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## Genome-wide analysis of Polycomb targets in *Drosophila*

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**Polycomb Group (PcG) complexes are multiprotein assemblages that bind to chromatin and establish chromatin states leading to epigenetic silencing<sup>1,2</sup>. PcG proteins regulate homeotic genes in flies and vertebrates but little is known about other PcG targets and the role of the PcG in development, differentiation and disease. We have determined the distribution of the PcG proteins PC, E(Z) and PSC and of histone H3 K27 trimethylation in the *Drosophila* genome. At more than 200 PcG target genes, binding sites for the three PcG proteins colocalize to presumptive Polycomb Response Elements (PREs). In contrast, H3 me3K27 forms broad domains including the entire transcription unit and regulatory regions. PcG targets are highly enriched in genes encoding transcription factors but receptors, signaling proteins, morphogens and regulators representing all major developmental pathways are also included.**

The components of PcG complexes are products of PcG genes, first discovered as critical regulators of the eight homeotic genes in *Drosophila*. Immunostaining of *Drosophila* polytene chromosomes, however, revealed PcG proteins at about 100 cytological loci<sup>3</sup>, implying a much larger number of target genes. Functional analysis has identified PREs as DNA sequences able to recruit PcG proteins and establish PcG silencing of neighboring genes<sup>4,5</sup>. Two separate kinds of PcG complexes bind to PREs. The PRC1-type generally includes a core quartet of proteins: PC, PSC, PH and dRing<sup>6</sup>. The PRC2-type complexes include the E(Z) methyltransferase, which methylates histone H3 lysine 27 (K27)<sup>7,8,9,10</sup>. Mono- and dimethylated K27 is widely distributed in the genome but PcG sites characteristically contain me3K27<sup>11</sup>. The activity of the E(Z) complex is essential for stable silencing<sup>3,12,13</sup> and it has been proposed that H3 me3K27 recruits the PRC1 complex through the specific affinity of the PC chromodomain for me3K27<sup>8,11</sup>. However the relationship between PRC1 and PRC2 complexes, between their binding sites and histone methylation and between binding, methylation and gene expression is not well understood and, perhaps therefore, still controversial. To characterize the genomic distribution of three PcG proteins: PC, PSC and E(Z), and of histone H3 me3K27, we used chromatin immunoprecipitation (ChIP) coupled with analysis of immunoprecipitated DNA with a high density genomic tiling microarray (see Supplementary Material for details). Since PcG target genes may be repressed in some tissues and active in others, we used a cultured cell line to minimize heterogeneity.

Viewed at the scale of a chromosome arm, the distributions of PC, PSC, E(Z) and me3K27 coincide at a number of distinct binding peaks, which we will call "PcG sites", that correspond to 70% of the bands reported in salivary gland polytene chromosomes stained with the corresponding antibodies (Fig. 1). Considering the developmental difference between salivary gland cells and the embryo-derived tissue culture cells, this suggests that a majority of target sites are occupied in a large percent of cells. The overall coincidence of the PcG proteins and K27 methylation gives us confidence that the immunoprecipitations are highly specific and the binding results are valid. Nevertheless, to minimize false positives, we have focused our analysis on the PcG sites that showed

simultaneous binding of two or more proteins, each above 2-fold enrichment. Of 149 PcG sites detected, 95 showed strong binding of all four proteins (“strong PcG sites”) while in 54 sites the binding was lower and below threshold for one of the proteins, (“weak PcG sites”) (Fig. 1, Suppl. Figs. 1-5, Suppl. Table 1). At higher resolution, most PcG sites involve two or more genes, often sharing structural or functional similarities. Thus: *engrailed* and *invected*<sup>14</sup>; the PcG genes *ph-p* and *ph-d*<sup>15</sup>; the *Dorsocross* T-box gene cluster<sup>16</sup>; the muscle NK homeobox gene cluster<sup>17</sup>; the *wingless* cluster; the two homeotic complexes ANT-C and BX-C.

The Bithorax Complex (BX-C), the best known PcG target, is a cluster of three homeotic genes, *Ubx*, *abd-A* and *Abd-B*, responsible for segmental identity in the abdomen and posterior thorax. The most prominent features are two sharp binding peaks for all three PcG proteins at the sites of the *bx* and *bxd* PREs that control *Ubx*<sup>4,18</sup> (Fig. 2). No peak is detected over the *Ubx* proximal promoter although the entire gene displays a low but significant level of PC. A series of lower peaks emerge in the *abd-A* region and part of the *Abd-B* gene. Some of these correspond to the known PREs *iab-2*<sup>19</sup>, *iab-3*, and *Mcp*<sup>20</sup>. In contrast, the H3 me3K27 distribution oscillates rapidly above a high plateau that covers *Ubx* and *abd-A* but not *Abd-B*. We used RT-PCR to determine the mRNA levels corresponding to the three genes. *Ubx* and *abd-A* transcription in these cells is very low but distinctly above background. *Abd-B* is well transcribed, at levels 300 times higher than *Ubx*. This pattern of activity is reflected by the distribution of both PcG proteins and me3K27. We note that in the *Abd-B* regulatory region, the previously characterized *Fab-7*<sup>21</sup> and *Fab-8*<sup>22</sup> PREs neither bind PcG proteins nor are methylated in these cells. The *Abd-B* gene has five distinct promoters. A sharp resurgence of both methylation and PcG protein binding in the region of the most upstream *Abd-B* promoter suggests that, in contrast to the other four promoters, this one might be repressed in our cells. RT-PCR analysis using primers specific for mRNAs initiating from each promoter confirms that the most upstream promoter is silent and the other four active. These results support the view that binding of PcG proteins to PREs is associated with transcriptional quiescence while robust transcriptional activity is accompanied by lack of binding to the PREs<sup>23</sup> and lack of K27 methylation over the transcription unit. However, we note that even in the case of *Ubx* and *abd-A*, PcG protein binding and methylation are not incompatible with measurable levels of transcription.

