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A Polycomb Group Protein Is Retained at Specific Sites on Chromatin in Mitosis

Nicole E. Follmer, Ajazul H. Wani, Nicole J. Francis*

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, United States of America

Abstract

Epigenetic regulation of gene expression, including by Polycomb Group (PcG) proteins, may depend on heritable chromatin states, but how these states can be propagated through mitosis is unclear. Using immunofluorescence and biochemical fractionation, we find PcG proteins associated with mitotic chromosomes in Drosophila S2 cells. Genome-wide sequencing of chromatin immunoprecipitations (ChIP–SEQ) from mitotic cells indicates that Posterior Sex Combs (PSC) is not present at well-characterized PcG targets including Hox genes in mitosis, but does remain at a subset of interphase sites. Many of these persistent sites overlap with chromatin domain borders described by Sexton et al. (2012), which are genomic regions characterized by low levels of long range contacts. Persistent PSC binding sites flank both Hox gene clusters. We hypothesize that disruption of long-range chromatin contacts in mitosis contributes to PcG protein release from most sites, while persistent binding at sites with minimal long-range contacts may nucleate re-establishment of PcG binding and chromosome organization after mitosis.

Introduction

Epigenetic mechanisms, including those used by the essential PcG proteins, mediate stable inheritance of gene expression patterns through mitotic divisions. During mitosis, chromosomes undergo dramatic structural and biochemical changes and transcription is repressed. Binding of many transcription factors and chromatin regulators is disrupted in mitosis through post-translational modification of the proteins or their chromatin substrate [1–3]. Some transcription factors and chromatin proteins have been shown to persist on mitotic chromosomes to facilitate reactivation or prevent derepression of genes in G1, in a phenomenon termed “mitotic bookmarking” [4–9]. In most cases, however, how gene regulatory information is preserved through mitosis is not understood.

PcG proteins are required to maintain gene silencing during development and in differentiated cells (reviewed in [10–15]). These proteins assemble into multiprotein complexes with an array of enzymatic and structural effects on chromatin (for detailed reviews on the biochemistry of PcG proteins, see [16,17]). Genes regulated by the PcG thus likely have unique chromatin features including histone and protein modifications, tightly bound PcG proteins, and a locally altered chromatin structure. In Drosophila, Polycomb Response Elements (PREs), functional binding sites for PcG proteins [18], also participate in long range interactions, which are disrupted when PcG proteins are depleted [19,20]. Long range interactions are influenced by insulator sequences, which are found near many well-studied PREs [21–24]. Insulator sequences restrict enhancer-promoter interactions, and delineate chromatin loops and large-scale domains [25]. Insulators function by binding several proteins, including CTCF, BEAF, Su(HW), Mod(mgl4) and CP190 [25]. The status of long range interactions in mitosis is not known.

The extensive biochemical characterization of PcG proteins has not yet elucidated how regulation by these proteins can be maintained through mitosis. One model is that PcG proteins remain bound to mitotic chromosomes. An alternative model is that most PcG proteins are released from mitotic chromosomes but certain proteins or chromatin features mark their binding sites through mitosis to direct re-establishment of PcG protein binding after mitosis [26]. In Drosophila, immunofluorescence and live cell imaging studies have provided evidence for loss of PcG proteins from mitotic chromosomes, and, in some cases, for retention of some PcG proteins [27–30]. Here, we use immunofluorescence, biochemical fractionation and ChIP to analyze PcG protein localization to chromatin in mitosis in Drosophila S2 cells. We describe persistent association of PcG proteins with mitotic chromosomes but loss at most specific binding sites. A class of PcG binding sites that persists in mitosis is described, which we hypothesize has unique functions in chromatin organization and heritable gene regulation.

Results

Polycomb Group proteins are not excluded from mitotic chromosomes

We analyzed PcG protein localization to mitotic chromosomes in Drosophila S2 cells, a well-characterized cell line derived from embryos, by immunofluorescence. In interphase, Polycomb (PC), PSC, and dRING (dR) are predominantly nuclear, while in mitotic cells, they are distributed throughout the cell body, and
PcG proteins are not detected at PREs in pure populations of mitotic cells

Our analysis indicates that PcG proteins are associated with chromatin in mitotic cells but does not indicate whether they remain bound to target genes. To address this question, we used chromatin immunoprecipitation (ChiP). Pure populations (≥95%) of mitotic cells were isolated from cells stained with Phalloidin, which is a reliable marker of mitotic cells. To control for the FACs procedure, we sorted untreated cells with antibodies to histone H3. At least 95% of control H3-sorted cells are H3-FITC positive in the post-sorting analysis (Figure 3B). Starting with G2/M populations that are ~66% mitotic, we obtain H3S10p-sorted cells that are ≥95% mitotic (Figure 3C). We used biotinylated antibodies to PSC and streptavidin-coated beads for ChiP-qPCR to avoid isolation of the antibodies used for sorting (and associated chromatin). To analyze the distribution of PH, we used a stable S2 cell line expressing low levels of biotinylated PH instead of antibodies (Figure S1A–S1C).

