab-7* PREs and at the *Ubx* promoter but, as in the case of PcG proteins, Trx is bound to all of these sequences at comparable levels in *Ubx^{OFF}* and in *Ubx^{ON}* chromatin (Fig. 3). In sharp contrast, Ash1 is only bound to *Ubx* in *Ubx^{ON}* chromatin, where it is localized ~1 kb downstream of the *Ubx* transcription start site (Fig. 3, F10). We note that Ash1 signals at PREs are at background levels (Fig. 3, F4/F5, F13, F18), a result that is in disagreement with the recently reported binding of Ash1 to the *bx*d PRE (Sanchez-Elsner et al. 2006). Taken together, our experiments show that Trx and Ash1 show very distinct binding patterns, consistent with them being part of distinct protein complexes (Papoulas et al. 1998; Petruk et al. 2001). Trx behaves like PcG proteins, it is constitutively bound at PREs in the ON and OFF state, whereas Ash1 is bound downstream of the transcription start site, but only in the ON state (see Fig. 7 below, for summary).

The X-ChIP profile of nucleosomes across the Ubx gene is not uniform

We next wanted to analyze the relationship between the binding of PcG and trxG proteins and the status of histone lysine methylation in *Ubx* chromatin in the ON and OFF state. As a first step toward this experiment, we performed X-ChIP analyses with antibodies that recognize different portions of unmodified H3, H4, or the linker histone H1. With each of these antibodies, we detect comparable levels of histones at all regions except at the PREs and at the *Ubx* promoter. Specifically, we observe a sixfold to 10-fold reduction of histone signals in the region of the *Ubx* PREs in both *Ubx^{OFF}* and

Figure 2. PhoRC, PRC1, and PRC2 are constitutively bound at PREs. X-ChIP analysis on wild-type wing (black bars) and haltere/third-leg (orange bars) imaginal discs that represent *Ubx^{OFF}* and *Ubx^{ON}* chromatin, respectively. Each graph shows the result from at least three independent immunoprecipitation reactions performed with an antibody against the indicated PcG protein. X-ChIP signal levels are presented as percentage of input chromatin precipitated for each region. Regions are numbered as shown in Figure 1B and a simplified map is shown for reference. PhoRC subunits Pho and dSfmbt; PRC1 subunits Ph, Psc, Pc, and Sce/Ring; and PRC2 subunits Su(z)12 and Pcl are all bound at comparable levels at the *bx*, *bx*, and *iab-7* PREs in both *Ubx^{OFF}* and *Ubx^{ON}* chromatin. Note that Ph is also bound at comparable levels at the promoter in both chromatins. However, note also that there are minor differences with respect to the levels of binding. First, binding of all PRC1 and PRC2 subunits at the *bx* PRE is about twofold reduced in *Ubx^{ON}* chromatin compared with *Ubx^{OFF}* chromatin. Second, at *Ubx*, low-level binding of Pc outside of PREs is at least threefold higher than in control regions F19–F21, but is reduced in the promoter and coding regions F9–F12 in *Ubx^{ON}* chromatin. For results obtained with preimmune serum, see SOM1.

Ubx^{ON} chromatin (Fig. 4A, F4, F5, F13; note, we also find similar results for H2A; data not shown). Histone signals are also reduced at the *Ubx* promoter, where the reduction is consistently more pronounced in *Ubx^{ON}* chromatin (sixfold to 10-fold) than in *Ubx^{OFF}* chromatin (twofold) (Fig. 4A, F9). The observation that lower levels of core and linker histones are detected at PREs and at the promoter could have different reasons. One possibility would be that the histone epitopes at PREs and at the promoter are masked because of binding of PcG, trxG, or other protein complexes, and that nucleosomes present in these regions can therefore not be detected by immunoprecipitation. However, it is important to note that the same observations were made with different antibodies that recognize different epitopes on H3, H4, H2A, and H1. Another possible explanation for the reduced histone signals at PREs and the *Ubx* promoter could thus be that these regions are depleted of nucleosomes.

Differential histone lysine trimethylation patterns mark the OFF and ON state

We next performed immunoprecipitations with antibodies against methylated H3-K4. Recent studies showed that H3-K4me3 is highly localized in the vicinity of transcription start sites of active genes (e.g., Ng et al. 2003; Bernstein et al. 2005; Wirbelauer et al. 2005). X-ChIP analysis with antibodies against H3-K4me3 (Fig. 5A) and H3-K4me2 (data not shown) shows that both of these modifications are localized at and 1 kb downstream of the transcription start site in *Ubx^{ON}* chromatin, but not in *Ubx^{OFF}* chromatin (Fig. 5A, wt, F9–F10; data not shown). H3-K4me3 signals thus correlate with transcriptional activity at the *Ubx* promoter. Note that the relative H3-K4 trimethylation at the transcription start site (F9) and in the 5' coding region (F10) is at comparable levels if normalized to H3 signals.

Figure 3. Trx is constitutively bound at PREs and the promoter, but Ash1 is selectively bound in the 5' coding region of the active *Ubx* gene. X-ChIP analysis on wild-type wing (black bars) and haltere/third-leg (orange bars) imaginal discs was performed and is presented in the same manner as in the experiments shown in Figure 2. Trx is bound at comparable levels at the *bx*d, *bx*, and *iab-7* PREs and the *Ubx* promoter in both *Ubx*^{OFF} and *Ubx*^{ON} chromatin. Ash1 is specifically bound at F10, 1 kb downstream from the *Ubx* transcription start site, but only in *Ubx*^{ON} chromatin. Note that Ash1 signals at other regions (i.e., PREs) are not significantly higher than in the control regions F19–F22.

