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SUZ12 Is Required for Both the Histone Methyltransferase Activity and the Silencing Function of the EED-EZH2 Complex

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Summary

Recent studies have revealed the intrinsic histone methyltransferase (HMTase) activity of the EED-EZH2 complex and its role in Hox gene silencing, X inactivation, and cancer metastasis. In this study, we focus on the function of individual components. We found that the HMTase activity requires a minimum of three components—EZH2, EED, and SUZ12—while AEBP2 is required for optimal enzymatic activity. Using a stable SUZ12 knockdown cell line, we show SUZ12 knockdown results in cell growth defects, which correlate with genome-wide alteration on H3-K27 methylation as well as upregulation of a number of Hox genes. Chromatin immunoprecipitation (ChIP) assay identified a 500 bp region located 4 kb upstream of the HoxA9 transcription initiation site as a SUZ12 binding site, which responds to SUZ12 knockdown and might play an important role in regulating HoxA9 expression. Thus, our study establishes a critical role of SUZ12 in H3-lysine 27 methylation and Hox gene silencing.

Introduction

Polycomb group (PcG) and trithorax group (trxG) proteins have long been known to be part of the cellular memory system (Francis and Kingston, 2001; Simon and Tamkun, 2002). Both groups of proteins are involved in maintaining the spatial patterns of homeotic box (Hox) gene expression, which are established early in embryonic development by transiently expressed segmentation genes. In general, PcG proteins are transcriptional repressors that maintain the "off state," and trxG proteins are transcriptional activators that maintain the "on state." Recent demonstration that members of PcG and trxG proteins contain intrinsic histone methyltransferase (HMTase) activity raises the possibility that PcG and trxG proteins participate in cellular memory through methylation of core histones (Beisel et al., 2002; Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Milne et al., 2002; Muller et al., 2002; Nakamura et al., 2002).

Biochemical and genetic studies have provided compelling evidence that *Drosophila* PcG proteins function in at least two distinct protein complexes, the Polycomb repressive complex 1 (PRC1) and the ESC-E(Z) complex, although the compositions of the complexes may be dynamic (Otte and Kwaks, 2003). Recent studies in *Drosophila* (Czermin et al., 2002; Muller et al., 2002) and mammalian cells (Cao et al., 2002; Kuzmichev et al., 2002) have demonstrated that the ESC-E(Z)/EED-EZH2 complexes have intrinsic histone methyltransferase activity. Although the compositions of the complexes isolated by different groups are slightly different, they all contain EZH2, SUZ12, EED, and RbAp48 or their *Drosophila* homologs. In addition, the complexes have strong activities toward H3-lysine 27, although the physiological relevance of the minor in vitro activity of the complexes toward H3-lysine 9 reported by some groups remains to be shown (reviewed in Cao and Zhang, 2004).

The mechanism by which histone methylation participates in transcriptional regulation is best illustrated by the HMTase SUV39H1 and its functional partner HP1. Substantial evidence supports a model where SUV39H1 and its *S. pombe* homolog Clr4 methylate lysine 9 of histone H3, which creates a binding site for subsequent recruitment of HP1 through its chromo domain (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Rea et al., 2000). Similarly, H3-K27 methylation by the ESC-E(Z)/EED-EZH2 complexes has been proposed to help recruit the PRC1 complex through specific recognition of the methylated lysine 27 of H3 by the chromo domain of the Polycomb (Pc) protein (Cao and Zhang, 2004), a core component of the PRC1 complex (Francis et al., 2001). Four lines of evidence are consistent with this notion. First, in vitro studies demonstrated that the Pc chromo domain has higher affinity toward lysine 27 methylated histone peptide when compared with its nonmethylated counterpart (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002). Second, chromatin immunoprecipitation (ChIP) coupled with RNAi experiments have demonstrated that loss of ESC-E(Z) binding on the Polycomb-responsive element (PRE) of the Ubx gene correlates with loss of H3-K27 methylation and concomitant loss of Pc binding (Cao et al., 2002). Third, recent structural studies have revealed that the amino acids of H3 preceding lysine 27 contribute to the specific recognition of the methylated lysine 27 by Pc chromo domain (Fischle et al., 2003; Min et al., 2003). Finally, amino acid substitutions that abolish E(Z) HMTase activity also eliminate its ability to contribute to PcG silencing of the Ubx gene in wing imaginal discs (Muller et al., 2002). Collectively, these data support that the HMTase activity of E(Z) plays an important role in recruiting the PRC1 complex as well as in Hox gene silencing.