We analyzed several PcG binding sites within the Bithorax Complex (BX-C) of Hox genes: bs, bxd, Fab-7 and MCPFREs, and two sites within the engrailed (en) locus (Figure 3D). PSC localizes to each of these sites in control, H3-sorted cells. In H3S10p-sorted, mitotic cells, however levels of PSC at these sites are indistinguishable from the level at a negative site (Figure 3E). In contrast, histone H3 is present at similar levels in mitotic and control cells. PH behaved similarly to PSC in a smaller number of experiments (not shown). We conclude that PSC is not detected at PREs in the BX-C and at the en locus in mitotic cells.

Genome-wide binding profiles of PSC and PH reveal reduced chromatin binding in mitosis

To determine if PSC and PH are bound to any specific sites on mitotic chromosomes, we carried out ChiP-SEQ. Immunoprecipitated and input DNA from FACs-sorted mitotic and control cells was sequenced to generate genome-wide binding profiles for both PSC and PH. Between 4.9–16.3 million reads were uniquely mapped to the genome for each sample. 4,831 and 4,629 binding sites were identified using the MACS algorithm at a 5% false discovery rate (FDR) [34]. Two biological replicates of PSC binding profiles from control cells are in good agreement (Pearson’s correlation coefficient, \( r = 0.97 \)). We also find that the PSC and PH binding profiles are nearly identical, indicating a very high degree of colocalization for the two proteins (\( r = 0.96 \) (control); \( r = 0.97 \) (mitotic) and Figure S1D–S1F). A high degree of colocalization of PSC and PH has been observed by others by polytenic chromosome staining and non-genome-wide ChiP-chip [35, 36].
Another ChIP-SEQ study in S2 cells found almost complete colocalization of PH and PSC at PSC sites, but many PH only sites were also described [37]. We carried out only one ChIP-SEQ experiment with PH but included the data in our analysis because of the high overlap with our two biological replicates with PSC.

We compared our data for PSC with two published studies in S2 cells, a ChIP-chip study of PSC done by the modENCODE consortium, and a ChIP-SEQ study identifying overlapping sites for PSC, PC, PH, and TRX done by Enderle et al. PSC overlaps with 69% of PcG binding sites described by Enderle et al. and with 24% of the sites described by modENCODE (Table 2). The Enderle et al. dataset has 52% overlap with the modENCODE data. All three studies used different antibodies to the PcG protein; some of the differences in the datasets may be due to differences in sequencing vs. microarray technology [38]. Our PSC dataset also overlaps 27% of peaks in BG3 cells, and 56% of peaks from Kc.
Polycomb Proteins on Chromatin in Mitosis

A. Propidium Iodide

- Control
  - 2N: G1: 18%
  - 4N: S: 66%
  - 4N: G2/M: 16%

- G2/M
  - 2N: G1: 1%
  - 4N: S: 5%
  - 4N: G2/M: 94%

B. Hoechst stained

- % mitotic cells
  - Control: 15 ± 2.3%
  - G2/M: 80 ± 2.3%

C. H3-S10p-FITC stained

- % of cells
  - FITC: 68%

D. Cell lysis

- Non-ionic detergent
  - 5 min, 4°C
  - 5 min at 1.3k xg, 4°C

- Soluble proteins
  - S1
    - 10 min, 20k xg, 4°C
  - S2
    - 30 min, 4°C
    - 5 min, 17k xg, 4°C

- Chromatin fraction
  - S3
  - P3

E. H3

- Control
  - TCE: S2, S3, P3
  - G2/M: S2, S3, P3

- % H3 in fraction

- Control: 100 ± 2%
  - G2/M: 100 ± 2%

F. β-tubulin

- Control
  - TCE: S2, S3, P3
  - G2/M: S2, S3, P3

- % β-tubulin in fraction

- Control: 100 ± 2%
  - G2/M: 100 ± 2%

G. Control vs G2/M

- Protein expression
  - PC, PSC, dR, E(Z), PH, Su(Z)12, PHO, CRM, dCBP

- Control vs G2/M

- * indicates significant difference

H. Protein in chromatin fraction

- % protein in chromatin fraction

- PC, PSC, dR, E(Z), PH, SU(Z)12, PHO, CRM, dCBP

- Control vs G2/M

- * indicates significant difference
Figure 2. PcG proteins fractionate with mitotic chromosomes. A) Representative FACS profiles of propidium iodide stained cells, showing DNA content. Results of cell cycle analysis are shown in the upper right. Representative profile of a control culture (top panel) and a G2/M (colchicine treated) culture (bottom panel) shows that colchicine treated cells are ~95% G2/M. B) Mitotic index of colchicine-treated G2/M cells determined by counting Hoechst-stained cells with condensed chromosomes shows that G2/M cells are ~60–70% mitotic. C) Representative western blot of fractions for histone H3 (E) or obtained by condensed chromosome counts. D) Schematic diagram of fractionation protocol used, adapted from [32] E, F) Representative western blots of fractions for PcG proteins and dCBP for control and G2/M cells reveal that PcG proteins fractionate with mitotic chromatin. H) Graph of quantification of fraction P3 for CODE consortium [39,40].