We next analyzed H3-K27 trimethylation. Earlier studies using nonquantitative X-ChIP analysis reported localized H3-K27me3 enrichment at the *bx*d PRE and flanking regions in imaginal wing discs or in S2 cells (Ringrose et al. 2004; Wang et al. 2004). In contrast to those studies, we find widespread H3-K27 trimethylation across the whole *Ubx* gene (Fig. 5A, wt). In particular, in *Ubx*^{OFF} chromatin, H3-K27me3 signals are almost uniform across the whole gene (Fig. 5A, wt). The extent of reduction of H3-K27me3 signals at PREs and at the *Ubx* promoter correlates very well with the reduction of H3 signals in these regions, (cf. Figs. 5A [wt] and 4).

Interestingly, in *Ubx*^{ON} chromatin, H3-K27me3 signals in the upstream control region are comparable to those seen in *Ubx*^{OFF} chromatin (Fig. 5A, wt, F1–F7). However, H3-K27me3 signals are reduced to background levels at the promoter and in the 5' coding region (F9–F12) in the ON state, suggesting that lack of H3-K27 trimethylation in these regions may be critical to permit transcription of the *Ubx* gene. Previous studies (Ebert et al. 2004) and our immunological analysis of imaginal disc cell clones that are homozygous for *E(z)* or *Su(z)12*-null mutations (SOM2) show that all H3-K27 methylation in *Drosophila* is generated by PRC2. These results

therefore provide strong evidence that PRE-tethered PRC2 does not only methylate nucleosomes in the vicinity of PREs, but that it methylates extended stretches of flanking chromatin.

We also tested for the presence of H3-K9me3 (Fig. 5A) and H4-K20me3 (Fig. 5B), two histone methylation marks that are characteristic for pericentric heterochromatin (Ebert et al. 2004; Schotta et al. 2004). No H3-K9me3 and only very low H4-K20me3 signals are detected in the euchromatic control regions F19–F21, but we find that H3-K9me3 is strongly enriched in the heterochromatic control region F22 (see Fig. 5A, wt, for H3-K9me3, and B for H4-K20me3). At the *Ubx* gene, the H3-K9me3 and H4-K20me3 profiles are strikingly similar to the H3-K27me3 profile (Fig. 5A,B). First, in *Ubx*^{OFF} chromatin, H3-K9me3 and H4-K20me3 are present at high levels across the whole *Ubx* locus (Fig. 5A,B). Second, in *Ubx*^{ON} chromatin, H3-K9me3 and H4-K20me3 signals are high in the upstream control region but, as in the case of H3-K27me3, they are reduced to low levels in the promoter and 5' coding region (Fig. 5A,B).

Figure 4. Histone profile across the *Ubx* gene. X-ChIP analysis on wild-type wing (black bars) and haltere/third-leg (orange bars) imaginal discs was performed and is presented in the same manner as in the experiments shown in Figure 2. Histone H3, H4, and H1 occupancy is uniform across the *Ubx* gene except at PREs (F4, F5, F13, and F18) and at the promoter (F9), where histone signals are reduced in both *Ubx*^{OFF} and *Ubx*^{ON} chromatin; see text for details.