As the examples in Figs. 2-4 show, strong genomic sites bind all three PcG proteins. The PSC and E(Z) peaks generally rise sharply and are contained within less than 2 kb while PC frequently forms a broader peak that may include shoulders or subsidiary peaks absent for E(Z) and PSC, and subsides to background more gradually. A statistical comparison of these binding peaks is shown in Supplementary Material Fig . We take these peak binding regions to correspond to PREs, which they in fact do in the cases where these are known. Presumptive PREs are frequently found near the promoter of the target gene but they are sometimes considerably further upstream. Additional binding peaks may be found within or downstream of the transcription unit. In contrast, the H3 me3K27 distribution at each site is very broad, forming a domain of tens or even hundreds of kb encompassing the transcription unit and regulatory regions of one or more genes but rather than a level plateau it consists of a series of deep oscillations.

The strong binding peaks or putative PREs are often associated with low values or troughs in the methylation profile (Fig. 3a,b,d). In contrast, at weak binding sites (Fig. 3c) and at secondary peaks the PC distribution frequently echo methylation peaks. Overall, their relationship does not support the idea that K27 methylation suffices to recruit PC binding. We propose instead that PC bound to the strong binding peaks, the presumptive PREs, is recruited by proteins that bind specifically to those sequences. The weaker PC binding peaks and tails that mirror the methylation profile near PREs may represent a second mode of PC binding mediated by the interaction of the chromodomain with H3 me3K27.

If the E(z) methyltransferase is localized at the PRE, how is the extensive methylation domain produced? As described elsewhere (Kahn et al., submitted), we envision a looping mechanism in which interaction of PRE-bound complexes with flanking chromatin is mediated by the PC chromodomain. We suppose that methylation domains initiated by a PRE might spread bidirectionally until they encounter “active” chromatin, characterized by histone acetylation or H3 K4 methylation, marks typical of transcriptionally active genes. Alternatively, specific features might shape the methylation domain either positively, by attracting the methyltransferase complex or negatively, by blocking productive interactions with the PRE. For example, we have found that insulator complexes block the spread of H3 K27 methylation from a PRE (Kahn et al., submitted). As in the case of the *Abd-B* gene or of CG7922 and CG7956 genes in Fig. 4a, sudden drops in me3K27 levels are generally associated with transcriptional activity. Are insulators involved in protecting CG7922 and CG7956 from silencing or is the activity of these two genes simply epigenetically maintained from the time the cell line was originally established? Further work is required to answer this question.

In many cases, the presumptive PRE lies between divergently transcribed genes such as *dco* and *Sox100B* (Fig. 4). Which of the two is the PRE target? Since PREs can act at distances of 20-30 kb, the proximity of PcG peaks to a promoter is not a reliable guide. We propose that the methylation domain is the clue to the target of PcG regulation. A PcG peak lying close to a promoter is not considered to regulate that promoter if the gene is not included in the methylation domain. When multiple genes are included in the methylation domain, it is likely that they are all affected by PcG regulation. We will distinguish however between genes that contain methylation as well as one or more PcG proteins and genes that contain only methylation.

Are PREs defined by characteristic sequence motifs? While our analysis of the sequences underlying the binding peaks will be presented elsewhere, we note that Ringrose et al.<sup>24</sup> devised an algorithm based on GAGA factor, PHO and Zeste binding motifs to identify sequences likely to represent PREs in the *Drosophila* genome. This algorithm correctly predicts a number of our strong PcG binding sites (27%) and a few of the weaker sites (7%), overall 20%; however it fails to predict the majority of our PcG sites and hence PREs. The reverse is also true: only 22% of the PREs predicted by Ringrose et al. bind PcG proteins in our experiments (Supplementary Fig. 7). Taken together these data suggest that additional criteria are necessary to predict most PREs reliably.

The 95 “strong binding sites” in the genome include a total of 392 genes. Of these, 186 contain both PcG binding and methylation, the remainder are found within broad methylation domains associated with PcG proteins binding but do not bind PcG proteins over their own promoter or transcription unit. They may represent genes not directly targeted but affected by the spread of methylation. An analysis of their ontology indicates that these two classes are in fact very different. Transcription regulators constitute 64.5% ( $p=1.0 \times 10^{-108}$ . For these and other statistical calculations see Supplementary Material) of the first set but only 4.0% ( $p=1.0$ ) of those that contain only me3K27. Since transcription regulators constitute only 4.3% of the total genes in the annotated *Drosophila* genome<sup>25</sup>, these comparisons strongly suggest that 1) transcription regulator genes are preferred PcG targets; 2) genes that only include the tails of a methylation domain are probably not the primary target of PcG regulation. A similar preference is also seen among the “weak binding sites”. These include a total of 74 genes containing both PcG proteins and methylation, 28.4% ( $p=2.34 \times 10^{-10}$ ) of which encode transcription regulators. Flanking genes containing only methylation include only 5.7% ( $p=1.0$ ) transcription regulators (For more details see: Supplementary Fig. 8 and Supplementary Table 2). While transcription regulators are preferred PcG targets, secreted proteins, growth factors or their receptors and signaling proteins are also represented. PcG target genes include components of all the major differentiation and morphogenetic pathways in *Drosophila*. Since these pathways and their genes are highly conserved between flies and mammals, we think it likely that these results will be useful for the identification of mammalian PcG targets.