Table 1. Quantification of cellular fractionation.

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Normalized percent protein in each fraction. doi:10.1371/journal.pgen.1003135.t001

Polycomb Proteins on Chromatin in Mitosis

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Normalized percent protein in each fraction. doi:10.1371/journal.pgen.1003135.t001

cells identified in ChIP-chip experiments done by the modENCODE consortium [39,40].

Using the same peak calling parameters as for control cells, we analyzed PSC and PH ChIP-SEQ data from mitotic cells. For PSC, we used an FDR of 5% for mitotic peaks, and identified 566 mitotic peaks, 39% of which overlap the modENCODE or Enderle datasets for PSC in asynchronous cells. For PH, the signal to noise in our profile from mitotic cells was lower than for PSC. Using a less stringent cutoff, 149 peaks were identified for PH in mitotic cells, 93% of which are also mitotic peaks of PSC. Due to the lower quality of this dataset, however, it was not included in subsequent analysis. The identified peaks for both PSC and PH are a subset of the peaks from control cells; no new peaks were identified in mitotic cells (Figure 4A). Average profiles of PSC in control and mitotic cells at “control only” sites (sites that do not persist in mitosis) confirm that sequenced reads are reduced at these sites in mitotic cells relative to control cells (Figure 4B, left panel). Average profiles of PSC in control and mitotic cells at “mitotic sites” (sites that persist in mitosis) reveals, on average, a reduction in sequence reads at these sites in mitosis (Figure 4B, right panel).

Examination of the PSC binding profile at the BX-C and ora locus confirms the results observed by qPCR, which is that PSC binding is reduced in these regions in mitosis (Figure 4C). However, other peaks throughout the genome are clearly retained (Figure 4A, 4B, 4D). To determine whether the peaks that persist in mitosis are simply the largest peaks, we ranked control peaks by p-value, number of reads within the peak, peak height, or by fold over background and graphed the percentage of corresponding mitotic peaks per decile (Figure 5A). While the mitotic peaks tend to correspond to the higher ranked control peaks (30–40% of mitotic peaks correspond to peaks in the top decile of control peaks), they are not simply the highest ranked peaks. Instead, PSC is retained at specific sites. Visual inspection of the binding profiles also indicates that specific peaks are retained in mitotic cells despite the disappearance of neighboring peaks that are of similar size (Figure 4D). These data indicate that mitotic sites are unlikely to arise from contamination of the sorted cells with non-mitotic cells (up to 5%). To further validate this conclusion, we created an average profile using 5% of the control reads in peak regions and compared it with the total reads from mitotic cells (Figure 5B); the 5% control profile shows much less enrichment then the averaged mitotic profiles. We conclude that PSC is specifically retained at certain sites in mitosis.

We used qPCR to validate 8 peaks that are present in both control and mitotic cells (Figure 5C, 5D). We found that PSC is detectable at all of these sites in both control and mitotic cells but that the signals are lower in mitotic cells, consistent with the decreased number of reads. It is possible that the mitotic binding profiles observed reflect differing accessibility of chromatin in mitosis, rather than differences in PSC binding. To address this possibility, we compared ChIP for histone H3 in control and mitotic cells. At PREs, where the PSC ChIP signal is lost in mitotic cells, H3 signals are identical in control and mitotic cells (Figure 3D). At sites where PSC is retained in mitosis, we observe a slight decrease in the H3 ChIP signals, although these differences are not statistically significant (Figure 5E). These data argue against a general decrease in chromatin accessibility in mitotic cells, although we cannot completely exclude the possibility that access is differentially reduced for specific proteins at specific sites.

In our hands, the eight non-PRE sites tested by qPCR that are bound by PSC in both control and mitotic sorted cells are not detectable in asynchronously growing S2 cells that have not been sorted. Three of these eight sites were identified by Enderle et al. [2011] in their ChIP-SEQ analysis of unsorted S2 cells, however. This suggests many of the sites identified in our analysis, including some of those which are retained in mitosis, may be less accessible than well characterized PREs and thus may have distinct properties. Binding at these sites is specific for PSC, however, as no antibody ChIP controls give low signal at these sites. The detection of PSC at these sites is also not likely to be due to non-specific binding of the PSC antibody to these sites since bioin-tagged PH is detected at the same sites (i.e. without any ChIP antibodies). We carried out a series of control experiments with two of these sites to determine which aspect of the sorting
Figure 3. PSC binding is not detected at PREs in mitotic cells. A) Schematic diagram of the FACS sorting protocol to isolate mitotic cells based on H3S10p immunoreactivity. Control cultures were sorted with antibodies to histone H3. B) Representative FACS profiles of a control cell culture stained with α-H3 primary antibody and a FITC-conjugated secondary antibody before FACS sorting to isolate FITC-positive cells (left) and after sorting (right). Quantification of percentage FITC-positive cells is indicated and shows that post-sorted populations are ~95% purely FITC-positive. C) Representative FACS profiles of G2/M cells stained with FITC-conjugated α-H3S10p antibody before (left) and after (right) sorting. Quantification of percentage FITC-positive cells is indicated and shows that post-sorted populations are ~95% purely FITC-positive. D) Schematic diagram of part of the BX-C and the engrailed locus. Gray boxes indicate PREs. E) ChIP-qPCR for PSC and H3 in H3-sorted and H3S10p-sorted cells shows PSC binding is lost at these PREs in mitotic cells, while H3 binding remains the same between control and mitotic cells. *P<0.05 (two-tailed Student’s t-test comparing mitotic and control).