Figure 5. Differential H3-K27, H3-K9, and H3-K4 trimethylation is controlled by PeG and trxG HMTases. (A, left) X-ChIP analysis on wing (black bars) and haltere/third-leg (orange bars) imaginal discs from wild-type (top row), *ash1^{22/ash1²²}* (middle row), and *E(z)⁶¹/E(z)⁶¹* (bottom row) animals was performed and is presented in the same manner as in the experiments shown in Figure 2. *E(z)⁶¹* homozygotes completed embryogenesis at 18°C (permissive temperature) and were then reared for 96 (–120) h at the 30°C (restrictive temperature). It is important to note that X-ChIP signals are not normalized to histone levels; reduced histone signals at PREs and in the *Ubx* promoter shown in Figure 4 need to be taken into account. In wild-type animals, H3-K4me3 (top left) is highly enriched at the transcription start site and 1 kb downstream (F9–F10) of the active *Ubx* gene in haltere/third-leg discs; take into account the reduced H3 signal at F9 (Fig. 4). In wild-type animals, H3-K27me3 and H3-K9me3 signals are highly enriched across the entire *Ubx* locus [F1–F17] at the inactive *Ubx* gene in wing discs, but both signals are very strongly reduced in the promoter and coding region (F8–F17) of the active *Ubx* gene in haltere/third-leg discs. Note that H3-K9me3, but not H3-K27me3, is highly enriched in the heterochromatic control region F22. In *ash1²²* mutant animals, H3-K4me3 signals are severely reduced in haltere/third-leg imaginal discs, whereas H3-K9me3 and H3-K27me3 signals are strongly increased in the promoter and coding region (F8–F17) in haltere/third-leg discs where they reach levels comparable to those seen in wing discs. In *E(z)⁶¹* mutants, H3-K4me3 signal at the transcription start site and 1 kb downstream (F9–F10) is present not only in haltere/third-leg, but also in wing discs. Note the strong reduction of H3-K27me3 and H3-K9me3 signals in the promoter and 5' coding region (F8–F11) in *E(z)⁶¹* mutant wing discs. The *E(z)⁶¹* allele used in this experiment is not a null mutation, the observed H3-K27me3 signals may be due to incomplete disruption of *E(z)* activity (see text and SOM2). (Right) Wing (W) and haltere (H)/third-leg (L) imaginal discs from wild-type, *ash1²²/ash1²²*, or *E(z)⁶¹/E(z)⁶¹* larvae used for X-ChIP were stained with antibody against Ubx protein. In haltere/third-leg discs from *ash1²²* homozygotes, ~80% of the cells show loss of Ubx protein signal. In wing discs from *E(z)⁶¹* homozygotes, high-level misexpression of *Ubx* is detected in ~20% of cells; in haltere/third-leg discs, Ubx signals are comparable to those in wild-type larvae. The larger size of *ash1* mutant haltere discs probably reflects partial transformation into wing discs. *E(z)⁶¹* homozygous wing and haltere discs are generally smaller than wild-type discs if larvae are grown at the restrictive temperature as described above. (B,C) Presence of H4-K20me3, H3-K9me2, and H4-K20me1 at *Ubx*. X-ChIP analysis on wild-type wing (black bars) and haltere/third-leg (orange bars) imaginal discs was performed and is presented in the same manner as in the experiments shown in Figure 2. The H4-K20me3 profile is very similar to the H3-K27me3 and H3-K9me3 profiles; note the reduction of H4-K20me3 signals in the promoter and coding region in the ON state. H3-K9me2 is strongly enriched in the heterochromatic control region F22, but no significant enrichment of H4-K20me1 is detected at Ubx and in the other control regions. H4-K20me1 is specifically enriched in the coding region in *Ubx^{ON}* chromatin and only background levels of H4-K20me1 signal are detected in *Ubx^{OFF}* chromatin.

Thus, we find that in the OFF state, three “repressive” histone lysine modifications—H3-K27me3, H3-K9me3, and H4-K20me3—are present throughout the *Ubx* gene, while in the ON state all three marks are restricted to the upstream control region and are specifically absent at the *Ubx* promoter and in the coding region. The localization of these modifications is summarized in Figure 7, below. It is intriguing that high levels of H3-K27me3, H3-K9me3, and H4-K20me3 trimethylation are present at the PBX enhancer (F1) in the ON state. PBX is strictly required for *Ubx* transcription in imaginal discs (White and Wilcox 1985). These results therefore suggest that high levels of H3-K27, H3-K9, and H4-K20 trimethylation at the PBX enhancer do not interfere with the ability of this enhancer to promote transcriptional activation in the ON state.

Loss of H3-K4me3 and gain of repressive histone trimethylation in ash1 mutants

To investigate the role of *trxG* HMTases, we next performed X-ChIP analysis in larvae that are homozygous for *ash1*²², an *ash1*-null mutation (Tripoulas et al. 1996). *ash1*²² homozygotes develop into pupal stages and show loss of *Ubx* expression in most cells in the haltere and third-leg imaginal disc, while a small fraction of cells still maintain apparently undiminished expression of *Ubx* (Fig. 5A; cf. LaJeunesse and Shearn 1995; Klymenko and Müller 2004). In such *ash1*²² mutant haltere and third-leg discs, H3-K4me3 signals in the promoter and 5' coding region are fivefold reduced (Fig. 5A, *ash1*[−]). Complementing this observation, we find that in *ash1*²² mutant haltere and third-leg discs, the “repressing” H3-K27 and H3-K9 trimethylation in the *Ubx* promoter and coding region is increased to levels that are comparable to those observed in wing discs (Fig. 5A, *ash1*[−]). This suggests that Ash1 function prevents Pc-repressive H3-K27 and H3-K9 trimethylation in the promoter and coding region in the ON state. The reduction of H3-K4me3 signal in *ash1* mutants is consistent with Ash1 trimethylating H3-K4 as reported by earlier studies (Beisel et al. 2002; Byrd and Shearn 2003; Sanchez-Elsner et al. 2006).

We note that studies from the Sauer lab reported that Ash1 also methylates H3-K9 and H4-K20 (Beisel et al. 2002) and, according to Sanchez-Elsner et al. (2006), Ash1 tri-methylates H3-K4, H3-K9, and H4-K20 in the region of the *bxd* PRE and at the *Ubx* promoter in haltere/third-leg discs. Our results are at odds with several of the data reported by Sanchez-Elsner et al. (2006). For example, we do not find H3-K4me3 at the *bxd* PRE and we do not detect H3-K9me3 and H4-K20me3 at the *Ubx* promoter in wild-type haltere/third-leg discs. Moreover, our observation that *ash1* mutant haltere/third-leg discs show a gain of H3-K9 trimethylation at the *Ubx* promoter is difficult to reconcile with the proposed H3-K9 HMTase activity of Ash1.