Finally, two PcG target sites, the *polyhomeotic* site and the *Psc-Su(z)2* site<sup>26,27</sup>, are conspicuous because they contain PcG genes. These genes must be active to ensure the functioning of the PcG mechanisms and in fact their protein and RNA products are present in these cells (data not shown). As has been noted previously<sup>15,7</sup>, the role of PcG complexes at these genes cannot be to silence but at best to downregulate expression. The *polyhomeotic* locus (Fig. 4b) is one of two sites in the entire genome that appear to bind PC but lack appreciable levels of E(Z) and of K27 methylation (Fig. 4c). In contrast, the *Psc-Su(z)2* region appears to be well methylated in Sg4 cells and binds both PC and E(Z) at multiple peaks over a 100 kb region containing only these two genes (Fig. 4a). We conclude that PcG mechanisms do not invariably lead to transcriptional silencing and are compatible with moderate levels of transcription.

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## Methods

All experiments utilized the Sg4 derivative of the *Drosophila* S2 cultured cell line<sup>28</sup>. To compensate for sequence-based variation in both hybridization and amplification efficiency, control arrays (hybridized with the input chromatin, amplified in parallel) were used and intensity ratios (ChIP to control) were computed. To reduce the impact of isolated, aberrant probes, these probe-level ratios were then smoothed by taking a trimmed mean over a sliding 675 bp window (see Materials and Methods for details).

Details of the chromatin immunoprecipitations, probe preparation, microarray methods, data analysis, real time PCR and RT-PCR are given in the Supplementary Materials.

The microarray data, in the form of cel files submitted to ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>), have Accession Number E-MEXP-535. The Accession Number contains also .sgr files for displaying the binding profiles in the Affymetrix Integrated Genome Browser.

## Figure Legends

### **Figure 1. Distribution of PcG proteins and H3 me3K27 mark on chromosome 3R.**

The DNA yield of a typical ChIP reaction was PCR-amplified before microarray hybridization. The microarray results are computed in terms of the ratio between the ChIP value and the input DNA value from a control microarray. The plots show the trimmed mean ratio over a sliding window of 675 bp along the genomic DNA sequence. In the resulting profile, all binding peaks exceeding a ChIP/Input ratio of 2 were considered significant and genomic sites that bound all four proteins simultaneously (“strong PcG sites”, black circles) or more than two proteins (“weak PcG sites”, empty circles) were further annotated (see Supplementary Table 1). The colored boxes above indicate cytological binding sites of the PcG proteins indicated in salivary gland polytene chromosomes. Here and in other figures the scale indicates Release 4 genomic coordinates.

**Figure 2. PcG and me3K27 profile at the Bithorax Complex.** Strong PcG site N12 of Figure 1 corresponds to the Bithorax Complex. The gaps in the binding profiles upstream of *bxd*-PRE or in the following figures are due to the lack of unique oligonucleotides in the underlying DNA sequence. The positions and the exon structure of the FlyBase annotated transcripts are shown above (transcription left to right) and below the kb scale (transcription right to left). The blue boxes indicate known PREs. The positions of short amplicons used to quantify the the Hox transcripts are indicated by red arrows. The histogram below shows transcription levels relative to *RpL32* (*rp49*). The data from two independent experiments are averaged and error bars indicate the standard deviation. The U-up1 amplicon, corresponding to a nontranscribed sequence upstream of the *Ubx* promoter sets the RT-PCR background detection level.

**Figure 3. Profiles of me3K27 and PcG proteins at representative PcG sites. a,** The cluster of *wingless* (*wg*)-related genes (Chromosome 2L, strong PcG site N 10). The

methylation profile shows a deep trough, corresponding to a sharp PcG binding peak (presumptive PRE), surrounded by peaks that are partly echoed by secondary peaks of PC. In some cases, a corresponding secondary peak of E(Z) is also seen as shown in **b**, in the upstream region of the *knirps-like* (*knrl*) gene from the *knirps* (*kni*) gene cluster (Chromosome 3L, strong PcG site N 14). **c**, The binding of PC and me3K27 over the 5' part of the *Epidermal growth factor receptor* (*Egfr*) gene (Chromosome 2R, weak PcG site N 5) illustrates the pattern seen in “weak PcG sites” where these two proteins are well correlated but not accompanied by appreciable binding of E(Z) or PSC. **d**, Heat map representation of me3K27 enrichment around putative PRE sites. The PREs were aligned at the point of maximum *name of factor??* crosslinking intensity (0 bp, center). The color (red=zero, white=highest) indicates the frequency of me3K27 enrichment (log<sub>2</sub>) among 167 presumptive PREs. The data show that the PRE core is depleted of me3K27.

Figure 4. **NK homeodomain cluster and *dco-Sox100B* site.** **a**, The distribution of PcG proteins and me3K27 along the 93DE cluster (Chromosome 3R, strong PcG site N15). Genes *tin*, *bapm* *lbl*, *lbe*, *C15* and *slouch* are conserved NK homeobox genes involved in mesoderm differentiation. The extensive methylation domain drops to background over the CG7922 and CG7956 genes. To test whether this drop corresponds to transcriptional activity, transcription levels were measured by RT-PCR. **b**, Transcription levels, relative to *RpL32* transcript levels, show that CG7922 and CG7956 are active while surrounding genes are silent. **c**, *dco-Sox100B* genes (Chromosome 3R, strong PcG site N23). A presumptive PRE equidistant from the two divergently transcribed genes acts only upon *Sox100B*, as indicated by the methylation domain

**Figure 5. PcG proteins bind to *ph* and *Psc-Su(z)2* polycomb group genes.** Chromatin profiles of PcG proteins and me3K27 over **a**, *Psc-Su(z)2* (Chromosome 2R, strong PcG site N8) and **b**, *ph* loci (X chromosome, weak PcG site N5). **c**, The strikingly low me3K27 signal at the *ph* locus is also evident from the immunostaining of salivary gland polytene chromosomes.