doi:10.1371/journal.pgen.1003135.g003
procedure allows us to detect PSC at them (Figure 6). PSC was not detected at the trx or 14-3-3e genes in cells that were either subjected to the staining procedure (with or without inclusion of antibody), which involves incubation with detergent containing buffers, or FACS sorted without antibody. In contrast, binding at these sites is observed when cells are sorted with anti-H3K27me3, or anti-tubulin, although the signal from the tubulin sorted cells is reduced. Together, these results suggest the FACS procedure, and the antibodies used for sorting both contribute to detection of PSC at these sites. The exact cause of the increased accessibility is not yet clear. Two results are thus apparent from the genome wide analysis: 1) PSC is lost at a majority of its binding sites in mitosis; and 2) PSC is retained at specific sites.

PSC is retained at sites across the genome and at cell cycle genes in mitosis

Because the mitotic binding sites for PSC did not include well-known target sites we analyzed them in several ways to understand their potential significance. Visual examination of the distribution of mitotic PSC sites over the chromosomes revealed that peaks are present on all chromosomes in control and mitotic cells, except for chromosome 4 where all peaks are lost in mitosis (Figure S2A). Quantification of the percentage of total sites per chromosome confirmed that the fraction of sites per chromosome remained relatively constant among the chromosomes between the mitotic and control binding sites except for chromosome 4 (Figure S2B). Analysis of the distribution of persistent sites within each chromosome arm suggests loss of sites from large regions along the chromosomes (Figure S2C) although the significance of this is not clear.

We mapped the genes in control and mitotic cells for which PSC lay within 2 kb of the transcription start site (TSS). The TSSs of 3,007 and 497 genes are bound by PSC in control and mitotic cells, respectively (mitotic site gene list, Table S1, and data not shown). We performed GO analysis on these gene lists using DAVID [41,42]. As expected, many PSC-bound genes in both control and mitotic cells encode transcriptional regulators and those involved in development and differentiation (mitotic site, transcriptional regulator list, Table S2, and data not shown). Both gene lists are enriched for genes involved in the cell cycle. Interestingly, these cell cycle related genes are enriched in the PSC mitotic gene list when analyzed with the PSC control sites as background (mitotic site, cell cycle related gene list, Table S3) while genes encoding transcriptional regulators are not. The functional significance of this finding is unclear. Finally, PSC is retained at four PcG and seven Trithorax Group (TrxG) genes (PcG: Asx, Sfmbt, E(Pc); trx, ase, fi(1)h, E(bx), uts, sbf, mod(mdg4), Table S1).

PSC binding sites overlap with insulator proteins and chromatin domain borders

Next we compared the binding profiles of PSC from both control and mitotic cells with all chromatin-bound protein profiles from Drosophila S2 cells published by the modENCODE consortium [39] (not shown). Several proteins exhibited a high degree of overlap with binding profiles for PSC in both control and mitotic cells including the insulator proteins CP190, BEAF, and the mitotic spindle protein Chromator [43] (Table 3). Overlap with these proteins is higher for mitotic sites than total sites, suggesting overlapping sites are preferentially retained in mitosis. To confirm this overlap, we compared PSC peaks with additional datasets for CP190 and BEAF [43] (Table 3). These three proteins were recently identified as proteins that demarcate borders between physical and functional domains that exist in the Drosophila genome [44]. Mapping of physical contacts among chromosomal regions across the Drosophila genome revealed that chromosomes are partitioned into physical domains defined by their high intra-regional contacts. These domains correlate well with functional domains characterized by the binding profiles of various histone modifications and chromatin proteins within them. Borders are the regions between these domains, and, conversely to the physical domains, are identified by their paucity of long-range interactions. We find that PSC binds 88% of domain borders in control cells, which comprises 26% of total PSC binding sites (Figure 7A). Interestingly, 46% of all mitotic PSC sites overlap borders, indicating that these sites are preferentially retained in mitosis. 34% and 51% of the PSC peaks at border sites in control and mitotic cells, respectively, are PSC sites that have been previously described in the modENCODE or Enderle datasets. Average profiles from control and mitotic cells show enrichment of PSC at domain borders (Figure 7B). As expected Chromator, CP190, BEAF and CTCF are enriched at PSC peaks at domain borders in both mitotic and control cells, with greater enrichment on average at sites that are bound by PSC in mitotic cells (Figure 7C). The same pattern was observed at the ANT-C and the Pse/Su(z)2 complex (Figure 7E, 7F). Thus, at least some large domains of PcG protein binding are flanked by persistent peaks in mitosis. PcG binding sites within these clusters (none of which persist in mitosis) engage in extensive long-range interactions [20,45,46,44], suggesting an inverse correlation between long range interactions and PcG protein persistence through mitosis.