In addition to Hox gene silencing, Eed-Ezh2-mediated H3-K27 methylation was also demonstrated to participate in X inactivation (Plath et al., 2003; Silva et al., 2003). Recruitment of the Eed-Ezh2 complex to Xi and subsequent trimethylation on H3-K27 occurs during the initiation stage of X inactivation and is dependent on Xist RNA. Furthermore, recent studies suggest that Ezh2 and its associated H3-K27 methyltransferase activity may also be involved in germline development and stem cell pluripotency (Cao and Zhang, 2004). For example, Ezh2 and its associated H3-K27 methyltransferase activity was found to differentially mark the pluripotent epiblast cells and the differentiated trophectoderm (Erhardt et al., 2003). Consistent with a role of Ezh2 in maintaining the

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epigenetic modification patterns of pluripotent epiblast cells, Cre-mediated deletion of Ezh2 resulted in loss of H3-K27 methylation in these cells (Erhardt et al., 2003). Finally, studies in prostate and breast cancer cell lines and tissues have revealed a strong correlation between the levels of EZH2 and SUZ12 and the invasiveness of these cancers (Bracken et al., 2003; Kirmizis et al., 2003; Kleer et al., 2003; Varambally et al., 2002), indicating that dysfunction of the EED-EZH2 complex may contribute to cancer.

Given that the EED-EZH2 complex-mediated H3-K27 methylation participates in so many important processes, it is important to dissect the function of individual components and to understand how the enzymatic activity is regulated. In this study, through reconstitution of the HMTase complex and subcomplexes, we determined that the enzymatically active complex requires a minimum of three components: EZH2, EED, and SUZ12. In addition, AEBP2, a potential DNA binding protein, greatly stimulates the enzymatic activity. Using a stable SUZ12 knockdown cell line, we demonstrate that SUZ12 is important for H3-K27 methylation and Hox gene silencing. Finally, chromatin immunoprecipitation (ChIP) assay demonstrates that a region 4 kb upstream of the transcription start site of HoxA9 gene responds to SUZ12 knockdown and may contribute to HoxA9 silencing. Thus, our work defines the minimum requirement for EED-EZH2 enzymatic activity and establishes a critical role for SUZ12 in H3-K27 methylation and Hox gene silencing.

Results

Reconstituted EED-EZH2 Complex Has Similar Enzymatic Activity and Substrate Specificity as that of the Native Complex

Previously, we, as well as others, have purified a multisubunit EED-EZH2/ESC-E(Z) complex, from HeLa cell and *Drosophila* embryos, respectively, capable of methylating nucleosomal H3 at lysine 27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). The EED-EZH2 complex that we purified includes EZH2, SUZ12, EED, RbAp48, and AEBP2 (Cao et al., 2002). To dissect the function of individual subunits and to obtain large amounts of purified enzyme complex for detailed functional analyses, we attempted to reconstitute the enzymatic activity using recombinant proteins. To this end, Sf9 cells were coinfected with baculoviruses expressing Flag-EED, EZH2, SUZ12, RbAp48, and AEBP2. Flag-EED and associated proteins were purified by affinity chromatography followed by gel filtration through which free Flag-EED and partial complex were removed from the five component complex (Figure 1A). Histone methyltransferase assay and silver staining of an SDS-polyacrylamide gel containing the column fractions indicated that the five components copurify with the enzymatic activity as a protein complex of about 400 kDa (Figure 1B). To assess the HMTase activity of the reconstituted complex relative to that of the native EED-EZH2 complex, the fractions between 50 and 53 were pooled and different amounts of the pooled recombinant complex were compared with fixed amounts of the native EED-EZH2 complex. Results shown in Figure 1C demonstrate that the reconstituted complex and the native complex had comparable HMTase activity when equal amounts of enzyme were compared (lane 1 with lanes 3 and 4). To characterize the reconstituted complex further, different forms of histone H3 were subjected to methylation. Similar to our previous results using the native complex (Cao et al., 2002), the preferred substrate for the reconstituted enzyme complex was histone H3 in oligonucleosome form (Figure 1D). Based on the above results, we conclude that the reconstituted EED-EZH2 complex and the native complex have similar enzymatic activity and substrate specificity.

