H3K36 methylation antagonizes PRC2 mediated H3K27 methylation

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Running title: Crosstalk between H3K27 and H3K36 methylation

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H3K27 methylation mediated by the histone methyltransferase complex PRC2 is critical for transcriptional regulation, Polycomb silencing, Drosophila segmentation, mammalian X chromosome inactivation and cancer. PRC2-mediated H3K27 methylation can spread along the chromatin and propagate the repressive chromatin environment; thus, chromatin components that antagonize PRC2's activity are important for restraining Polycomb silencing. Here we report that in HeLa cells, H3 histones unmethylated at K36 are mostly methylated at K27, with the exception of newly synthesized H3. In addition, K27me3 rarely co-exists with K36me2 or K36me3 on the same histone H3 polypeptide. Moreover, PRC2 activity is greatly inhibited on nucleosomal substrates with pre-installed H3K36 methylation. These findings collectively identify H3K36 methylation as a chromatin component that restricts the PRC2-mediated spread of H3K27 methylation. Finally, we provide evidence that the controversial histone lysine methyltransferase Ash1, a known Trithorax group protein that antagonizes Polycomb silencing \textit{in vivo}, is an H3K36-specific dimethylase, not an H3K4 methylase, further supporting the role of H3K36 methylation in antagonizing PRC2-mediated H3K27 methylation.

Histone modifications play a vital role in defining the conformation and function of their associated chromatin (1-4). Histone H3K27 methylation mediated by the PRC2 complex (5-8) is critical for transcriptional regulation, Polycomb silencing, Drosophila segmentation, mammalian X inactivation and cancer (9-12). Interestingly, PRC2 not only catalyzes H3K27 methylation, it also recognizes methylated H3K27 (13, 14) and becomes allosterically activated upon recognition (14), thus facilitating the spread of H3K27 methylation along the chromatin and creating a regional repressive environment. However, this spreading event must be restrained \textit{in vivo} by chromatin components that antagonize PRC2 function.

The Set domain protein Ash1 is a Trithorax group protein that antagonizes Polycomb silencing...
in Drosophila (15, 16). Interestingly, it functions as an “anti-repressor” rather than as a “co-activator” in maintaining Hox gene expression (16). Moreover, the presence of Ash1 at Hox loci prevents H3K27 trimethylation (17, 18). Therefore, Ash1 has a well-established role in antagonizing PRC2-mediated H3K27 methylation in vivo, which implies that the chromatin-modifying event mediated by Ash1 likely involves one of the chromatin components that antagonize H3K27 methylation. However, the biochemical role of Ash1 in mediating histone lysine methylation is highly controversial. Ash1 was first reported to be a histone methyltransferase specific for H3K4, H3K9 and H4K20 (19). Ash1 was also reported to be a histone methyltransferase specific for H3K36 (20). In addition, two other independent studies reported Ash1 to be a histone H3K4-specific methyltransferase (21, 22). Therefore, the exact chromatin modification that Ash1 generates and thereby potentially antagonizes PRC2-mediated H3K27 methylation remains controversial. Nevertheless, when new nomenclature was assigned to chromatin-modifying enzymes, Ash1 was termed "KMT2H," which recognizes Ash1 as a H3K4-specific methyltransferase (23).

Here we report that in HeLa cells, H3K27me3 rarely co-exists with H3K36me2 or H3K36me3 on the same histone H3 polypeptide. In contrast, H3 histones unmethylated at K36 are mostly methylated at K27, suggesting crosstalk between H3K27 and H3K36 methylation in vivo. Moreover, pre-existing H3K36 methylation effectively inhibits PRC2-mediated H3K27 methylation in vitro. Finally, we demonstrate that Ash1, a known PRC2-antagonizing factor, is indeed a H3K36-specific methyltransferase. Thus, we conclude that H3K36 methylation antagonizes PRC2-mediated H3K27 methylation by direct inhibition of PRC2's enzymatic activity.