Loss of repressive histone trimethylation in E(z) mutants

We next explored how removal of the PcG HMTase *E(z)* affects histone methylation. *E(z)*-null mutants die as lar-

vae with extremely poorly developed imaginal discs, and we therefore performed X-ChIP analysis in larvae that are homozygous for the temperature-sensitive allele *E(z)*⁶¹. When *E(z)*⁶¹ mutant embryos were allowed to complete embryogenesis at the permissive temperature and were then shifted to the restrictive temperature during larval development (96 h at 30°C), they show high-level misexpression of *Ubx* in ~20% of wing disc cells (Fig. 5A; Jones and Gelbart 1990). HOX misexpression in *E(z)*⁶¹ mutant larvae at the restrictive temperature is thus less severe than in clones of *E(z)* protein-null mutant cells (Müller et al. 2002), suggesting that the *E(z)*⁶¹ protein still retains some *E(z)*⁺ function. Even with this residual activity, we find almost complete loss of H3-K27 trimethylation at the promoter and in the 5' coding region (F9–F11) in *E(z)*⁶¹ mutant wing discs; H3-K27me3 signals in these regions drop to the levels of H3-K27me3 signals seen in haltere/third-leg discs; i.e., in *Ubx*^{ON} chromatin [Fig. 5A, *E(z)*[−]]. However, H3-K27me3 signals are only slightly reduced in the 3' coding region in wing discs and they are largely unchanged in the upstream control region in both wing and haltere/third-leg discs [Fig. 5A, *E(z)*[−]]. Thus, the promoter and 5' coding region show the most severe loss of H3-K27me3 signals in *E(z)*⁶¹ mutants. It is possible that H3-K27me3 loss in this region is more dramatic than in other regions because F9, F10, and F11 are located far away from PRE-tethered PRC2. However, distance is unlikely to be the only explanation, since F16, a region as distant from the *bx* PRE as F9 and F10, still shows considerable levels of H3-K27 trimethylation in *E(z)*⁶¹ mutant wing discs. Perhaps the loss of H3-K27me3 at the promoter and 5' coding region is exacerbated by exchange of histones due to initiation of (abortive) transcription, a possibility that we have not explored.

Interestingly, *E(z)*⁶¹ mutant wing discs also show localized loss of H3-K9me3 signals in the same regions where H3-K27me3 signals are lost (F9–F11) but, again, the levels of H3-K9 trimethylation in the upstream control region in wing and haltere/third-leg discs is largely unchanged and only slightly reduced in the 3' coding region in wing discs [Fig. 5A, *E(z)*[−]].

In vitro, *Drosophila* PRC2 preferentially methylates H3-K27 in nucleosomes and it only poorly methylates H3-K9 (Müller et al. 2002). We cannot exclude the possibility that, in vivo, PRC2 methylates both H3-K27 and H3-K9, but we think it is equally possible that a different, presently unidentified HMTase is responsible for H3-K9 methylation at *Ubx*. For example, H3-K27 trimethylation of HOX gene chromatin by PRC2 may be a prerequisite for subsequent trimethylation by a H3-K9-specific HMTase. We also note that *E(z)*⁶¹ mutant wing discs show significant H3-K4me3 signals in the promoter and 5' coding region, consistent with the observed transcriptional activation in *E(z)*⁶¹ mutant cells [Fig. 5A, *E(z)*[−]].

Finally, we also analyzed the binding of the PRC1 subunits Pc and Ph in *E(z)*⁶¹ mutant discs. We find that in both wing and haltere/third-leg discs, Pc and Ph binding at PREs is unaffected (SOM3) (data not shown).

The repressed Ubx gene is extensively trimethylated at H3-K27, H3-K9, and H4-K20

The results described above establish that trimethylation at H3-K27, H3-K9, and H4-K20 at the *Ubx* promoter and coding region tightly correlates with transcriptional repression. We next wanted to explore to what extent these histone lysine residues are in the tri-, di- or monomethylated state. We performed X-ChIP in wild-type animals using antibodies specific for dimethylated H3-K9 and for di- or monomethylated H4-K20 and H3-K27, respectively.

For H3-K9me2 we find strong signal in the heterochromatic control region F22, but no signals are detected at the *Ubx* gene in either *Ubx*^{OFF} or *Ubx*^{ON} chromatin (Fig. 5C). In the case of H4-K20, we find that H4-K20me1 is strongly enriched in the transcribed region (F10–F17) in *Ubx*^{ON} chromatin, but that in *Ubx*^{OFF} chromatin, signals are at low levels throughout the gene (Fig. 5C). Similarly, H4-K20me2 signals are also low in all regions (data not shown). With antibodies against H3-K27me2 and against H3-K27me1, we find that signals at the *Ubx* gene in *Ubx*^{OFF} chromatin are even lower than in the control regions F19–F22 (data not shown).

Taken together, these observations support the idea that in the OFF state, the whole *Ubx* gene is extensively trimethylated at H3-K27, H3-K9, and H4-K20 and that only a minor fraction of nucleosomes may be mono- or dimethylated at these sites. In the ON state, the promoter and coding region lack nucleosomes that are trimethylated at H3-K27, H3-K9, and H4-K20 and nucleosomes of lower methylated states—e.g., H4-K20me1—are present instead (see Fig. 7, below, for summary).