Table 2. Overlap of PSC binding sites with published datasets.

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<td>196/800 (25%)</td>
<td>1562/2274 (69%)</td>
</tr>
<tr>
<td>modENCODE PSC</td>
<td>800</td>
<td>-</td>
<td>278/2274 (12%)</td>
</tr>
<tr>
<td>Enderle, et al. PcG</td>
<td>2274</td>
<td>257/800 (32%)</td>
<td>-</td>
</tr>
</tbody>
</table>

PcG, Polycomb Proteins on Chromatin in Mitosis

Enderle et al.

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Figure 4. Genome-wide analysis reveals PSC is retained at specific sites on mitotic chromosomes. A) Venn diagram depicting overlap of peaks of PSC binding in H3-sorted (control) cells versus H3S10p-sorted (mitotic) cells shows that a subset of PSC binding sites are retained in mitosis and no new sites are bound in mitosis. Visual inspection of sequenced reads at the one peak exclusively in mitotic cells reveals it is likely not a PSC binding site. B) Normalized read density of PSC in control cells (gray line) and mitotic cells (red line) in 50 bp windows averaged over control-only PSC binding sites (left panel) or over all mitotic PSC binding sites (right panel) shows persistent yet reduced binding in mitosis genome-wide. C) Sequence tracks from ChIP-SEQ showing PSC binding control cells (top track, gray) and in mitotic cells (bottom track, red) over the BX-C and the engrailed locus confirm loss of binding at PREs within these loci in mitotic cells. PSC binding is lost globally across the BX-C. Y-axis is normalized reads per million (RPM)/10 bp. Chromosome position and gene models are shown at the bottom. D) Sequence tracks for PSC binding in control cells (top track, gray) and in mitotic S2 cells (bottom track, red) over a 400 kbp region of chromosome 2 show persistent mitotic binding sites. Y-axis is normalized reads per million (RPM)/10 bp. Chromosome position and gene models are shown at the bottom.

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Figure 5. Validation of PSC peaks in control and mitotic cells. A) Plot of the percentage of mitotic PSC peaks per decile of corresponding ranked control PSC sites indicates mitotic peaks tend to be, but are not exclusively, the highest ranked peaks. Control PSC peaks are ranked by p-value, number of sequence reads per peak, peak height and fold over background. B) Average profile plot at control peak regions (left panel) or mitotic peak regions (right panel) for PSC in control cells (gray line), PSC in mitotic cells (red line) and 5% of the PSC profile in control cells in peak regions (gray dashed line) shows that the average plot for PSC in mitotic cells is greater than that for 5% of the PSC profile in control cells, indicating the mitotic peaks are likely not due to contamination of the mitotic population with up to 5% of interphase cells. C) ChIP-qPCR for PSC in control cells.
H3K27me3 persists at PREs in the BX-C and en gene in G2/M cells

If PSC and PH binding is lost at target genes in mitosis, memory of repression may be carried by another PcG protein or the PcG-specific histone modification H3K27me3. To address this possibility ChIP assays were carried out on asynchronously growing (control) and colchicine treated (G2/M) S2 cells. PSC, PC, dR, SU(Z)12 binding and the PcG-specific histone modification H3K27me3 localization was analyzed for PREs in the BX-C and the en locus (Figure 9A). All PcG proteins and the H3K27me3 modification are bound at all target sites in control cells except for the en intron at which only PC and H3K27me3 are bound, but not at a negative site (Figure 9B). In G2/M cells PSC binding is reduced at PREs, consistent with our analysis of FACS sorted pure mitotic cells (compare Figure 9B to Figure 3E) and indicating that binding detected at PREs in G2/M cells is due to the presence of G2 cells in the population. Association of PHO, PC, SU(Z)12, and dR with PREs is reduced in G2/M cells similarly to PSC. Thus association of PHO, PC, SU(Z)12, and dR with these PREs may be lost in mitotic cells, although this will need to be confirmed with sorted mitotic cells. In contrast, the H3K27me3 modification is present at comparable levels at PREs in control and G2/M cells. S28 of histone H3 is phosphorylated in mitosis [47]. We do not know if the H3K27me3 antibody we used recognizes the H3K27me3/S28p double modification. It is therefore possible the level of H3K27me3 we see in G2/M cells is an underestimation, although we do not know if S28p is present on H3 at these sites. Nevertheless, our data indicates that the H3K27me3 modification most likely persists through mitosis, which is consistent with other reports [48–50].

Discussion

We set out to determine whether PcG-dependent repression might be propagated through mitosis in Drosophila cells by persistent binding of PcG proteins (Model 1, Figure 10A), or whether binding needs to be re-established after mitosis (Model 2, Figure 10B). Using three different methods (immunofluorescence, biochemical fractionation, and ChIP-SEQ), we show that PcG proteins are associated with mitotic chromosomes. ChIP-SEQ analysis, however, indicates that PSC and likely PH binding is retained at only a subset of interphase sites. Thus, the mitotic behavior of PcG proteins displays features of both Models 1 and 2, prompting consideration of new models (Figure 10C).