Physical Relationship among Components of the EED-EZH2 Complex

Of the five components present in the complex, EZH2 is believed to be the catalytic subunit because it is the only SET domain-containing protein. While it is known that EZH2 and EED interact directly (Sewalt et al., 1998; van Lohuizen et al., 1998), it is not clear how the other subunits associate with these two proteins to form the complex. In order to reconstitute subcomplexes so that the effect of a loss of a particular component on HMTase activity can be evaluated, it is important to define the precise spatial relationship among components of the complex. Toward this end, individual components, with the exception of EZH2 that we failed to express in *E. coli*, were expressed as GST fusion proteins and were used in GST pull-down assays. Consistent with previous reports (Sewalt et al., 1998; van Lohuizen et al., 1998), EZH2 was found to interact strongly with EED, but it did not seem to directly contact any of the other subunits under our assay conditions (Figure 2A, panel 2). In addition to EZH2, EED can also interact with SUZ12 and AEBP2 (Figure 2A, lane 3). RbAp48 appears to strongly interact with SUZ12 (last panel, lane 2) and weakly with AEBP2 and EED (panels 4 and 5, lane 4). It is interesting to note that AEBP2 is capable of self association (panel 5, lane 5), and this ability appears to be important for its interaction with RbAp48 since immobilized GST-RbAp48 can pull-down AEBP2 (panel 4, lane 4), but immobilized GST-AEBP2, which can not self associate, can not pull-down RbAp48 (last panel, lane 5). All the interactions were specific, as parallel pull-down assays using GST alone failed to detect the interactions (Figure 2A, lane 6). These interaction studies indicate that EZH2 associates with other components through EED, which in turn interacts with SUZ12 and AEBP2, both of which can interact with RbAp48 (Figure 2B).

AEBP2 is a zinc finger protein originally identified as a transcriptional repressor (He et al., 1999). Although it was identified as an integral component in our purified EED-EZH2 complex (Cao et al., 2002), it was not present in a similar complex purified by another group (Kuzmichev et al., 2002). Given its ability of incorporating into the complex (Figure 1B) and to interact with multiple subunits of the complex (Figure 2A, panel 5), we attempted to characterize its interaction with SUZ12 and RbAp48 further. GST pull-down assays using in vitro transcribed/translated full-length and deletion mutants of AEBP2 demonstrated that the N-terminal region of AEBP2 (1–87) is involved in RbAp48 interaction (Figure 2C, compare the middle two panels), while a C-terminal region (144–198) is involved in SUZ12 interaction (Figure 2C, compare the first two panels). These studies, in