General transcription factors and factors involved in transcriptional elongation are differentially bound in the ON and OFF state

Finally, we examined how the recruitment and localization of general transcription factors correlates with the histone methylation state at the active and inactive *Ubx* gene. We first analyzed the binding of TATA-binding protein (TBP) and the elongation factor Spt5 in wild-type animals. TBP is bound at the *Ubx* promoter in *Ubx*^{ON} chromatin and, albeit at two- to threefold lower levels, also in *Ubx*^{OFF} chromatin (Fig. 6, F9). This is consistent with earlier studies that showed that TBP is bound to repressed HOX promoters in *Drosophila* tissue culture cells (Breiling et al. 2001, 2004). In agreement with earlier studies that reported that TBP is bound to the *bxd* PRE in embryos (Dellino et al. 2004) we find that TBP is bound to the *bxd*, *bx*, and *iab-7* PREs in both *Ubx*^{ON} and *Ubx*^{OFF} chromatin (Fig. 6, F4, F5, F13, F18).

Spt5 and Spt4 exist in a complex called DSIF that regulates the processivity of RNA Pol II (Wada et al. 1998). In particular, studies on the *Drosophila hsp70* gene showed that Spt5 is already bound to the *hsp70* promoter before heat-shock induction and has a role in restricting elongation of paused RNA Pol II but that, after heat shock, Spt5 is required for efficient elongation of the released

Figure 6. Differential binding of components of the transcription apparatus. X-ChIP analysis on wild-type wing (black bars) and haltere/third-leg (orange bars) imaginal discs was performed and is presented in the same manner as in the experiments shown in Figure 2. TBP, Spt5, and Kismet are all bound at PREs in both *Ubx*^{OFF} and *Ubx*^{ON} chromatin; the significance of this binding is not known, but note the differential binding of all three proteins at the promoter and in the coding region. In the promoter region, F9, TBP, and Spt5 are both bound at the active and at the inactive *Ubx* gene, but note that the levels of binding are about threefold lower at the inactive *Ubx* promoter. Note also that in the coding region F10–F17, Spt5 is only present in *Ubx*^{ON} chromatin, probably reflecting the presence of elongating RNA Pol II complexes. At the promoter (F9), Kismet is only bound in *Ubx*^{ON} chromatin and no binding is detected in *Ubx*^{OFF} chromatin; we also detect binding Kismet in the 3' coding region F16 in *Ubx*^{ON} chromatin.

RNA Pol II (Saunders et al. 2003; Wu et al. 2003). Importantly, biochemical analyses suggest that both steps involve physical association of Spt5 with RNA Pol II (Krogan et al. 2002; Lindstrom et al. 2003). Presence of Spt5 in the coding region of genes thus serves as a marker for paused as well as for elongating RNA Pol II. At the *Ubx* transcription start site, we find that Spt5 is bound not only in *Ubx*^{ON} chromatin but also in *Ubx*^{OFF} chromatin, but, as in the case of TBP, Spt5 levels are about threefold lower at the inactive *Ubx* promoter (Fig. 6; F9). However, across the coding region we observe significant Spt5 signals only in *Ubx*^{ON} chromatin but not in *Ubx*^{OFF} chromatin (Fig. 6; F10–F17). These observations

are consistent with a model where Spt5 is associated with a stalled RNA Pol II at the *Ubx* promoter in the OFF state, but that elongating RNA Pol II complexes with Spt5 are only present in *Ubx*^{ON} chromatin. Unexpectedly, we also observe strong binding of Spt5 at the *bxd*, *bd*, and *iab-7* PREs at comparable levels in both *Ubx*^{ON} and *Ubx*^{OFF} chromatin (Fig. 6; F4, F5, F13, F18).

Finally, we analyzed binding of Kismet (Kis), a CHD family ATPase (Daubresse et al. 1999). Kis was originally classified as a trxG protein because of its role in HOX gene regulation, but recent studies suggest that it plays a more general role in transcription by facilitating an early step in transcriptional elongation (Srinivasan et al. 2005). Consistent with this role, we find that Kis protein is bound to the *Ubx* promoter region only in *Ubx*^{ON} chromatin, but not in *Ubx*^{OFF} chromatin (Fig. 6). Nevertheless, we also find Kis protein bound at the *bxd*, *bx*, and *iab-7* PREs, where it is bound at comparable levels in *Ubx*^{OFF} and *Ubx*^{ON} chromatin (Fig. 6).

The observation that TBP, Spt5, and Kis are all bound not only at the promoter but also at PREs is puzzling. It is tempting to speculate that PREs do not only serve as binding platforms for PcG protein complexes but may also constitute sites for assembly of different transcription factor complexes. However, it should be emphasized that TBP, Spt5, and Kis signals at PREs are comparable in *Ubx*^{ON} and *Ubx*^{OFF} chromatin; the binding at PREs therefore does not correlate with the differential binding that these proteins show at the promoter (Fig. 6). Binding of TBP, Spt5, and Kis at PREs may therefore reflect a distinct function of these proteins at PREs. In this context, it should also be pointed out that the trxG protein Ash1 is not bound at PREs (Fig. 3), supporting the idea that not all factors participating in HOX gene regulation bind to PREs.