By immunofluorescence, we observe PSC, PC, and dR associated with mitotic chromosomes, although at reduced levels compared with interphase cells (Figure 1). These results are consistent with some studies [51,28], and at odds with the conclusions of other investigators for PSC and PC [27]. Details in how material was prepared and quantified, as well as which cell types and developmental stages were analyzed may account for these differences. We therefore used an independent method, biochemical fractionation, to confirm that PcG proteins including PSC are associated with mitotic chromosomes (Figure 2). Our ChIP-SEQ results also demonstrate persistence of PSC on mitotic chromosomes (Figure 4).

The amount of PSC retained on mitotic chromosomes as determined by ChIP is lower than by the other methods. Based on comparisons of sequenced reads, it is only a few per cent of that bound to chromatin in control cells (Figure 4B). We cannot rule out the possibility that the condensation of chromatin reduces accessibility to the antibodies and thus decreases the signal in ChIP
that some of these sites flank PcG targets such as the chromatin domain borders and insulator proteins, and the finding transcribed genes, at least in part through recognition of (MSL) dosage compensation complexes on the proposed for establishing binding of the Male Specific Lethal persistent chromatin features (Figure 10). A similar model has been PREs that are marked with H3K27me3 and possibly other sites, PcG proteins could spread into the chromatin domain to PcG protein binding after mitosis (Figure 10). From these loading sites is that they function as nucleation sites for re-establishment of clusters (Figure 7), a possible model for the function of persistent PcG binding sites at chromatin domain borders is that they reflect an independent role of PcG proteins in demarcating large scale chromatin domains through mitosis.

PcG proteins and their functions are widely conserved. In mammalian cells, immunofluorescence studies report some PcG protein and H3K27me3 associated with mitotic chromosomes [48,58]. PcG proteins, including EZH2, the enzyme responsible for H3K27 methylation, are phosphorylated in a cell cycle dependent manner [59–64]. Cell-cycle dependent CDK-mediated phosphorylation of EZH2, which peaks in G2/M is important for maintaining PRC2 (the complex formed by EZH2) at target genes, possibly because it increases the affinity of PRC2 for non-coding RNAs. PRC2 also interacts and co-localizes with CTCF [24,65]. Interestingly, a careful study of PcG proteins through the cell cycle in mammalian cells found that PcG bodies, which are thought to be sites of long range interactions among PcG proteins in Drosophila [44], are reformed in G1 although some PcG proteins and H3K27me3 persist on chromosomes through mitosis [48]. It will therefore be interesting to compare ChIP-SEQ analysis of PcG proteins in mammalian mitotic cells with the observations presented here.

While this paper was being revised, a paper describing mitotic ChIP-SEQ for the transcription factor GATA-1 in FACS-sorted mammalian cells was published [66]. Persistent GATA1 binding was observed at about 10% of interphase sites in mitosis. Degradation of GATA1 in mitosis led to slower transcriptional activation of some genes with mitotic GATA1 binding and loss of repression at other targets where GATA1 is involved in negative regulation [66]. Thus, GATA1 seems to function similarly to previously described mitotic bookmarking factors [8,9] to ensure timely regulation of gene expression on mitotic exit. We do not know if PcG proteins carry out a bookmarking function at persistent sites, but our data indicate that at important PcG targets such as the Hox genes, PcG does not function as a bookmark.

In summary, we found that a key PcG protein, PSC, is lost from most sites, including well-characterized PREs, in mitosis. PSC is retained at specific sites, many of which overlap chromatin borders. We hypothesize that the persistent binding sites are important for re-establishing PcG-dependent chromatin structures and/or large scale chromatin domains after mitosis and may contribute to propagation of silencing.

| Table 3. Overlap of mitotic PSC sites with published insulator datasets. |
|---------------------------|-------------------------|-------------------------|
|                          | # of sites | overlap mitotic PSC |
| CP190, modENCODE        | 6467       | 518/566 (92%)        |
| CTCF, modENCODE         | 6227       | 499/566 (88%)        |
| CTCF, Wood et al.       | 6691       | 513/566 (91%)        |
| BEAF, modENCODE         | 4716       | 476/566 (84%)        |
| BEAF, Wood et al.       | 6135       | 532/566 (94%)        |
| Chromator, modENCODE    | 5319       | 494/566 (87%)        |

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Materials and Methods

Cell culture

_Drosophila_ S2 cells (Invitrogen, Carlsbad, CA) were cultured in ESF 921 media (Expression Systems, Woodland, CA) at a density between 1 and 7 × 10^6 cells/mL in shaking flasks at 27°C.

Antibodies

The affinity-purified anti-PSC antibody raised against PSC aa 521–869 was previously described [67]. Antibodies against PC, PH, PHO and SU(Z)12 were kind gifts from J. Mueller [68]. The antibody against dRing was a gift from R. Jones [69]. The anti-CRM antibody was a gift from W. Gehring [70], and the anti-dCBP antibody was a kind gift from A. Mazo [71]. The anti-E(Z) antibody, (dL-19), and the anti-β-tubulin antibody, (d-140) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-H3 antibodies, ChIP-grade ab1791 (for ChIP) and ab39655 (for sorting), and the anti-H3K27me3 antibody, ChIP-grade ab6002, were purchased from Abcam (Cambridge, MA). The antibody to H3Ser10p was purchased from Millipore (Billerica, MA).