Experimental Procedures

Histone methyltransferases

Recombinant human NSD2 set domain was expressed and purified from E. coli (Fig. S1A, ref. 24). Recombinant human Ash1 set domain was expressed and purified from E. coli (Fig. S1B, ref. 22). Recombinant full length Drosophila PRC2 was expressed and purified from Baculovirus (Fig. S1C).

Cell synchronization

HeLa cells were arrested with a double thymidine block (2 mM thymidine for 14 h, then release for 10 h, followed by a second 2 mM thymidine treatment for 12 h). The cells were then released into K8 ([13C6, 15N2] heavy isotope-labeled L-lysine) supplemented medium for labeling (25). Thymidine (2 mM) was again added into the medium 17 h after the cell cycle release to prevent the cells from advancing into one more S phase.

Histone preparation for MS analysis

Chromatin histones were isolated from cells using acid extraction. In brief, cells were lysed with lysis buffer (10 mM Tris-HCl pH8.0, 1.5 mM KCl, 2 mM MgCl2, 1 mM DTT and 1.5% NP40), and washed 3 times more in lysis buffer to remove non-chromatin histones. The chromatin histones were subsequently extracted with 0.2 M HCl according to the literature (26).

Mass spectrometric analysis and quantification

The histone samples were chemically propionylated (27) prior to mass spectrometry to improve detection of the methylated peptides. Propionylated H3 histones were digested with trypsin (Promega) at a substrate/enzyme ratio of 20:1 overnight at 37°C. Peptides were separated by an analytical capillary column (50 μm × 10 cm) packed with C18 reverse phase material (YMC 5...
μm spherical particles). HPLC-separated peptides were directly electrosprayed into an LTQ-Orbitrap mass spectrometer (Thermo). Data were acquired in an information-dependent mode, full mass spectrometry scan was from m/z 300-2000 with resolution R = 60,000. The top 8 abundant ions were selected and fragmented in the linear ion trap by electron transfer dissociation (ETD); all the fragment ions were scanned in the ion trap. Precursor ions were placed into an exclusion list from further selection for 20 sec.

For stable isotope labeling-based quantification, the search results from Mascot were analyzed by MSQuant (28) to calculate ratios for the heavy/light peptide pairs.

XICs (extracted ion chromatograms) were employed to calculate the approximate relative abundance of H3:K27-R40 peptides with different modifications. Xcalibur 2.0.7 software (Thermo) was used to extract the XICs from the mono-isotopic peaks of all the doubly, triply and quadruply charged H3:K27-R40 peptides. Mass tolerance was designated as 0.1Da, and mass precision was set to 2 decimals. XIC peaks of Isobartic ions were manually defined according to Mascot search results. For relative quantification, the sum of the XIC peak area from all the modification form of H3:K27-R40 peptide was defined as 100%.

**Mono-nucleosome preparation and immunoprecipitation**

Cells were pelleted and resuspended in lysis buffer (10 mM Tris-HCl [pH8.0], 250 mM Sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl, 0.5 mM DTT and 1 mM CaCl₂), and incubated at 37 °C for 80 min with MNase (TaKaRa) at 40 U/10⁷ nuclei. Digestion was stopped by adding EDTA to a final concentration of 20 mM and chilling at 4 °C. After centrifugation (10,000 g, 5 min), nuclear pellet was resuspended in 5 mM EDTA (10 min, 4 °C). A supernatant fraction containing mono-nucleosomes generated by centrifugation (10,000 g, 10 min) was subjected to further fractionation with a 24 ml Superose 6 gel filtration column (GE) in buffer containing 10 mM Tris-HCl [pH8.0], 100 mM KCl, 0.5 mM EDTA, 1 mM DTT and 10% Glycerol. The mono-nucleosome fractions were pooled for subsequent immunoprecipitation.

For immunoprecipitation, mono-nucleosomes were incubated with antibodies against H3K27me3 or H3K36me3 for 3 hrs at 4 °C, and then captured by the protein-A agarose beads. The beads were extensively washed with buffer containing 10 mM Tris-HCl [pH8.0], 500 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% Glycerol and 0.1% NP40. Immunoprecipitated mono-nucleosomes were then eluted with SDS/PAGE loading buffer.