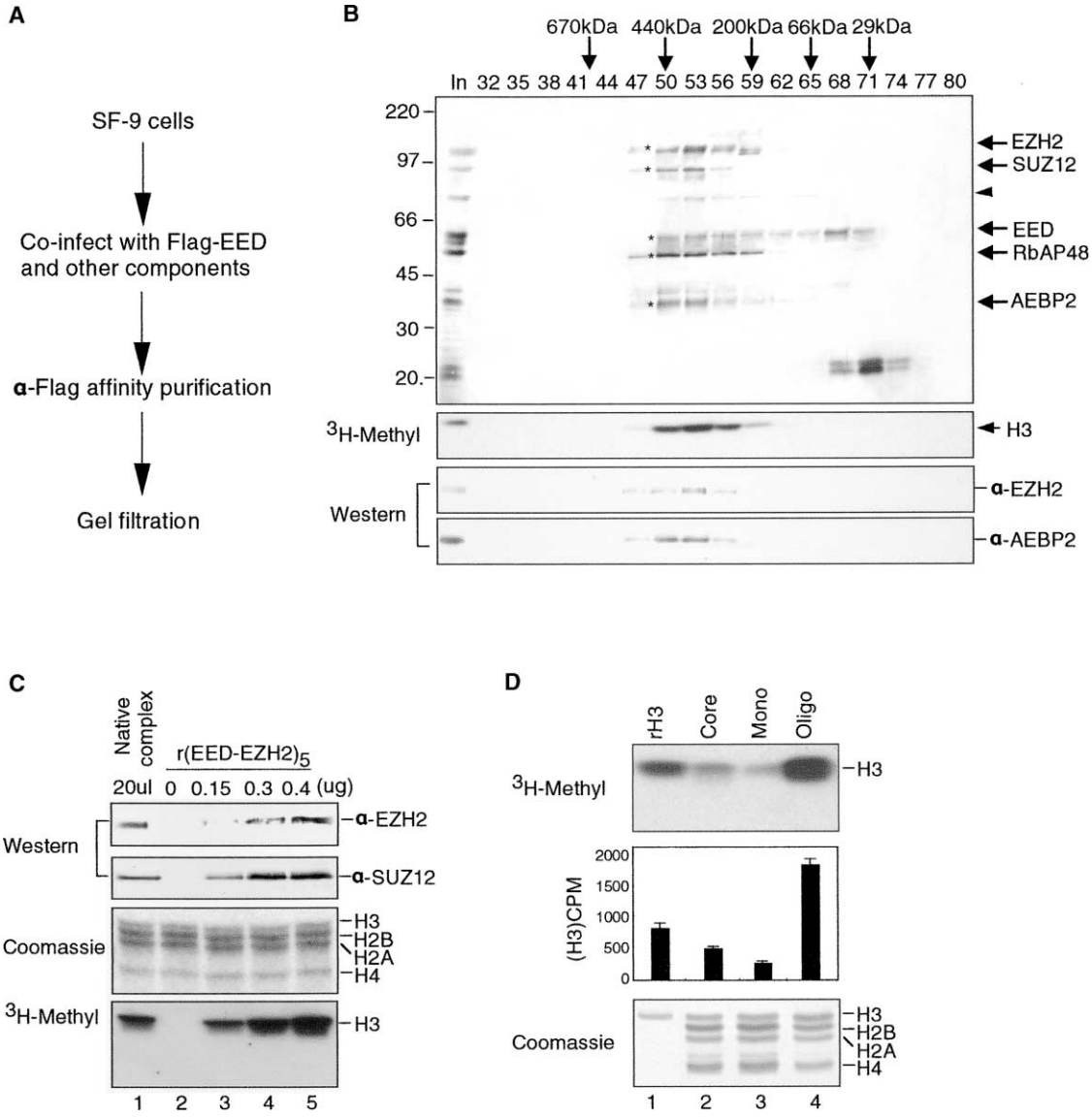

Figure 1. Reconstituted EED-EZH2 Complex Has Similar Enzymatic Activity and Substrate Specificity as that of the Native Complex

(A) Schematic representation of the steps involved in EED-EZH2 reconstitution.

(B) Silver staining of a polyacrylamide-SDS gel (top panel) and HMTase activity assay (second panel) of the fractions derived from the Superose 6 gel-filtration column. The five components of the recombinant EED-EZH2 complex are indicated by "*." The arrowhead indicates an EZH2 degradation product confirmed by Western blotting (third panel). The elution profile of the protein markers is indicated at the top. The positions of the protein size markers are indicated on the left. For reasons unknown, the migration of recombinant AEBP2 (35 kDa), confirmed by Western blotting (fourth panel), is different from that when it is in the native complex (65 kDa).

(C) Comparison of the HMTase activity (bottom panel) of native complex (lane 1) with varying amounts of reconstituted complex (lanes 3–5) (top two panels) when equal amounts of nucleosomal histone are used (third panel). The amount of complexes used is quantified by Western blotting of EZH2 and SUZ12 as indicated.

(D) Characterization of the substrate specificity of the recombinant EED-EZH2 complex. Equal amounts of the recombinant enzyme complex were used to methylate equal amounts of histone H3 alone or in octamer, mono-, and oligonucleosome forms (bottom panel). The top panel is an autoradiograph of the bottom panel. Quantification of the top panel is presented in the middle panel with error bars from two independent experiments.

combination with the functional studies described below, support AEBP2 being an integral component of the EED-EZH2 complex.

AEBP2 Stimulates and SUZ12 Is Required for HMTase Activity

After defining the spatial relationship among the five components, we attempted to define the minimum components required for the enzymatic activity. Given that Ezh2 and Eed are both required for H3-K27 methylation in vivo (Erhardt et al., 2003; Silva et al., 2003; Su et al., 2003) and based on the physical interactions defined in Figure 2, we attempted to reconstitute subcomplexes by omitting AEBP2, RbAp48, and SUZ12 individually or in combination. Using a similar two-step purification procedure outlined in Figure 1A, we reconstituted the EED-