Figure 7. PSC preferentially binds domain borders in mitosis. A) Venn diagram showing overlap between domain borders and PSC binding sites in control and mitotic cells shows border sites are preferentially retained in mitotic cells. B) Average profile plot of PSC in control and mitotic cells surrounding domain borders shows enrichment of PSC at these sites. C) Average profile plot of Chromator (Chro), CP190, BEAF or CTCF surrounding PSC peaks at borders in control and mitotic sites shows enrichment of these proteins at PSC peaks at domain borders. D) Sequence tracks from ChIP-SEQ showing PSC binding in control and mitotic cells over the BX-C and surrounding regions show persistent PSC peaks at borders flanking the locus in mitotic cells. Domain borders are indicated as vertical black bars below the tracks. PcG domains identified by Sexton et al. (2012) [44] are indicated by dashed lines, and the BX-C is indicated by brackets below the gene models. (E,F) Sequence tracks from the Psc/Su(z)2 locus (E) and ANT-C (F) showing PSC binding in control and mitotic cells in relation to borders reveal persistent PSC peaks at borders flanking these loci in mitotic cells.

doi:10.1371/journal.pgen.1003135.g007

Figure 8. Validation of PSC binding a domain borders in control and mitotic cells. A) ChIP-qPCR for PSC in control sorted cells at six domain border sites and a negative site confirms PSC binding at the sites identified by ChIP-SEQ but not the negative site in control cells. Flanking sites are included for four of the border sites to confirm that binding detected by qPCR coincides with the peaks observed by ChIP-SEQ. B) ChIP-qPCR for PSC in control cells at six domain border sites and a negative site confirms PSC binding at the some of the ChIP-SEQ identified sites but not the negative site in control cells. Flanking sites are included for four of the border sites.

doi:10.1371/journal.pgen.1003135.g008
Figure 9. PcG protein binding, but not H3K27me3, is reduced at PREs in G2/M cells. A) Schematic diagram of part of the BX-C and the en locus. Gray boxes indicate PREs. B) ChIP-qPCR for PcG proteins and H3K27me3 in control and G2/M cultures is consistent with lost of PcG proteins but retention of H3K27me3 at these PREs in mitotic cells. *P<0.05 (two-tailed Student’s t-test comparing control and G2/M). ^P<0.05 (two-tailed Student’s t-test comparing G2/M to no antibody, not shown).

doi:10.1371/journal.pgen.1003135.g009
Immunofluorescence and imaging

Immunofluorescence was performed as previously described [28].

1 \times 10^6 Drosophila S2 cells were plated on concanavalin A (0.5 mg/mL) coated coverslips in 6-well plates and allowed to attach overnight. For detergent-extraction, cells were first incubated in 1% digitonin, 20 mM HEPES, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM MgOAc, 1 mM EGTA for 5 min. on ice. The rest of the staining procedure was the same for detergent-extracted and unextracted cells: cells were washed at room temperature 2\times with 0.7% NaCl, incubated for 10 min. in PBS and permeabilized for 10 min. in PBS +1% triton-X 100. Cells were blocked for 30 min. at room temperature in 5% milk in PBS, rinsed in PBS and incubated with primary antibody diluted 1:200 in 1% BSA+PBS overnight at 4°C. Cells were then washed 3\times5 min. in PBS and incubated for 2 hrs. at room temperature in 1:200 dilution of secondary antibody in 1% BSA in PBS +0.1% Triton-X 100. Cells were washed 2\times5 min. in PBS and stained 10 min. with Hoechst (0.5 \mu g/mL), washed 5 min. in PBS and mounted on slides.

Cells were visualized on a Zeiss LSM700 inverted confocal microscope. 0.7 \mu m optical sections were taken using a 63\times objective. Laser power and gain were kept constant for images taken from the same slide. Images were quantified using ImageJ. A DNA mask was chosen by applying a threshold to the DNA channel using the Li method and selecting the outline of the DNA at the signal/background border. Average signal intensity for the PcG channel within this DNA mask was recorded to give PcGDNA, the PcG signal that overlaps DNA. A cell mask was chosen by applying a threshold to the PcG channel and selecting the outline of the cell at the signal/background border. A cytoplasmic mask was created by subtracting the DNA mask from the cell mask. Average signal intensity for the PcG channel within the cytoplasmic mask was recorded to give PcGcyto, the PcG signal in the cytosol. PcGDNA/PcGcyto were calculated and averaged for at least 10 each of mitotic and interphase cells.