**Methyl-lysine analog (MLA) reactions:**

MLA reactions were performed according to methods described in the literature (29, 30). All MLA reaction products were verified by MALDI-TOF mass spectrometric analysis for quality assurance.

**Histone lysine methyltransferase (HKMT) assays**

The reactions were performed as previously described (31). A 40-μl reaction mixture containing S-[methyl-³H] adenosyl-methionine (PerkinElmer Life Sciences), 1 μg recombinant...
oligo-nucleosomes and 0.2-0.5 μg enzyme in HKMT assay buffer (50 mM Tris, pH 8.5, 20 mM KCl, 10 mM DTT and 250 mM sucrose) was incubated for 1 h at 25°C. The reaction products were separated by 13% SDS/PAGE, transferred to PVDF membranes and then subjected to autoradiography. For quantification, the membranes were stained with Coomassie Blue G250 followed by liquid scintillation counting for each stained histone band.

Results

H3 histones unmethylated at K36 are mostly methylated at K27, except for newly synthesized histones

We initially intended to assess the establishment of histone lysine methylation on newly synthesized histones during the cell cycle using stable isotope labeling-based quantitative mass spectrometry (25, 32-34). In brief, HeLa cells were arrested by double thymidine block and then released into lysine-8 ([13C6, 15N2] heavy isotope-labeled L-lysine, abbreviated as K8) supplemented medium, which labels newly synthesized proteins. Histone samples were extracted at various time points after cell-cycle release and then subjected to quantitative mass spectrometric analysis (Fig. 1A). Nine hours after cell-cycle release, almost all cells advanced through S phase, as the K8/K0 ratio of histone H3 backbone peptides reached a stable plateau (Fig. 1B). When we examined the status of K27 and K36 methylation on newly synthesized histone H3 at the 9-h time point in detail, we found that the establishment of higher methylation states (such as H3K27me3 and H3K36me3) tended to lag behind label incorporation into histone backbones (Fig. 1C, Fig. S2), indicating that these marks are unlikely to be established in a replication-coupled manner. These observations are in agreement with previous reports (32, 33). The identities of all detected peptides were confirmed by MS/MS (Fig. S3). To our surprise, at the 9-h time point, when the K8/K0 ratio of H3 backbone peptides was around 0.85; the K8/K0 ratio of peptides carrying both unmodified K27 and unmodified K36 was 23.7 (Fig. 1C, 1D). This suggests that, except for newly synthesized H3, very few H3 histones are unmodified at both K27 and K36.

The above results are further supported by quantitative data at the 3-h time point. Three hours after the cell-cycle release, K8-labeled H3K27me0/K36me0 peptides were already 3.2-fold more abundant than their older K0-labeled counterparts, even though K8-labeled H3 histones were only 19% of K0-labeled older histones at this time (Fig. 1E). These data collectively suggest that in HeLa cells, H3 histones unmodified at K36 are either methylated or acetylated at K27. Given the fact that histone deacetylase inhibitors were not supplemented into the medium during this experiment and the relatively low histone acetylation levels in the samples (Fig. 2), the above observation implies that H3 histones unmethylated at K36 are mostly methylated at K27. This prompted us to test whether methylation at H3K27 and H3K36 might negatively regulate each other.

H3K27me3 rarely co-exists with H3K36me2 or H3K36me3 on the same histone H3 polypeptide in HeLa cells

Genome-wide profiling of histone modifications has demonstrated that repressed genes are enriched for H3K27me2/3, whereas active genes are enriched for H3K36me2/3 (35-39); this suggests that H3K27me2/3 and H3K36me2/3 exist in a spatially distinct manner and that they may antagonize each other. To test this idea, we performed immunoprecipitation experiments with antibodies specific for H3K27me3 or H3K36me3,
using purified mono-nucleosomes as the input material. Indeed, anti-H3K27me3 antibodies enriched K27me3 markers on the precipitated H3 histones; in contrast, H3K36me3 markers were depleted from these samples (Fig 2, lane 2). Consistently, H3 histones immunoprecipitated by antibodies against H3K36me3 displayed enriched H3K36me3 and depleted H3K27me3 (Fig 2, lane 3).