Taken together, we find that TBP and Spt5 are bound to the *Ubx* promoter in the ON and OFF state, whereas binding of Kis at the transcription start site and binding of Spt5 in the coding region are only observed in the ON state. The similarity with the situation at the inactive *hsp70* promoter (Saunders et al. 2003; Wu et al. 2003) is striking. The presence of TBP and Spt5 at the inactive *Ubx* promoter suggests that *Ubx* transcription is blocked at a late step of transcriptional initiation, and the absence of Kis suggests that the block occurs prior to the transition to transcriptional elongation. Repressive histone methylation at H3-K27, H3-K9, and H4-K20 may directly or indirectly participate in generating this block.

Discussion

In this study we used quantitative X-ChIP analysis to examine the chromatin of the HOX gene *Ubx* in its ON and OFF state in developing *Drosophila* larvae. Previous genetic studies had established that all of the PcG and trxG proteins analyzed in this study are critically needed to maintain *Ubx* OFF and ON states in the very same imaginal disc cells in which their binding to *Ubx* was analyzed here (LaJeunesse and Shearn 1995; Beuchle et al. 2001; Birve et al. 2001; Müller et al. 2002; Klymenko

and Müller 2004; Klymenko et al. 2006). The following conclusions can be drawn from the analyses reported in this study and are schematically summarized in Figure 7. (1) The PcG protein complexes PhoRC, PRC1, and PRC2 and the Trx protein are all highly localized at PREs, but they are all constitutively bound at comparable levels in the OFF and ON state. (2) The trxG protein Ash1 is only bound in the ON state, where it is specifically localized ~1 kb downstream of the transcription start site. (3) In the OFF state, PRC2 and other unknown HMTases trimethylate H3-K27, H3-K9, and H4-K20 over an extended 100-kb domain that spans the whole *Ubx* gene. (4) In the ON state, comparable H3-K27, H3-K9, and H4-K20 trimethylation is restricted to the upstream control regions and Ash1 selectively prevents this trimethylation in the promoter and coding region. (5) Repressed *Ubx* chromatin is extensively tri- but not di- or monomethylated at H3-K27, H3-K9, and H4-K20. (6) Trimethylation of H3-K27, H3-K9, and H4-K20 at imaginal disc enhancers in the upstream control region does not impair the function of these enhancers in the ON state. (7) TBP and Spt5 are bound at the *Ubx* transcription start site in the ON and

Figure 7. Schematic representation of PcG and trxG protein complex binding and histone methylation at the *Ubx* gene in the OFF and ON state. PhoRC, PRC1, PRC2, and Trx are constitutively bound at the *bxd* and *bx* PREs and Trx, TBP, and Spt5 are constitutively bound near the transcription start site. The twofold reduction in the level of PRC1 and PRC2 binding at the *bx* PRE in the ON state and the reduction in the level of TBP and Spt5 binding at the transcription start site in the OFF state are indicated by smaller symbols. Note that Ash1 is only bound in the ON state, 1 kb downstream of the transcription start site. Note also that binding of Kis at the transcription start site is only observed in the ON state. For simplicity, binding of TBP, Spt5, and Kis at other sites (i.e., PREs) is not shown. In the OFF state, H3-K27, H3-K9, and H4-K20 trimethylation is present across the whole *Ubx* gene. In the ON state, H3-K27, H3-K9, and H4-K20 trimethylation is present throughout the upstream control region, but is largely absent in the promoter and coding region and H4-K20me1 and H3-K4me3 are present instead.

OFF state, but Kis is only bound in the ON state. This suggests that in the OFF state, transcription is blocked at a late step of transcriptional initiation, prior to the transition to elongation.

Unexpectedly, our ChIP analysis by qPCR used in this study and in a similar study by the laboratory of Vincent Pirrotta (V. Pirrotta, pers. comm.) reveals that the relationship between PcG and trxG proteins and histone methylation is quite different from the currently held views. Specifically, X-ChIP studies by Cao et al. (2002), Ringrose et al. (2004), and Wang et al. (2004) reported that H3-K27 trimethylation is localized at PREs and this led to the model that recruitment of PRC1 to PREs occurs through H3-K27me₃ (i.e., via the Pc chromodomain). In contrast, our study and the study by Vincent Pirrotta and colleagues found H3-K27 trimethylation to be present across the whole inactive *Ubx* gene, both in wing discs (this study) and in S2 cells (V. Pirrotta, pers. comm.). We have not been able to detect specific enrichment of H3-K27 trimethylation at PREs; rather, we observed a reduction of H3-K27me₃ signals at PREs, consistent with the reduced signals of H3 that are detected at these sites. Consistent with our results, genome-wide analyses of PcG protein binding and H3-K27me₃ profiles in S2 cells revealed that, at most PcG-binding sites in the genome, PcG proteins are tightly localized, whereas H3-K27 trimethylation is typically present across an extended domain that often spans the whole coding region (Schwartz et al. 2006). How could the differences between our study and the earlier studies be explained? It should be noted that in contrast to the qPCR analysis used in our study here, the studies by Cao et al. (2002), Ringrose et al. (2004), and Wang et al. (2004) all relied on nonquantitative end-point PCR after 36 or more cycles to assess the X-ChIP results. It is possible that these experimental differences account for the discrepancies. In the following we shall base our discussion on the findings reported in this study.