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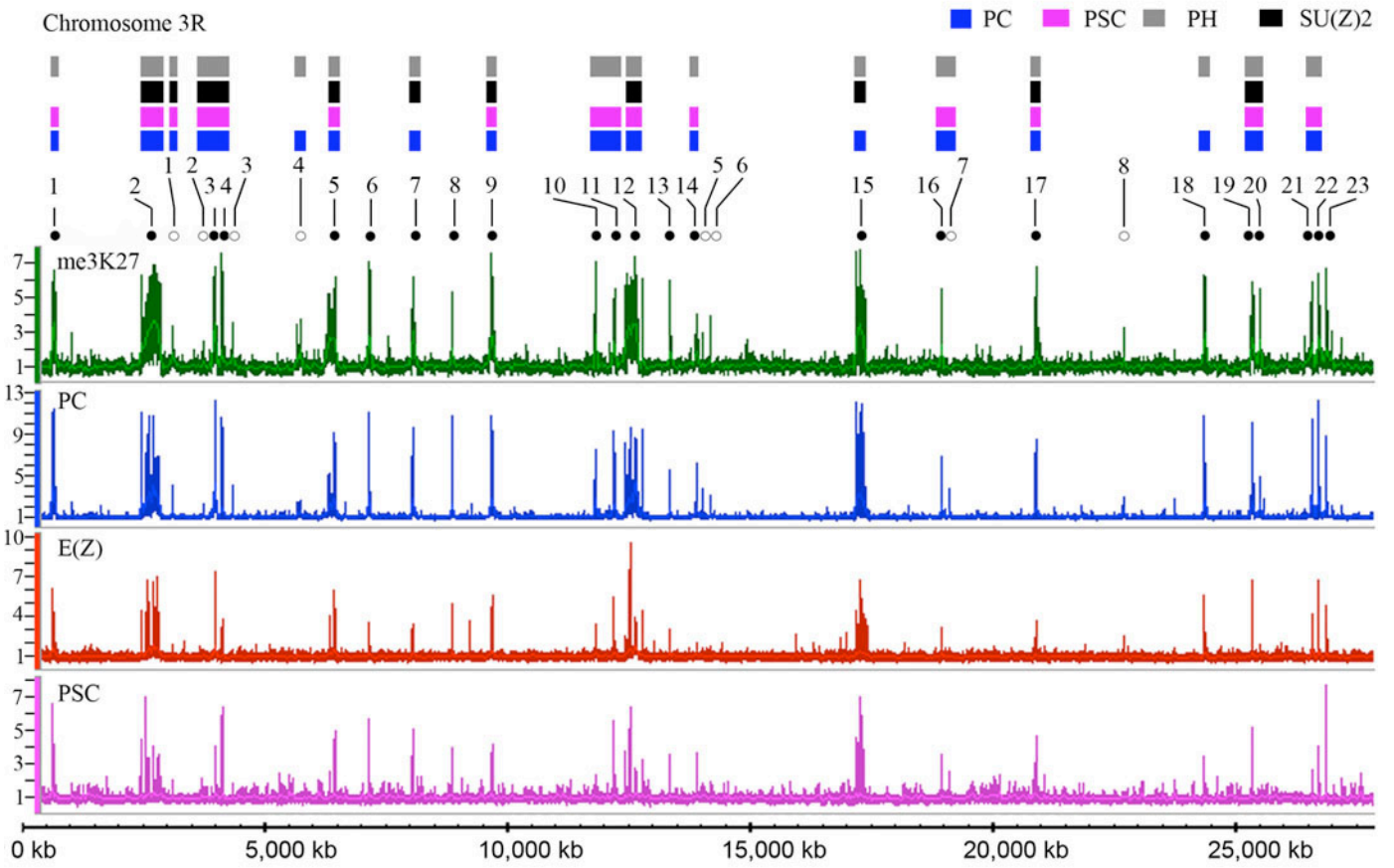