To determine the relative abundance of histone H3 polypeptides that harbor various degrees of K27 and K36 modification marks, mass spectrometric data were generated for the 0-h time point materials, representing HeLa cells at the G1/S boundary. XICs (extracted ion chromatograms) were employed to calculate the relative levels of H3:K27-R40 peptides with different modifications. As shown in Fig. 2A, we successfully separated most forms of H3:K27-R40 peptides carrying various combinations of K27 and K36 modifications, except for two groups: K27me0/K36me1 with K27me1/K36me0 and K27me2/K36me3 with K27me3/K27me2; these peptide pairs co-eluted in reverse phase chromatography and share identical masses. Despite this, the approximate abundance of each H3:K27-R40 peptide with a particular modification status was calculated (Fig. 3B). Only 2% of total H3 histones in HeLa cells at the G1/S boundary were unmodified at both K27 and K36, which is consistent with the earlier data shown in Fig. 1. Less than 5% of H3 histones simultaneously harbored both H3K27me3 and H3K36me2 (Fig. 3); astonishingly, H3 histones carrying both H3K27me3 and H3K36me3 were undetectable in HeLa cells (Fig. 1, Fig. 3).

The immunoprecipitation experiments (Fig. 2) suggest that K27me3 and K36me2/3 rarely co-exist within the same mono-nucleosomes; and the mass spectrometric analysis further suggests that K27me3 and K36me2/3 rarely co-exist on the same histone H3 polypeptides. These data collectively imply a potential negative crosstalk mechanism between these two modifications in vivo.

H3 pre-methylated at K36 inhibits PRC2-mediated K27 methylation in vitro

Because the above data suggest that K27 and K36 methylation on the same histone polypeptide might antagonize each other, we decided to test this directly with histone methyltransferases specific for H3K27 or H3K36 and nucleosome substrates pre-modified at H3K36 or H3K27. We first installed H3K36 methylation onto nucleosomes with recombinant human NSD2, a H3K36-specific dimethylase (24); the samples were then dialyzed against TE to remove SAM (S-adenosyl-methionine) and incubated at 37°C overnight to inactivate NSD2. Nucleosomes with pre-methylated H3K36 were subsequently reacted with reconstituted PRC2 complex (Fig. 4A). Interestingly, nucleosomes pre-treated with NSD in the presence of SAM were no longer methylated by PRC2, whereas nucleosomes pre-treated with NSD in the absence of SAM were (Fig. 4B). This provides direct evidence that PRC2 activity is inhibited by pre-existing H3K36 methylation. In contrast, Dot1L-mediated H3K79 methylation was not affected by pre-existing H3K36 methylation (Fig. 4C).

We also pre-installed dimethylation or trimethylation at H3K36 using MLA (methyl-lysine analogue) reactions (29), and then tested the enzymatic activity of PRC2. Consistently, PRC2 activity on nucleosomes with pre-methylated H3K36 were greatly reduced (Fig. S4A).
In a reciprocal experiment, we pre-installed trimethylation at H3K27 using MLA reactions and then tested the enzymatic activity of human HYPB, an H3K36-specific trimethylase (31). However, we did not observe any significant impact (Fig. S4B). Thus, we conclude that H3K36 methylation directly inhibits the H3K27 trimethylase PRC2, but H3Kc27me3 does not inhibit the H3K36 trimethylase HYPB.

The mechanism by which H3K36 methylation inhibits PRC2 enzymatic activity is not clear. Ultimately, this would require a crystal structure of PRC2 in complex with nucleosome substrates, which is currently unavailable. However, we do have a hint that the amino acids around K36 is important for PRC2 activity, since nucleosomes assembled with H3 histones harboring a K36A mutation greatly jeopardizes the enzymatic activity of PRC2 (Fig. S4C). This suggests that PRC2 makes contact with amino-acids around H3K36, and such contact is important for PRC2 activity. Therefore, mutating K36 (Fig. S4C) or methylating K36 (Fig. 4, Fig. S4A) likely impair PRC2’s enzymatic activity by restricting the binding of PRC2 towards its nucleosome substrates.