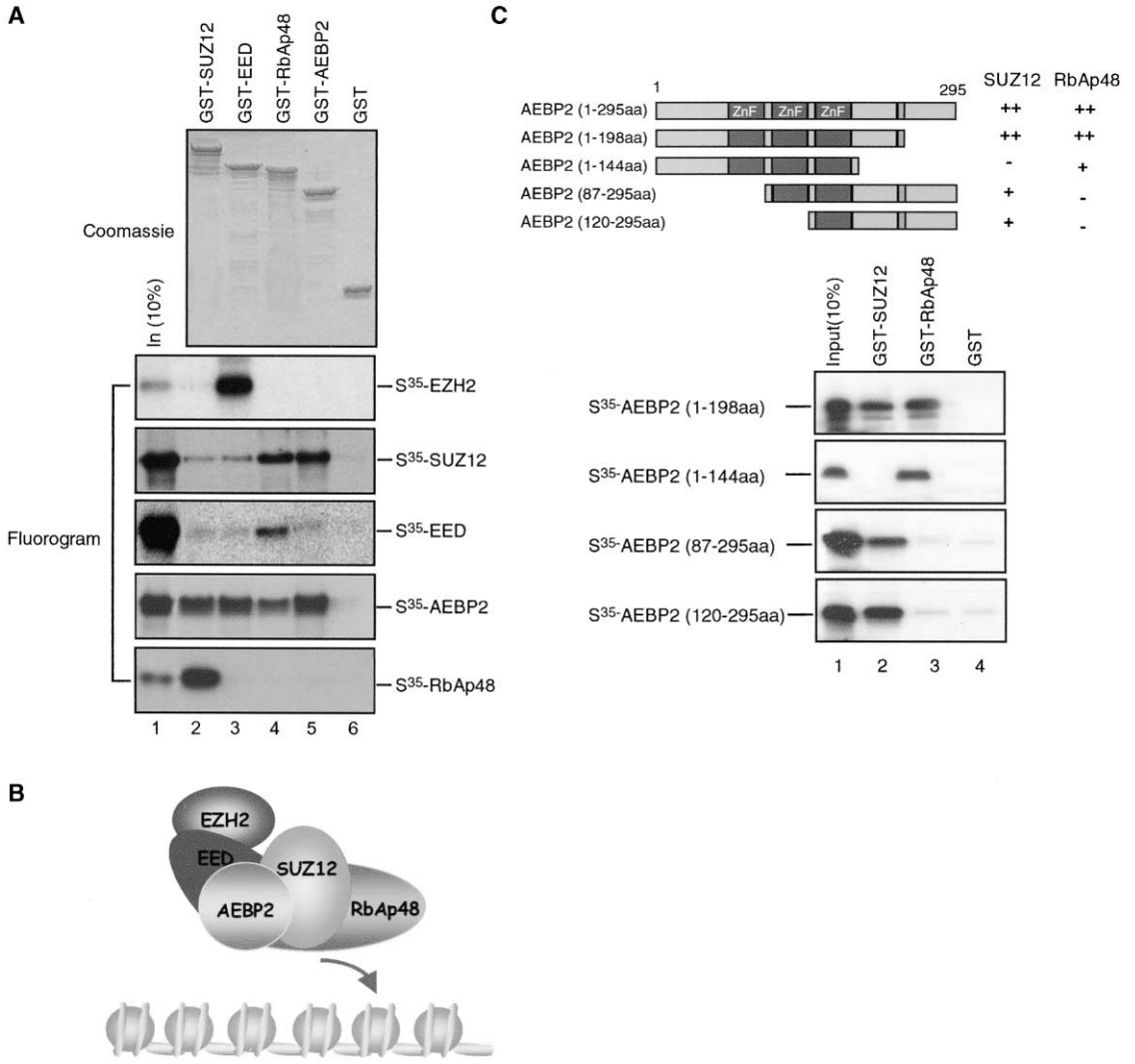

Figure 2. Protein-Protein Interactions among Components of the EED-EZH2 Complex

(A) GST pull-down assays using equal amounts of GST-fusion proteins (top panel) and in vitro-translated S^{35}-labeled proteins indicated on right (bottom panels). "In" represents 10% of the total input.

(B) Schematic representation of the interactions detected in (A).

(C) Mapping of the regions on AEBP2 involved in SUZ12 and RbAp48 interaction. Different deletion constructs (top panel) were in vitro transcribed/translated before being used in GST pull-down assays (bottom panels). The three zinc fingers on AEBP2 are indicated. The strength of the interaction is summarized on the right of the top panel. "++," "+," and "−" represent strong, weak, and no interaction, respectively.

EZH2 subcomplexes with four (omit AEBP2), three (omit AEBP2 and RbAp48), and two (omit AEBP2, RbAp48, and SUZ12) components, respectively. Silver staining revealed that these reconstituted subcomplexes are near homogeneity (Figure 3A). To evaluate the relative HMTase activities of these purified complexes, equal molar amounts of the complexes, as indicated by Western blot analysis of EZH2 (Figure 3B, top panel), were used to methylate equal amounts of nucleosomal substrates (Figure 3B, second panel). Results shown in Figure 3B (bottom two panels) indicate that a minimum of three components containing EED, EZH2, and SUZ12 are required for the HMTase activity (compare lanes 3 and 4). Addition of RbAp48 to the three-component complex increased the incorporation of SUZ12 (Figure 3A, compare lanes 2 and 3) resulting in an increased enzymatic activity, particularly at a higher enzyme concentration (Figure 3C). Importantly, addition of AEBP2 to the four-component complex significantly increased the HMTase activity (Figure 3B, compare lanes 1 and 2). We note that coinfection of EED, EZH2, and RbAp48 failed to form a stable complex (data not shown), supporting that incorporation of RbAp48 into the complex depends on its interaction with SUZ12 (Figure 2A). In addition, omission of SUZ12 also failed to form a four-component complex (data not shown).