Cell fractionation and chromatin isolation

Cell fractionation was carried out as described in [32] with minor changes. 3.5 \times 10^7 asynchronously-growing, control, or colchicine-treated, G2/M, cells were treated with 2 units of DNaseI and incubated on ice for 1 hr. for total cell extract (TCE) or fractionated by resuspension to 7 \times 10^7 cells/mL in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10%...
glycerol) plus 0.1% Triton-X 100, 1 mM DTT, 50 mg/mL TLCK, 10 mg/mL aprotinin, 16 mg/mL benzamidine, 10 mg/mL leupeptin, 2 mg/mL pepstatin, 10 mg/mL phenanthroline, and 0.2 mM PMSF, and incubated on ice for 5 min. The samples were centrifuged (1,300×g, 4 min, 4°C) to give pellet 1 (P1) and supernatant (S1). S1 was centrifuged (20,000×g, 15 min, 4°C) to give supernatant (S2) and pellet (P2). P1 was washed once in Buffer A and then lysed in Buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors as described above). Insoluble chromatin was collected by centrifugation (1,700×g, 4 min, 4°C) to give pellet (P3) and supernatant (S3). P3 was washed once in Buffer B, centrifuged again under the same conditions and resuspended in SDS loading buffer and sonicated for 5 sec. with a VibraCell sonicator (Sonics & Materials, Inc., Newtown, CT) using a microtip at 25% amplitude. Fractions were run on 8% or 15% SDS PAGE gels, transferred to nitrocellulose, blotted and developed using HRP. Blots were scanned on a Typhoon Imager and quantified with ImageQuant.

Cell synchronization and sorting
A detailed description of the synchronization and cell sorting protocol is described elsewhere [72]. Brieﬂy, cells were treated with 350 ng/ml (380 mM) colchicine for 15 hrs, harvested and then centrifuged twice (480×g for 5 min.) through a 20% sucrose cushion to remove cell debris. Fixed colchicine treated or asynchronously growing S2 cells were resuspended in 0.016% Triton-X 100, 1× PBS + protease inhibitors (which are used throughout) and incubated for 15 min. on ice. Cells were washed in 1% BSA, 0.1% Triton-X 100, 1× PBS and incubated with 2.7 µg/mL FITC-conjugated α-H3Ser10p antibody (colchicine-treated cells) or 3 µg/mL α-H3 antibody (control cells) at a concentration of 1×10^7 cells/mL and incubated on ice in the dark for 30 min. Cells were washed with 1% BSA, 0.1% Triton-X 100, 1× PBS. Control cells were incubated with FITC-conjugated secondary antibody for 30 min. and washed with 1% BSA, 0.1% Triton-X 100, 1× PBS. Cells were resuspended in 1× PBS, 10% horse serum and incubated overnight at 4°C in the dark. Cells were passed through a 40 µm filter and sorted by the FAS Center for Systems Biology Flow Cytometry Core on a MoFlo Legacy Cell Sorter (Beckman Coulter) to collect FITC-positive cells. Pre- and post-sorted cell populations were analyzed on an LSRII cell sorter (BD Biosciences).

Chromatin immunoprecipitation (ChIP)
ChIP was carried out as previously described [67]. Brieﬂy, Drosophila S2 cells were ﬁxed in 1% formaldehyde and quenched with 0.125 mM glycine, pH 7. Cells were washed with 1× PBS, wash buffer I (10 mM Heps, pH 7.6, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, plus protease inhibitors as described above), and wash buffer II (10 mM Heps, pH 7.6, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.01% Triton X-100, plus protease inhibitors as described above). Cells were centrifuged (250×g, 4 min, 4°C) and resuspended in sonication buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) to a cell concentration of 2×10^7 cells/mL in 2 mL and sonicated with 10×30 second pulses with 30 seconds between pulses using a Sonics VibraCell sonicator at 40% power. Following sonication, samples were centrifuged for 5 min. at full speed in a refrigerated microcentrifuge. The supernatant was used for ChIP. 100 µL of chromatin (corresponding to ~2×10^5 cells) were used for each reaction and were adjusted to 1× ChIP binding buffer (15 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.01% SDS). Protein A-agarose-ChIP was carried out as previously described [67]. Biotin-ChIP was carried out as follows:
line) in control cells averaged over control PH binding sites (left panel) or control PSC binding sites (right panel). F) Sequence tracks from ChIP-SEQ comparing PH binding (top track, black) and PSC binding (bottom track, gray, same as in Figure 4C) in control cells over the BX-C and the engrailed locus. Y-axis is normalized reads per million (RPM)/10 bp. Chromosome position and gene models are shown at the bottom. (TIF)

**Figure S2** PSC distribution on chromosomes in mitosis. A) Distribution of PSC binding sites across chromosomes in control (top panel) and mitotic (bottom panel) cells. X-axis is chromosomal position and Y-axis is peak height given by relative sequence reads. B) Quantification of PSC peaks per chromosome shows the percentage of peaks per chromosome remains the same, except for chromosome 4, where all peaks are lost. C) Plot of difference in % of binding sites per 5 Mb window between control and mitotic distributions for PSC across chromosomes. The locations of the ANT-C and BX-C on chromosome 3R are indicated. (TIF)

**Table S1** Genes bound by PSC in mitosis (within 2 kb of TSS). List of genes with PSC bound in mitosis. (XLS)

**References**