**Ash1 is an H3K36-specific dimethylase**

The inhibition of PRC2 activity by H3K36 methylation implies that at least one histone H3K36-specific methyltransferase might belong to the Trithorax group proteins. However, neither Drosophila HYPB nor Mes4, the two well-studied H3K36-specific methyltransferases (40), belongs to the Trithorax group of proteins. Thus, we decided to turn to the controversial histone methyltransferase Ash1, which is a well-known Trithorax group protein that antagonizes Polycomb silencing (15, 16). Although several groups reported that Ash1 is specific for H3K4 (19, 21, 22), or even H3K9 and H4K20 (19), one independent study reported that Ash1 is a H3K36-specific methyltransferase (20). Moreover, the Set domain of Ash1 is much more closely related to H3K36-specific methyltransferases (including HYPB and Mes4) than H3K4-specific methyltransferases such as Set1 or Trithorax (Fig. S4D).

Recombinant human Ash1 Set domain purified from *E. coli* displayed far greater activity on nucleosomal H3 than free H3 histones or mixed core histones (Fig. 5A). This is again a hint that Ash1 might be an H3K36-specific histone methyltransferase, since two other well-studied histone methyltransferases, HYPB and NSD2 were also reported to show far greater activity on nucleosomal H3 (24, 31); in contrast, H3K4 specific histone methyltransferases, like Set1 and MLL1 tend to have better enzymatic activity towards non-nucleosomal H3 histones (41, 42).

Wild-type nucleosomes or nucleosomes harboring mutant H3 histones with their K4, K9, K27 or K36 positions substituted with alanine were utilized as substrates for Ash1. K36A, but not the other mutations, completely abolished Ash1’s activity (Fig. 5B), indicating that Ash1 is a histone methyltransferase specific for H3K36. We also tested Ash1’s activity on nucleosomes containing different methyl-lysine analogues at position 36 (H3Kc36me0/1/2/3). Ash1 displayed robust activity on nucleosomes containing H3Kc36me0 and H3Kc36me1, but no activity on H3Kc36me2 or H3Kc36me3 (Fig. 5C), indicating that Ash1 is an H3K36-specific dimethylase. Our results are in agreement with one previous report (20) but disagree with three other reports (19, 21, 22).

Finally, Ash1 displayed comparable activity on wild-type nucleosomes and nucleosomes with pre-installed H3Kc27me3 (Fig. S4E), suggesting
that H3K27me3 does not impair Ash1’s enzymatic activity.

Discussion

PRC2-mediated H3K27 methylation can spread along chromatin in a self-propagating manner (14). To prevent unlimited H3K27 methylation spreading, certain chromatin components must exist to antagonize PRC2 function. In this report, we identify H3K36 methylation as one such chromatin component. We show that in HeLa cells, most H3 histones that are unmethylated at K36 are methylated at K27; in addition, H3 histones with K27me2 or K27me3 account for more than 70% of total H3 histones in G1/S arrested (Fig. 2) or asynchronous HeLa cells (43). These results suggest that besides targeted recruitment of PRC2 complex, the establishment of precise H3K27 methylation pattern may require chromatin components that antagonize PRC2-mediated H3K27 methylation, which play an important regulatory role in restraining the repressive environment.

The identification of H3K36 methylation as one of the chromatin components that antagonizes PRC2-mediated function provides new insights into the balance between activating and repressive chromatin modifications. The fact that Ash1 mediates H3K36 methylation, which in turn antagonizes PRC2-mediated H3K27 methylation, elucidates molecular mechanisms for Ash1-mediated Polycomb depression in Drosophila. Because Ash1’s enzymatic activity is highly controversial, we examined the experimental details of the four previous related reports (19-22) for potential explanations for the discrepancies. In the two studies that reported Ash1 as an H3K4-specific methyltransferase, GST-tagged H3 tail (22) or recombinant histone H3 (21) were used as substrates for determining site specificity. However, those substrates are not the native substrates of Ash1. In one previous study (20) and in this study, the native substrates of Ash1, nucleosomes, were used to determine site specificity, and both studies agree that Ash1 is a H3K36-specific methyltransferase (Fig. 4 and ref. 20). In the first report of Ash1’s enzymatic activity (19), nucleosomes were indeed used as the substrates. However, Edman degradation-based microsequencing was used to determine the methylation site, which unfortunately did not reach K36 because it is too far from the N terminus (19).