To further substantiate the observations described above, we assayed the HMTase activity of the reconstituted complexes using a wide range of enzyme concentrations. Results presented in Figure 3C not only confirmed the data shown in Figure 3B but also extended the conclusion to include a wide range of enzyme con-

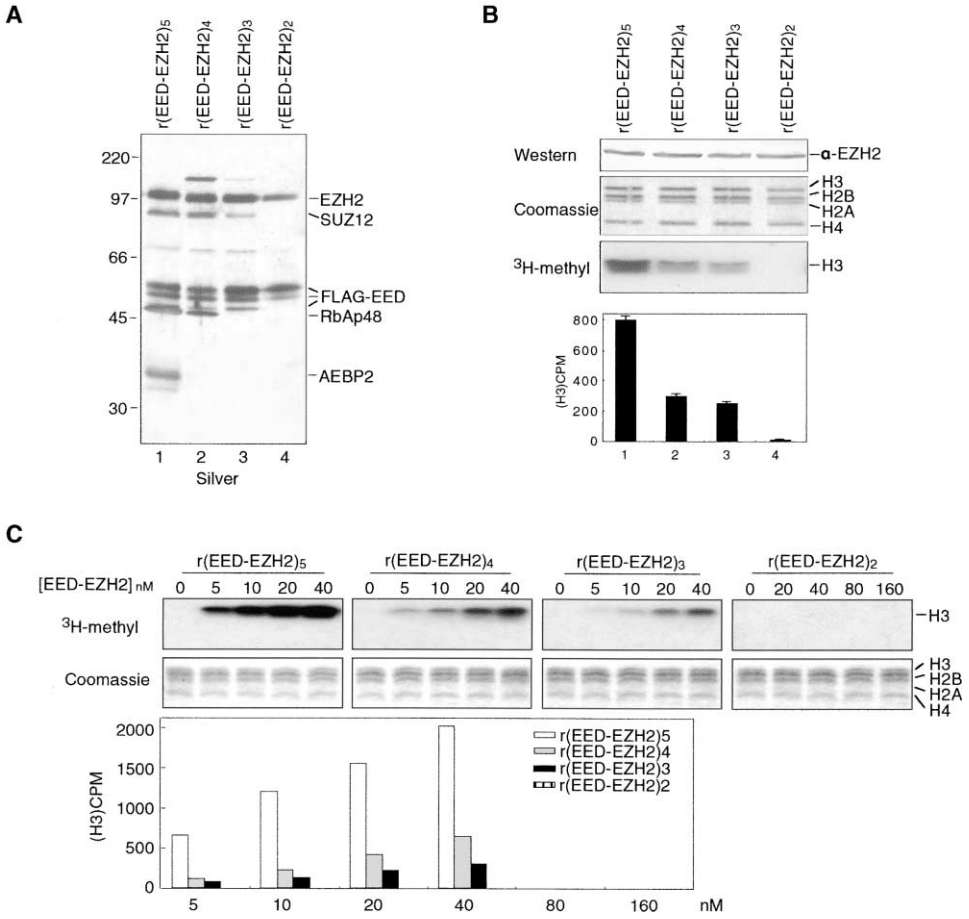

Figure 3. Characterization of the EED-EZH2 Complex and Subcomplexes

(A) Silver staining of a polyacrylamide-SDS gel containing the purified recombinant complex and subcomplexes. $(EED\text{-}EZH2)_5$ contains all five components; $(EED\text{-}EZH2)_4$ does not contain AEBP2; $(EED\text{-}EZH2)_3$ does not contain AEBP2 and RbAp48; $(EED\text{-}EZH2)_2$ only has EED and EZH2. The different forms of Flag-EED have been verified by Western blotting.

(B) Comparison of the enzymatic activity of the different recombinant complexes with 5, 4, 3, and 2 components. The complexes used were normalized so that each reaction contains equal amounts of EZH2 and chicken oligonucleosomes (top two panels). Reactions were performed with an enzyme/substrate ratio of 1:20. The enzymatic activity (third panel) and quantification (bottom panel) with error bars from two independent experiments are shown.

(C) Comparison of the enzymatic activity of the different recombinant complexes with a wide range of enzyme concentrations indicated at the top of the panels. The oligonucleosome substrate used in each reaction is about 200 nM. Quantification of the data on top panels is presented in the bottom panel.

centrations. It is apparent that although significant HMTase activity was detected at 5 nM enzyme concentration when the five-component complex was used, no activity was detected when the two-component complex was used even at a 160 nM enzyme concentration level. Again, a significant difference in enzymatic activity was observed when the five-component and four-component complexes were compared. Thus, we conclude that EZH2, EED, and SUZ12 are the minimum components required for HMTase activity and that AEBP2 significantly stimulates the HMTase activity of the four-component EED-EZH2 complex.