Localized binding of PcG protein complexes and widespread histone trimethylation

PhoRC, PRC1, and PRC2 are all tightly localized at PREs but they are all constitutively bound at the inactive and active *Ubx* gene. This suggests that recruitment of PcG complexes to PREs occurs by default. Although all three complexes are bound at comparable levels to the *bxd* PRE in the inactive and active state and PhoRC is also bound at comparable levels at the *bx* PRE, it should be pointed out that the levels of PRC1 and PRC2 binding at the *bx* PRE are about twofold reduced in the active *Ubx* gene compared with the inactive *Ubx* gene. Even though there is still high-level binding of PRC1 and PRC2 at the *bx* PRE, we cannot exclude that the observed reduction in binding helps to prevent default PcG repression of the active *Ubx* gene. It is possible that transcription through the *bx* PRE reduces PRC1 and PRC2 binding at this PRE. Transcription through PREs was recently proposed to serve as an “anti-silencing” mechanism that prevents

default silencing of active genes by PREs (Schmitt et al. 2005).

The highly localized binding of all three PcG protein complexes at PREs, together with earlier studies on PRE targeting of PcG protein complexes (Mohd-Sarip et al. 2002, 2005; Wang et al. 2004; Klymenko et al. 2006) supports the idea that not only PhoRC but also PRC1 and PRC2 are targeted to PRE DNA through interactions with Pho and/or other sequence-specific DNA-binding proteins. In the case of trxG proteins, the binding modes are more diverse. In particular, recruitment of Trx protein to PREs and to the promoter is also constitutive in both states but recruitment of Ash1 to the coding region is clearly only observed at the active *Ubx* gene. At present, we do not know how Trx or Ash1 are targeted to these sites. It is possible that a transcription-coupled process recruits Ash1 to the position 1 kb downstream of the transcription start site.

In contrast to the localized and constitutive binding of PcG protein complexes and the Trx protein, we find that the patterns of histone trimethylation are very distinct in the active and inactive *Ubx* gene. Our results also suggest that the locally bound PcG and trxG HMTases act across different distances to methylate chromatin. For example, H3-K4 trimethylation is confined to the first kilobase of the *Ubx* coding region where Ash1 and Trx are bound, whereas H3-K27 trimethylation is present across an extended 100-kb domain of chromatin that spans the whole *Ubx* gene. This suggests that PRE-tethered PRC2 is able to trimethylate H3-K27 in nucleosomes that are as far as 30 kb away from the *bxd* or *bx* PREs. Unexpectedly, we found that the H3-K9me₃ and H4-K20me₃ profiles closely match the H3-K27me₃ profile. At present it is not known which HMTases are responsible for H3-K9 and H4-K20 trimethylation, but our analysis of *E(z)* mutants indicate that these modifications may be generated in a sequential manner, following H3-K27 trimethylation by PRC2. The molecular mechanisms that permit locally tethered HMTases such as PRE-bound PRC2 to maintain such extended chromatin stretches in a trimethylated state are only poorly understood. However, a recent study showed that the PhoRC subunit dSfmbt selectively binds to mono- and dimethylated H3-K9 and H4-K20 in peptide-binding assays (Klymenko et al. 2006). One possibility would be that dSfmbt participates in the process that ensures that repressed *Ubx* chromatin is trimethylated at H3-K27, H3-K9, and H4-K20. For example, dSfmbt, tethered to PREs by Pho, may interact with nucleosomes of lower methylated states (i.e., H3-K9me_{1/2} or H4-K20me_{1/2}) in the flanking chromatin and thereby bring them into the vicinity of PRE-anchored HMTases that will hypermethylate them to the trimethylated state.

Repressive and antirepressive HMTases target the promoter and coding region

Our analyses suggest that H3-K27, H3-K9, and H4-K20 trimethylation in the promoter and coding region is critical for Polycomb repression. First, although H3-K27, H3-

K9, and H4-K20 trimethylation is present at the inactive and active *Ubx* gene, it is specifically depleted in the promoter and coding region in the active *Ubx* gene. Second, misexpression of *Ubx* in wing discs with impaired *E(z)* activity correlates well with loss of H3-K27 and H3-K9 trimethylation at the promoter and 5' coding region. It is possible that the persisting H3-K27 and H3-K9 trimethylation in the 3' coding region is responsible for maintenance of repression in those *E(z)* mutant wing discs cells that do not show misexpression of *Ubx*. Third, in haltere and third-leg discs of *ash1* mutants, the promoter and coding region become extensively trimethylated at H3-K27 and H3-K9, and this correlates with loss of *Ubx* expression. Previous studies showed that *Ubx* expression is restored in *ash1* mutants cells that also lack *E(z)* function (Klymenko and Müller 2004). Together, these findings therefore provide strong evidence that *Ash1* is required to prevent PRC2 and other HMTases from trimethylating the promoter and coding region at H3-K27 and H3-K9. The loss of H3-K4 trimethylation in *ash1* mutants is formally consistent with the idea that *Ash1* exerts its antirepressor function by trimethylating H3-K4 in nucleosomes in the promoter and 5' coding region, but other explanations are possible.

