Role of Histone H3 Lysine 27 Methylation in Polycomb-Group Silencing

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Polycomb group (PcG) proteins play important roles in maintaining the silent state of HOX genes. Recent studies have implicated histone methylation in long-term gene silencing. However, a connection between PcG-mediated gene silencing and histone methylation has not been established. Here we report the purification and characterization of an EED-EZH2 complex, the human counterpart of the Drosophila ESC-E(Z) complex. We demonstrate that the complex specifically methylates nucleosomal histone H3 at lysine 27 (H3-K27). Using chromatin immunoprecipitation assays, we show that H3-K27 methylation colocalizes with, and is dependent on, E(Z) binding at an Ultrabithorax (Ubx) Polycomb response element (PRE), and that this methylation correlates with Ubx repression. Methylation on H3-K27 facilitates binding of Polycomb (PC), a component of the PRC1 complex, to histone H3 amino-terminal tail. Thus, these studies establish a link between histone methylation and PcG-mediated gene silencing.

Maintenance of the spatially restricted expression pattern of HOX genes in both flies and vertebrates is controlled by PcG and trithorax group (trxG) proteins (1). Biochemical and genetic studies indicate that PcG proteins exist in at least two separate protein complexes, the Polycomb repressive complex 1 (PRC1) and the ESC-E(Z) complex. These two complexes function in a cooperative manner to maintain long-term gene silencing (2, 3). Although components of both protein complexes are required to maintain the silenced state, the function of PRC1 appears to depend on the ESC-E(Z) complex (4). Recent studies on the covalent modifications of the histone NH2-terminal tails have given rise to the “histone code” hypothesis (5). One of the covalent modifications, histone lysine methylation, has emerged as an important player in regulating gene expression and chromatin function (6). Histone lysine methylation occurs on lysines 4, 9, 27, 36, and 79 of H3 and on lysine 20 of H4. Biochemical and genetic studies indicate that methylation of different lysine residues, with the exception of H3-K79 (7–9), is catalyzed by different SET domain–containing proteins (6).

References and Notes

6. Materials and methods and supporting text are available as supplementary material on Science Online.
7. I. Maritanov et al., unpublished data.
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Materials and Methods

Supporting Text

Figs. S1 to S6

References and Notes

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To understand the function of histone methylation, we attempted to identify histone methyltransferase (HMTase) using a systematic biochemical approach. During purification of the H4-K20–specific HMTase SET8 (10), we noticed that both the 0.3 M and 0.5 M P11 fractions derived from HeLa cell nuclear pellet contained high levels of HMTase activity toward nucleosomal histone H3 (Fig. 1A). To identify the enzyme(s) present in the 0.5M P11 fraction, we fractionated the proteins in a DEAESPW column, which separated the HMTase activities into two peaks (Fig. 1B). The present study focuses on the second peak. After fractionation on Phenyl Sepharose and Hydroxypatite columns (Fig. 1C) (11), the active fractions were further purified through a gel filtration Superose 6 column. Analysis of the fractions derived from this column indicates that the HMTase activity elutes between fraction 47 and 50 with an estimated mass of about 500 kD (Fig. 1D). Silver staining of an SDS-polyacrylamide gel containing these fractions revealed that six major polypeptides copurify with the enzymatic activity. Because the largest protein band neither cofractionates with the HMTase
activity in the Hydroxyapatite column (fig. S1), nor coimmunoprecipitates with the other components (fig. 1F), we conclude that it is not a part of the HMTase protein complex.

To identify the proteins that copurify with the HMTase activity, we excised the protein bands and analyzed them by a combination of peptide mass fingerprinting and mass spectrometric sequencing (10). In addition to RhAp48, a polyepitope present in many protein complexes involved in histone metabolism, several human Pcg proteins, including EZH2 (12), SUZ12 (13), and EED (14), were identified in the HMTase complex (Fig. 1E). A zinc finger transcriptional repressor named AEBP2 was also identified (15). Whether this protein is involved in targeting the complex remains to be determined. EZH2 contains a SET domain, a signature motif for all known histone lysine methyltransferases except the H3-K79 methyltransferase DOT1 (7), and their respective homologs in Caenorhabditis elegans, Drosophila man, and Drosophila (9). Whether this complex has no detectable HMTase activity, indicating that either a posttranslational modification or other components in the complex are required for the HMTase activity. This is consistent with previous results in which a partial EZH2 protein containing the SET domain was used (16).