Finally, we would like to point out that the H2A ubiquitination E3 ligase dRing, PSC and the histone H3K36me2 demethylase dKDM2 form a Polycomb complex, which suggests another interesting interaction between H3K36 methylation and Polycomb silencing (44).

Acknowledgments

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References


Figure legends

Figure 1. Except for newly synthesized histone H3, H3 moieties unmodified at both K27 and K36 rarely exist in HeLa cells. (A) Experimental scheme. (B) Almost all HeLa cells advanced through S phase 9 h after cell cycle release, as indicated by the K8/K0 ratio of H3 backbone peptides. (C) Summary table for the K8/K0 ratio of H3 backbone peptides and H3:K27-R40 peptides with various modifications at the 9-h time point. Data for three independent repeat experiments were provided individually. Mean value and standard deviation of the three repeats were also calculated. (D) Mass spectra of an H3 backbone peptide and an H3K27me0/K36me0 peptide at the 9-h time point. (E) Mass spectra of an H3 backbone peptide and an H3K27me0/K36me0 peptide at the 3-h time point.

Figure 2. Immunoprecipitation experiments showing that H3K27me3 and H3K36me3 markers rarely co-exist in the same mono-nucleosomes.

Figure 3. Approximate abundance of each H3:K27-R40 peptide with a distinct modification in HeLa cells at the G1/S boundary. (A) XICs of all detected H3:K27-R40 peptides with various degrees of modifications and their relative abundance. (B) Summary table of Fig. 3A. Data for three independent repeat experiments were provided individually. Mean value and standard deviation of the three repeats were also calculated.

Figure 4. PRC2 activity is inhibited by pre-methylated H3K36. (A) Experimental scheme. (B) PRC2 activity is inhibited by pre-methylated H3K36. (C) Dot1L activity is not affected by pre-methylated H3K36.

Figure 5. Ash1 is a nucleosomal H3K36-specific dimethylase. (A) Ash1 prefers nucleosomal H3 as its substrates. (B) H3K36A, but not the other mutant nucleosomes, abolishes Ash1’s enzymatic activity. (C) Enzymatic activity of Ash1 on nucleosomes with H3Kc27me0/1/2/3.
Figure 1

### Figure 1

#### A

HeLa cells → Double Thymidine → Cells arrested at G1/S

Thymidine - Lys-8 (K8) → Cells went through 1st S phase

Thymidine + Lys-8 (K8) → Cells arrested before 2nd S phase

#### B

![Graph showing K8/K0 ratio over time](image)

- **K8/K0 ratio**
  - **0 h**
  - **3 h**
  - **6 h**
  - **9 h**
  - **12 h**
  - **15 h**
  - **18 h**
  - **21 h**
  - **24 h**
  - **27 h**

- **K8/K0 ratio**
  - **Exp1**
  - **Exp2**
  - **Exp3**
  - **Mean Value**

- **K8/K0 ratio (9 h after release)**
  - **K8/K0 ratio**
  - **Mean Value**

- **Peptide**
  - **K(pr)LPFQR, 2H**
  - **K8/K0=0.19**

- **H3 backbone peptides**
  - **K8/K0=0.85 ± 0.01**

- **H3 backbone peptides**
  - **K8/K0=23.7 ± 0.64**

- **K8/K0 ratio**
  - **Exp1**
  - **Exp2**
  - **Exp3**
  - **Mean Value**

#### C

- **H3 backbone peptides**
  - **K(pr)LPFQR**
  - **K8/K0=0.19**

- **H3 backbone peptides**
  - **K(pr)SAPATGGVK(pr)K(pr)PHR, 3H**
  - **K8/K0=23.7 ± 1.05**

- **Note:** H3K27me0/K36me1 and H3K27me1/K36me0 peptides, as well as H3K27me2/K36me3 and H3K27me3/K36me2 peptides co-elute on the reverse phase chromatography and share identical mass; thus we were unable to quantify their K8/K0 ratio individually.