But how might H3-K27, H3-K9, and H4-K20 trimethylation in the promoter and coding region repress transcription? Our observation that TBP and Spt5 are also bound to the promoter in the OFF state suggests that these methylation marks do not prevent assembly of the basic transcription apparatus at the promoter. However, the nucleosome remodeling factor *Kis* is not recruited in the OFF state, and transcription thus appears to be blocked at a late step of transcriptional initiation prior to elongation. We found that the low-level binding of Pc in the coding region correlates with the presence of H3-K27 trimethylation; i.e., Pc and H3-K27me₃ are both present in the OFF state, but are absent in the ON state. One possible scenario would thus be that H3-K27 trimethylation in the promoter and coding region permits direct recruitment of PRC1. According to this view, locally recruited PRC1 would then repress transcription; e.g., by inhibiting nucleosome remodeling in the promoter region (Shao et al. 1999; King et al. 2005). However, several observations are not easily reconciled with such a simple "recruitment-by-methylation" model. First, peak levels of all PRC1 components are present at PREs and, apart from Pc, very little binding is observed outside of PREs. Second, excision of PRE sequences from a PRE reporter gene during development leads to a rapid loss of silencing (Sengupta et al. 2004), suggesting that transcriptional repression requires the continuous presence of PREs and the proteins that are bound to them. A second, in our opinion more plausible scenario would therefore be that DNA-binding factors first target PcG protein complexes to PREs, and that these PRE-tethered complexes then interact with trimethylated nucleosomes in the flanking chromatin in order to repress transcription. For example, it is possible that bridging interactions between the Pc chromodomain in PRE-tethered PRC1 and H3-K27me₃-marked chromatin in the promoter or coding region per-

mit other PRE-tethered PcG proteins to recognize the chromatin interval across which they should act, e.g., to inhibit nucleosome remodeling in the case of PRC1 or to trimethylate H3-K27 at hypomethylated nucleosomes in the case of PRC2.

Concluding remarks

The analysis of a HOX gene in developing *Drosophila* suggests that histone trimethylation at H3-K27, H3-K9, and H4-K20 in the promoter and coding region plays a central role in generating and maintaining of a PcG-repressed state. Contrary to previous reports, our findings provide no evidence that H3-K27 trimethylation is specifically localized at PREs and could thus recruit PRC1 to PREs; we find widespread H3-K27 trimethylation across the whole transcription unit. The data presented here provide evidence that PREs serve as assembly platforms for PcG protein complexes such as PRC2 that act over considerable distances to trimethylate H3-K27 across long stretches of chromatin. The presence of this trimethylation mark in the chromatin that flanks PREs may in turn serve as a signal to define the chromatin interval that is targeted by other PRE-tethered PcG protein complexes such as PRC1. The results reported here also provide a molecular explanation for the previously reported antirepressor function of *trxG* HMTases; selective binding of *Ash1* to the active HOX gene blocks PcG repression by preventing PRC2 from trimethylating the promoter and coding region. It is possible that the extended domain of combined H3-K27, H3-K9, and H4-K20 trimethylation creates not only the necessary stability for transcriptional repression, but that it also provides the molecular marks that permits PcG repression to be heritably maintained through cell division.

Materials and methods

Antibody production

For this study, antibodies against Pc_{15–78} and against Pho_{324–520} were raised in rabbits. Epitopes for antibody production were expressed as 6xHis-tagged fusion proteins in *Escherichia coli*. Antibodies against Pc_{15–78} and Su(z)_{12,448–802} (Müller et al. 2002) were affinity-purified on the same epitope used for immunization.

Immunostaining of discs

Staining of imaginal discs was performed as described (Beuchle et al. 2001).

X-ChIP from *Drosophila* imaginal discs using qPCR analysis

The immunoprecipitation protocol is a modified version of the X-ChIP protocol described in Orlando et al. (1997) and is described in SOM4. Details about the quantitative analysis of immunoprecipitated material are also described under SOM4. The antibodies used in this study are listed in Table 1.

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Table 1. List of antibodies used in ChIP and immunostaining experiments

Antibody	Antibody reference or source
Ash 1	Gift from Allen Shearn, described in Tripoulas et al. (1996)
dSfmbt	Described in Klymenko et al. (2006); (epitope: AA531-980)
Histone H1	Gift from J. Kadonaga
Histone H3	Abcam (ab 1791)
Histone H4	Abcam (ab 10156)
H3-K4me3	Gift from Bryan Turner, described in Schübeler et al. (2004)
H3-K9me2	Upstate Biotechnology (UBI #07-441)
H3-K9me3	Upstate Biotechnology (UBI #07-442)
H3-K27me3	Upstate Biotechnology (UBI #07-449)
H4-K20me1	Upstate Biotechnology (UBI #07-440)
H4-K20me3	Upstate Biotechnology (UBI #07-463)
Kismet	Gift from J.W. Tamkun, described in Srinivasan et al. (2005)
Pc	This study (see Materials and Methods)
Pcl	Gift from R. Jones, described in O'Connell et al. (2001)
Ph	Gift from H.W. Brock, described in DeCamillis et al. (1992)
Pho	This study (see Materials and Methods)
Psc	Gift from V. Pirrotta, described in Poux et al. (2001)
Ring/See	Gift from S.E. Bickel, described in Balicky et al. (2003)
Spt5	Gift from J. Lis, described in Saunders et al. (2003)
Su(z)12	Described in Müller et al. (2002) (see Materials and Methods)
TBP	Gift from J. Kadonaga
Trx	Gift from R. Paro, unpubl.; AA2388-2674, affinity-purified rabbit polyclonal
Ubx	Gift from R. White, described in White and Wilcox (1984)

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