Although mammalian EZH2 and EED, and their respective homologs in Drosophila and Caenorhabditis elegans, are known to interact directly (2, 3), the presence of SUZ12 in such a complex has not been previously reported. To verify that these proteins are components of the same protein complex, we generated antibodies against each of these proteins. Western blot analysis of the column fractions derived from the last two columns indicated that these proteins copurify with the HMTase activity (Fig. 1, C and D). To further confirm that the copurified proteins exist as a single protein complex, we immunoprecipitated the last column fractions (51 to 53) with an antibody to SUZ12. As shown in Fig. 1F, all five proteins coimmunoprecipitated. Because a protein complex containing Drosophila ESC and E(Z), respective homologs of EED and EZH2, has been previously named the ESC-E(Z) complex, we refer to the human counterpart as the EED-EZH2 complex. Although both EED-EZH2 and ESC-E(Z) complexes physically associate with HDACs (17, 18), our purified complex neither contains any HDAC polypeptide nor possesses detectable HDAC activity. It is possible that a different protein complex containing EED, EZH2, and HDAC may exist. Alternatively, HDACs may be recruited to target sites through direct interaction with EED, yet may not exist as a stable subunit of EED-EZH2 complexes. Further work is needed to differentiate these possibilities.

To characterize the substrate specificity of the EED-EZH2 complex, we subjected equivalent amounts of histone H3 that exist alone, in complex with other core histones, and in mononucleosomes or forms to methylation by equal amounts of the enzyme. As shown in Fig. 2A, the EED-EZH2 complex was capable of methylating all forms of histone H3, but showed a strong preference for H3 in oligonucleosome forms.

We next attempted to identify the residue methylated by the EED-EZH2 complex. Because oligonucleosomes are preferred substrates, they were subjected to methylation by the EED-EZH2 complex in the presence of S-adenosyl-L-[methyl-3H]methionine (3H-SAM). After purification, the labeled H3 was subjected to microsequencing followed by liquid scintillation counting. Neither K4 nor K9 released numbers of counts clearly greater than background. However, a small radioactive peak was detected in cycle 27 (Fig. S2). Given that the recovery efficiency decreases with each microsequencing cycle, the detection of a small peak on cycle 27 indicates that K27 is likely to be the site targeted by the EED-EZH2 complex. To confirm this possibility, we mutated each of the five potential methylation sites on H3 and compared the ability of the H3 mutants to be methylated by SVU39H1 was also analyzed. Mutation of K27 completely abolished the ability of H3 to serve as a substrate, whereas mutations of other sites had little effect (Fig. 2B, top panel). As expected, only mutation of K9 affected the SVU39H1-mediated H3 methylation (Fig. 2B, middle panel). These data, in combination with the in vivo results presented in Fig. 3, allow us to conclude that K27 is the predominant site, if not the only site, that is targeted for methylation by the EED-EZH2 complex.

To gain insight into the function of H3-K27 methylation in vivo, we generated a polyclonal antibody against a dimethyl-K27 H3 peptide. This antibody is highly specific for mK27 when evaluated by peptide competition and enzyme-linked immunosorbent assay (Fig. S3A). Western blot analysis with the H3-mK27–specific antibody demonstrated that K3-K27 methylation occurs in a variety of multicellular organisms, including human, chicken, and Drosophila. However, it does not appear to occur in the budding yeast Saccharomyces cerevisiae (Fig. S3B).