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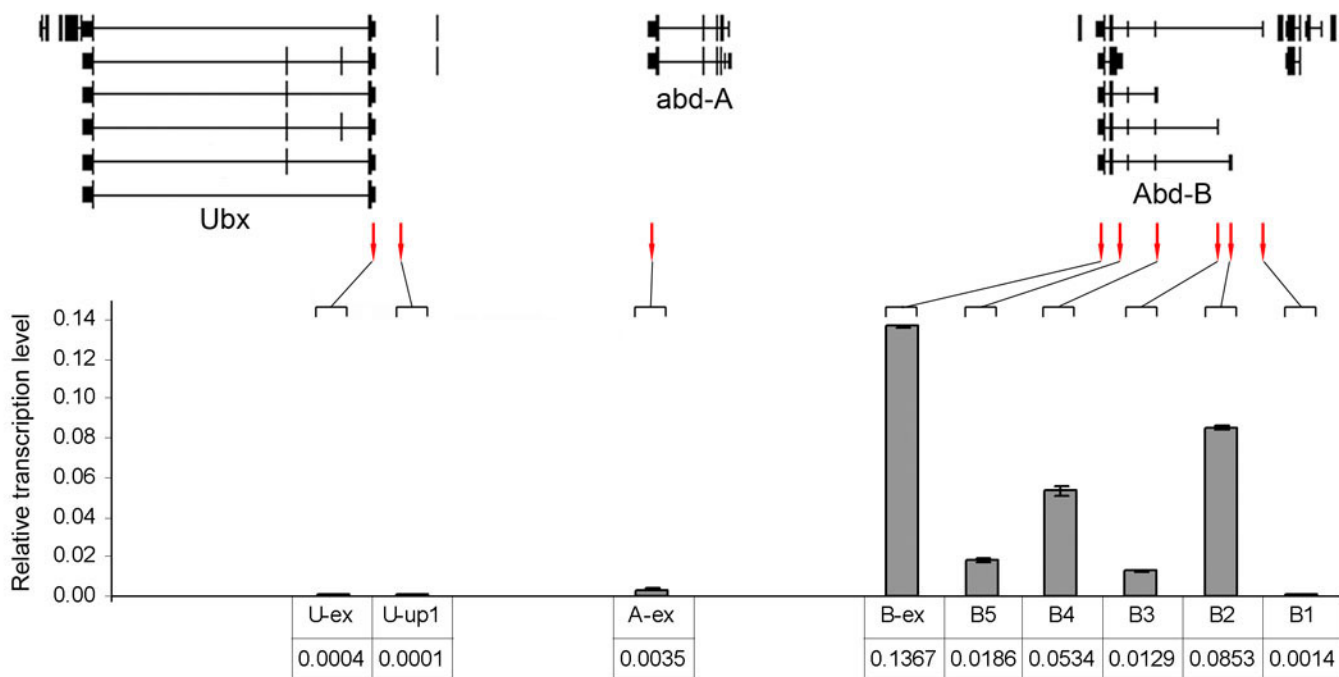
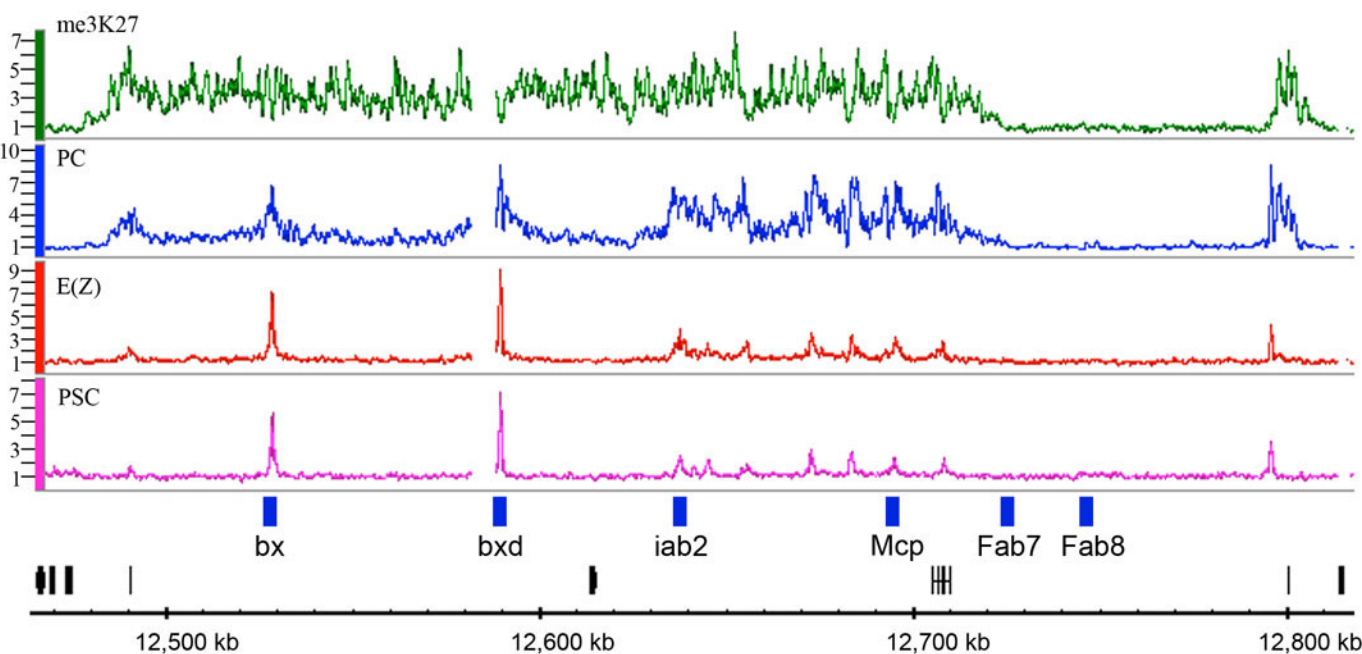