- **H3K27me3/K36me3 peptides were not detected.**

#### D

- **9 h**

- **H3 backbone**
  - **K(pr)LPFQR, 2H**, **K8/K0=0.84**

- **H3K27me0/K36me0**
  - **K(pr)SAPATGGVK(pr)K(pr)PHR, 3H**, **K8/K0=23.7**

#### E

- **3 h**

- **H3 backbone**
  - **K(pr)LPFQR, 2H**, **K8/K0=0.19**

- **H3K27me0/K36me0**
  - **K(pr)SAPATGGVK(pr)K(pr)PHR, 3H**, **K8/K0=3.2**

- **Relative Abundance**
  - **m/z**
  - **K0**
  - **K8**

- **Relative Abundance**
  - **m/z**
  - **K0**
  - **K8**

- **Relative Abundance**
  - **m/z**
  - **K0**
  - **K8**

- **Relative Abundance**
  - **m/z**
  - **K0**
  - **K8**

- **Relative Abundance**
  - **m/z**
  - **K0**
  - **K8**

- **Relative Abundance**
  - **m/z**
  - **K0**
  - **K8**
Figure 2
**Figure 3**

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### A

![Graph showing time in minutes and relative abundance on the y-axis, with peaks at specific m/z values](image)

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### B

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* These peptides could not be distinguished from each other due to co-migration on the reverse phase chromatography and identical mass.

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Figure 4
Figure 5
**Supplementary Figure legends**

Figure S1. Coomassie blue staining of histone methyltransferases used in this study. (A) Recombinant human NSD2 Set domain; (B) Recombinant human Ash1 Set domain; (C) Reconstituted Drosophila PRC2 complex.

Figure S2. Mass spectra of all peptides listed in Fig. 1C.

Figure S3. MS/MS spectra of all H3 peptides carrying various methylation status at K27 and K36.

Figure S4. (A) H3Kc36me2 and H3Kc36me3 inhibits PRC2 activity; (B) H3Kc27me3 does not affect HYPB’s activity; (C) H3K36A mutation reduces PRC2 activity; (D) The Set domain of Ash1 is more closely related to H3K36 methyltransferases like HYPB or Mes4 than H3K4 specific methyltransferase such as Set1 or Trithorax; (D) H3Kc27me3 does not affect Ash1’s activity.
Figure S1
H3K27me3/K36me0: K(me3)SAPATGGVK(pr)K(pr)PHR, 3H⁺

H3K27me0/K36me1: K(pr)SAPATGGVK(pr-me1)K(pr)PHR, 3H⁺

H3K27me1/K36me1: K(pr-me1)SAPATGGVK(pr-me1)K(pr)PHR, 3H⁺

Figure. S3 Page 2
H3K27me2/K36me1: K(me2)SAPATGGVK(pr-me1)K(pr)PHR, 3H^+

H3K27me3/K36me1: K(me3)SAPATGGVK(pr-me1)K(pr)PHR, 3H^+

H3K27me0/K36me2: K(pr)SAPATGGVK(me2)K(pr)PHR, 3H^+

Figure. S3 Page 3
H3K27me1/K36me2: K(pr-me)SAPATGGVK(me2)K(pr)PHR, 3H^+ 

H3K27me2/K36me2: K(me2)SAPATGGVK(me2)K(pr)PHR, 3H^+ 

H3K27me3/K36me2: K(me3)SAPATGGVK(me2)K(pr)PHR, 3H^+ 

Figure. S3 Page 4
Figure. S4

Note: all proteins in Fig. S4D are Drosophila origin.