The Reconstituted EED-EZH2 Complexes Methylate Nucleosomal Histone H3 at Lysine 27

Several groups have independently purified and characterized the EED-EZH2/ESC-E(Z) HMTase complexes (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). However, the properties of the enzyme complexes appear to have some differences. For example, while the EED-EZH2 complex purified by our group has a clear preference for oligonucleosome over octomer substrates (Cao et al., 2002), another group observed the opposite preference using a similar HeLa complex devoid of AEBP2 (Kuzmichev et al., 2002). To determine whether the presence of AEBP2 in the complex can alter the substrate preference, we analyzed substrate preference using the reconstituted EED-EZH2 complex devoid of AEBP2. Results shown in Figure 4A indicate, like that of the AEBP2-containing complex (Figure 1D), the four-component EED-EZH2 complex also shows clear preference for oligonucleosome over octomer. Similar substrate preference was also observed with the three-component EED-EZH2 complex (data not shown). Therefore, we conclude that neither AEBP2 nor RbAp48 are involved in substrate-specificity determina-

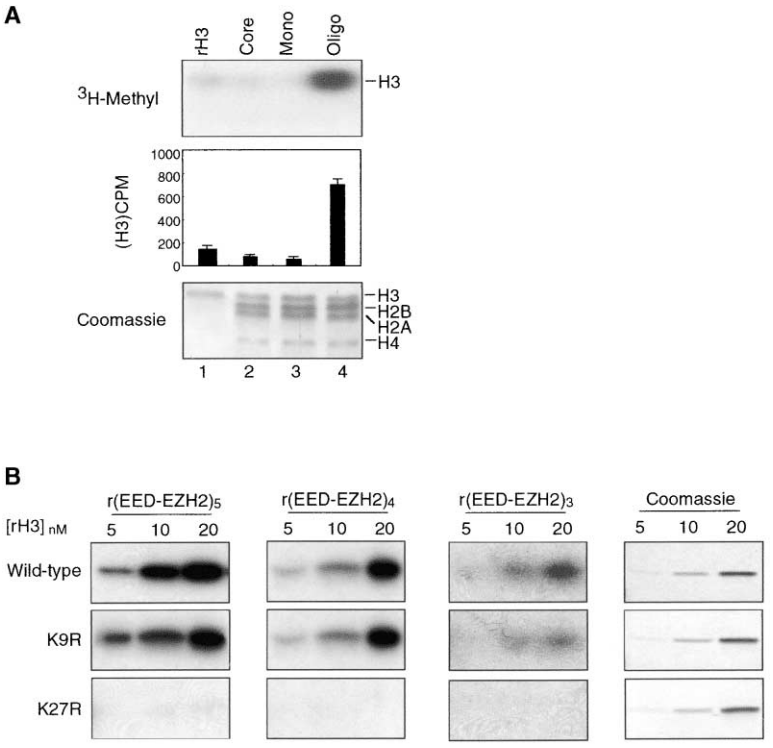

Figure 4. Substrate Preference and Site Specificity of the Reconstituted EED-EZH2 Enzyme Complexes

(A) The EED-EZH2 complex with or without AEBP2 has the same substrate preference. Equal amounts of the four-component recombinant enzyme complex were used to methylate equal amounts of histone H3 alone or in octamer, mono-, and oligonucleosome forms (bottom panel). The top panel is an autoradiograph of the bottom panel. Quantification of the top panel is presented in the middle panel with error bars from two independent experiments.

(B) H3-K27 is the target site for the reconstituted enzyme complexes with five, four, and three components. Three levels (indicate on top of the panels) of wild-type or mutant H3 were methylated with different reconstituted complexes with an enzyme/substrate ratio of 1:20. The activities were shown by autoradiography (first three columns).

tion. Instead, EZH2 or its associated EED and SUZ12 are likely involved in this process.

In addition to substrate preference, there is also some discrepancy about the lysine residues that the enzyme complexes methylate. Although the human EED-EZH2 complex and its *Drosophila* ESC-E(Z) counterpart were reported to methylate H3-K27 only (Cao et al., 2002; Muller et al., 2002), H3-K9 HMTase activity was also reported for similar complexes by two other groups (Czermin et al., 2002; Kuzmichev et al., 2002). This discrepancy is likely the result of either subtle differences in the complex composition, for example the presence or absence of AEBP2, or the minor H3-K9 HMTase activity was simply due to contamination. To differentiate these two possibilities, we determined the lysine residues methylated by the different reconstituted complexes. To this end, three levels of recombinant wild-type or mutant H3 were subjected to a histone methyltransferase assay using the enzyme complexes containing five, four, or three components. Like that of the native complex (Cao et al., 2002), mutation on K27 resulted in undetectable HMTase activity for the five-component complex (Figure 4B, left column). Similar results were obtained when the four- or three-component complex was used (Figure 4B, middle two panels). Therefore, AEBP2 does not limit the ability of the four-component EED-EZH2 complex to methylate H3-K9, if it has such a capability. However, we note that mutation on H3-K9 does appear to cause a small reduction in the ability of H3 to serve as a substrate for the reconstituted EED-EZH2 complexes. How the mutation causes the reduction in HMTase activity remains to be determined. In conclusion, our current and previous results (Cao et al., 2002) do not support that H3-K9 is a target site for the EED-EZH2 complex.