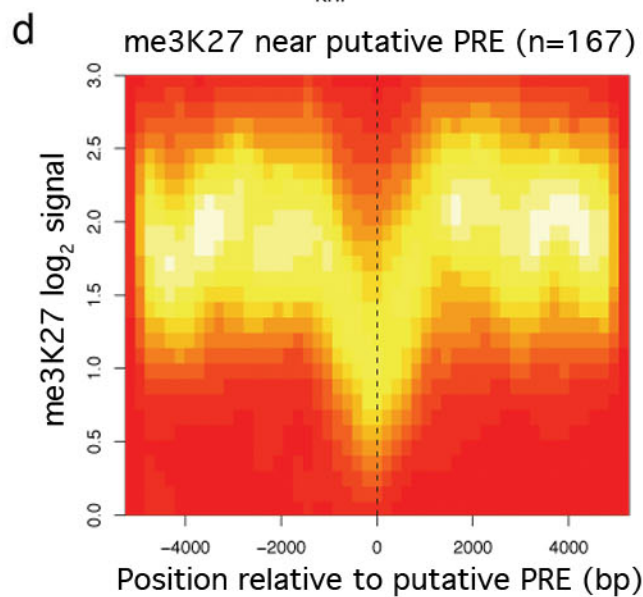
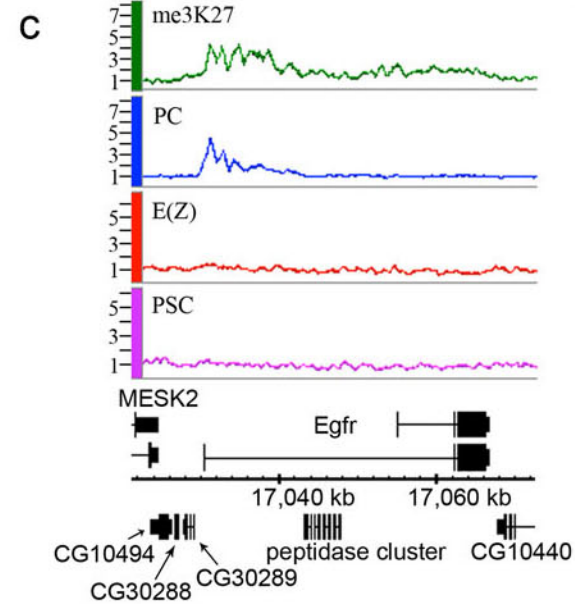
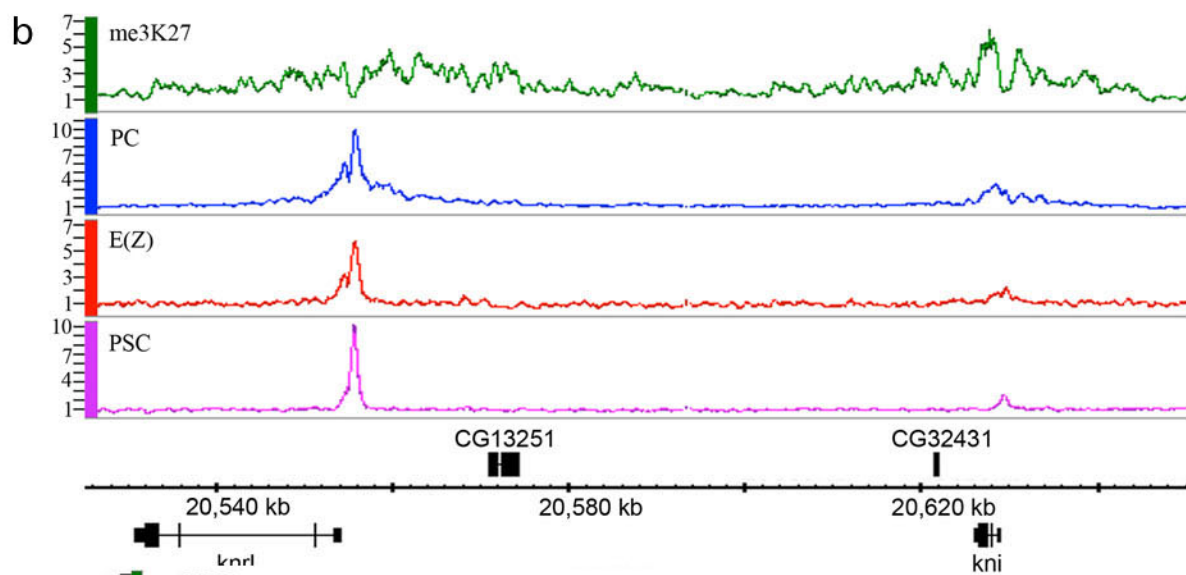
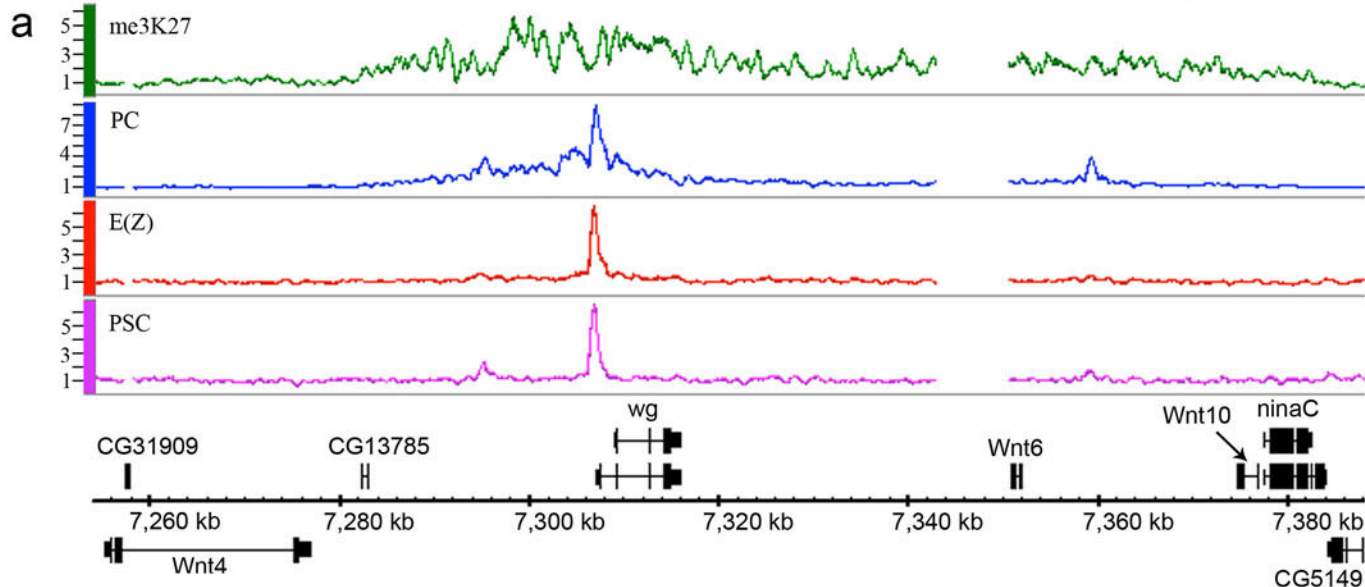