Given that both H3-K27 methylation as well as the EED-EZH2 complex exist in Drosophila, we examined whether the ESC-E(Z) complex is responsible for H3-K27 methylation in this organism. Previously, we have characterized several E(z) temperature-sensitive mutant alleles, one of which, E(z)694, contains a Cys-to-Tyr substitution (C603Y) in the cysteine-rich region immediately preceding the SET domain (19). When reared continuously at 18°C (permissive temperature), E(z)694 homozygotes exhibit no detectable mutant phenotype and maintain wild-type expression patterns of HOX genes, such as Ubx (19, 20). However, at 29°C (restrictive temperature), E(z)694 produces multiple homeotic phenotypes due to derepression of HOX genes (20), which correlates with loss of polytene chromosome binding by the E(Z)694 protein (19) and disruption of chromosome binding by Polycomb (PC) and other PRC1 components (4, 21). Given that chromosome binding by E(Z)694 protein is abolished at 29°C (19), K3-K27 methylation should be correspondingly reduced in the mutants at 29°C, if E(Z) is responsible for H3-K27 methylation. Western blot analysis (Fig. 2C)
showed loss of E(Z)61 and PC binding to this tene chromosome binding by both E(Z)61 and mutant E(z)61 (lanes 3 and 4) Drosophila embryos were probed with H3-mk9 (top panel)– or H3-mk9 (middle panel)–specific antibodies. (B) Map of the Ubx upstream regulatory region, including locations of the major PRE (22) and regions amplified by polymerase chain reaction (PCR) in ChIP assays. The scale above the map is according to a published numbering system (24) with the Ubx promoter at −32. The box indicates the position of PREz, and the horizontal lines below the map indicate the PCR-amplified regions. Restriction sites in this region are labeled as follows: E, Eco Ri; H, Hind III; P, Pst; K, Kpn I. (C) Colocalization of E(Z), H3-K27 methylation, and PC to the PRE in mock-transfected S2 cells (left panel) and concomitant reduction of E(Z), H3-K27 methylation, and PC binding to the PRE in esc-RNAi-treated cells (right panel). [D] ChIP assays demonstrate colocalization of E(Z) binding and H3-K27 methylation in E(z)61 wing imaginal discs at 18°C (left panel) and loss of binding in wing discs at 29°C (right panel). (C and D) Antibodies used in each assay are indicated on the left. Genomic DNA from each S2 cell culture or pooled collection of wing imaginal discs was amplified by PCR as a control for efficiencies of PCR primers. Numbers below the panels indicate the PCR primers used in each ChIP assay. Lanes 1 to 9 corresponding to the primer regions amplified are indicated in (B); lane 10 is a PCR product of Rpl140 promoter, which served as a negative control.

To examine the relation between E(Z) binding, H3-K27 methylation, and Ubx gene repression in vivo, we dissected wing imaginal discs from homozygous E(z)61 larvae that had been either reared continuously at 18°C or shifted from 18°C to 29°C −48 hours before dissection, and analyzed E(Z) binding and H3-K27 methylation in the same Ubx PRE region by ChIP. Consistent with previous studies demonstrating disruption of polytene chromosome binding by both E(Z)61 and PC proteins at 29°C (19), ChIP analysis showed loss of E(Z)61 and PC binding to this PRE at restrictive temperature (Fig. 3D, right panel). In addition, H3-K27 methylation colocalizes with E(Z) binding at permissive temperature, but is lost along with E(Z) binding at 29°C. In contrast, similar changes in H3-K9 methylation were not observed under the same conditions (Fig. 3D). Under normal conditions, Ubx is not expressed in wing discs due to PcG-mediated silencing (24). Similar inactivation of an E(z) temperature-sensitive allele during larval development has been shown to result in derepression of Ubx in wing discs (25). Thus, Ubx PRE-associated nucleosomes appear to be targeted by E(Z)-mediated H3-K27 methylation, which correlates with PC binding and repression of Ubx. Collectively, these data suggest that H3-K27
methylation plays an important role in the maintenance of Ubx gene silencing.

The chromodomain of the heterochromatin protein HP1 specifically binds to H3 tails that are methylated at K9 by the HMTase SUV39H1 (26, 27). Given that PC contains a chromodomain and that loss of E(Z) function abolishes H3-K27 methylation as well as PC binding to the Ubx PRE (Fig. 3, C and D), it is possible that methylation of H3-K27 by ESC-E(Z) facilitates PRE binding by PC, analogous to the effect of H3-K9 methylation on nucleosome binding by HP1 (26, 27). To test this possibility, we generated Drosophila PC using the rabbit reticulocyte transcription/translation-coupled system and incubated it with biotinylated H3 peptides with or without K27 methylation in the presence of streptavidin-conjugated Sepharose beads. After extensive washing (see supplementary text), the beads were boiled with SDS loading buffer and resolved in SDS-polyacrylamide gels. "In" represents 10% of the total input used for the pull-down assays. "B" and "Ft" represent bound and flow-through, respectively. Numbers below each lane are quantified signals relative to inputs. (B) Model depicting the relation between ESC-E(Z)–mediated H3-K27 methylation and PcG silencing. PRE is represented by a thick line.

Collectively, our studies support a model in which ESC-E(Z)–mediated H3-K27 methylation serves as a signal for the recruitment of the PRC1 complex by facilitating PC binding (Fig. 4B). Recruitment of PRC1 in turn prevents the access of nucleosome remodeling factors, such as SWI/SNF (2, 32), leading to the formation of a repressive chromatin state (Fig. 4B). Although this model is attractive, it does not exclude the possibility that protein–protein interaction also contributes to the recruitment of PRC1 to PREs. Indeed, a recent study indicates that PC transiently associates with the ESC-E(Z) complex during early embryogenesis (33). Our studies established a correlation between H3-K27 methylation and PcG silencing. Further work is needed to establish the exact role of H3-K27 methylation in PcG silencing.