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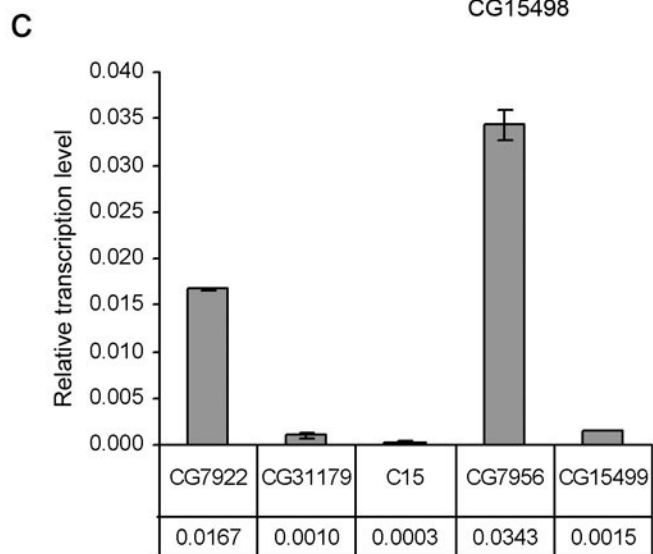
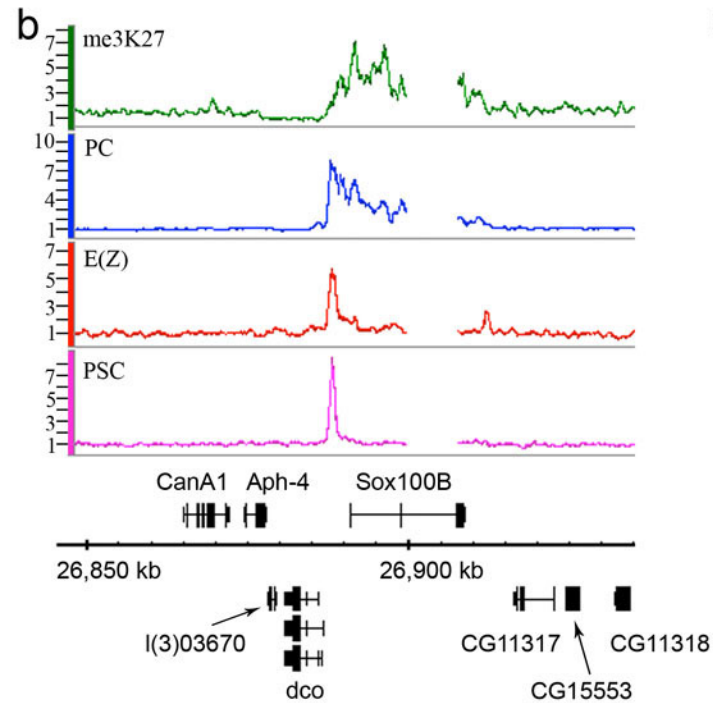
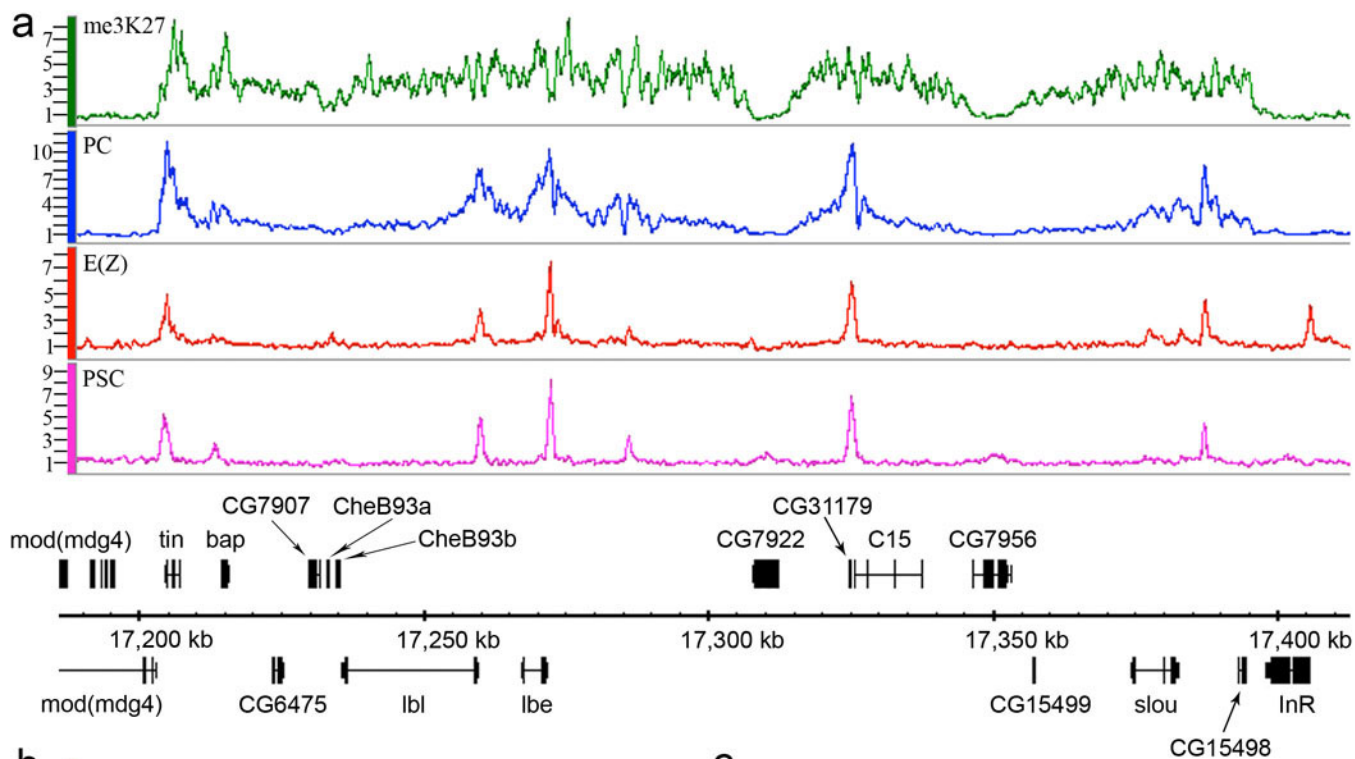
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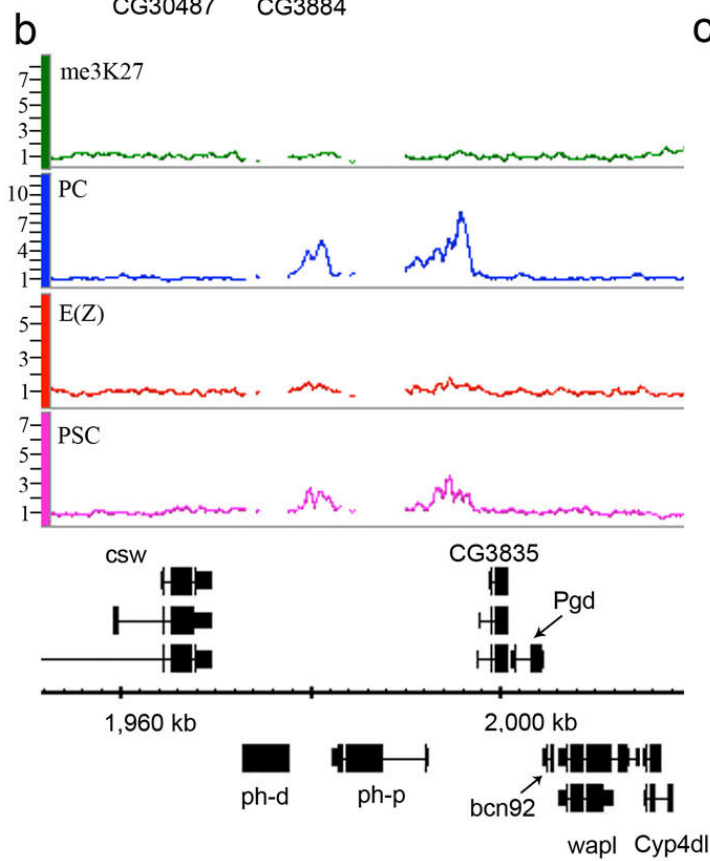
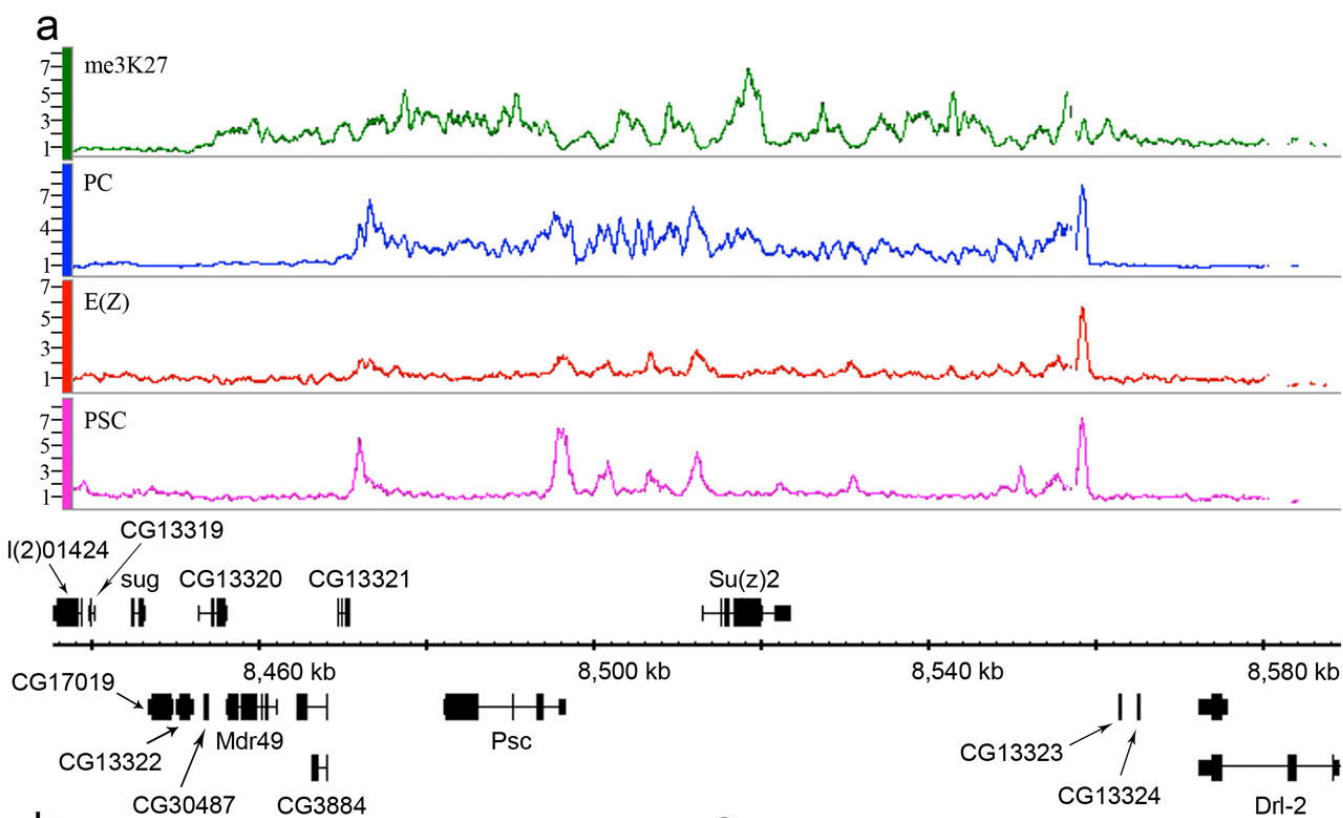












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