SUZ12 Contributes to H3-Lysine 27 Trimethylation In Vivo

Having established a role for SUZ12 in H3-K27 methylation in vitro, we attempted to demonstrate the same function in vivo. Toward this end, we generated a stable SUZ12 knockdown cell line using a vector-based siRNA approach as previously described (Wang et al., 2003). Characterization of the cell line indicates that about 75% knockdown at the protein level was achieved (Figure 5A, compare lanes 3 and 5). Quantitative RT-PCR revealed that about 65% knockdown is achieved at the mRNA level (Figure 5A, right panel). Like that of siRNA transfection for the EZH2 and EED components of the EED-EZH2 complex (Bracken et al., 2003; Varambally et al., 2002), vector-based SUZ12 knockdown resulted in changes in cellular morphology and slower growth (Figure 5B). To evaluate the effects of SUZ12 knockdown on H3-K27 methylation in vivo, equivalent amounts of histones, isolated from the SUZ12 knockdown cells and cells from a parallel empty vector transfection, were subjected to Western blotting analysis using antibodies specific for methylated lysine 9 or lysine 27 of histone H3. Results shown in Figure 5C indicated that SUZ12 knockdown resulted in a significant decrease on the trimethyl-K27 level but had little affect on the trimethyl-K9 level (Figure 5C, third and fourth panels). Interestingly, an increase in monomethyl-K27 and a moderate decrease in dimethyl-K27 were also observed (Figure 5C, the top two panels). The fact that SUZ12 knockdown does not affect EZH2 level (Figure 5A) in combination with the requirement of SUZ12 for H3-K27 methyltransferase activity in vitro (Figure 3B) allows us to conclude that SUZ12 directly contributes to H3-K27 methylation.

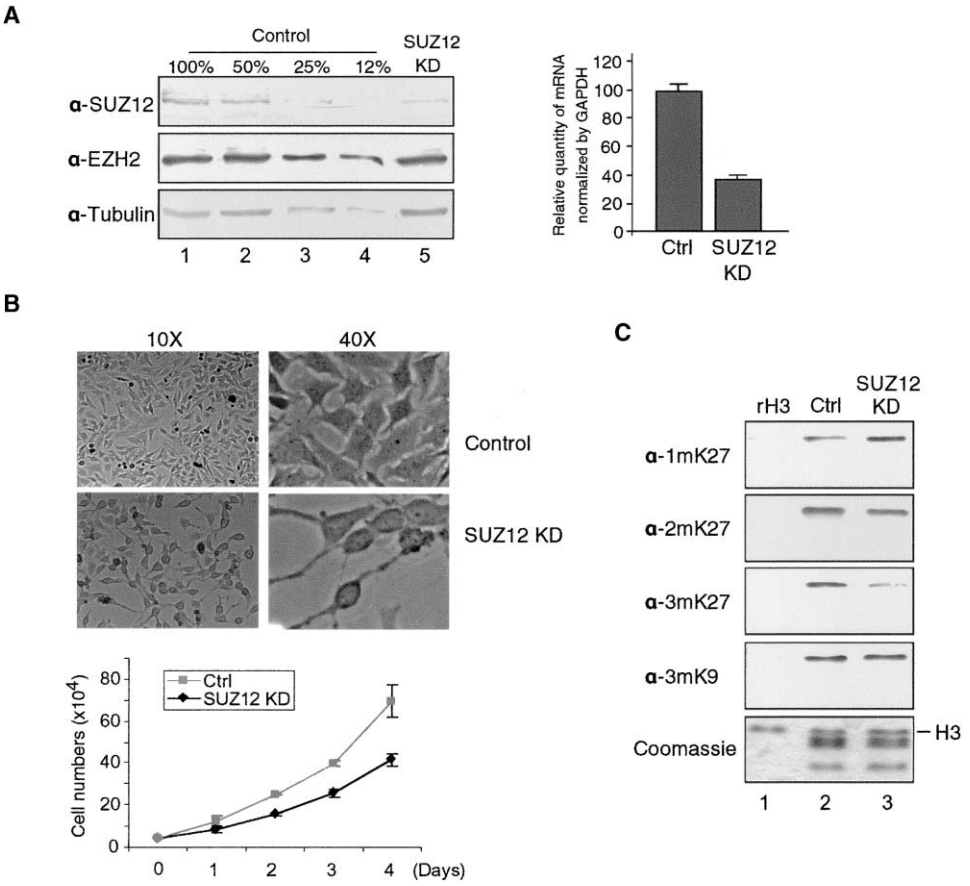

Figure 5. SUZ12 Is Important for Cell Growth and H3-K27 Methylation In Vivo

(A) Western blot (left panel) and quantitative RT-PCR (right panel) analysis of a SUZ12 stable knockdown cell line and a parallel mock knockdown cell line. Tubulin serves as a loading control for Western blotting. GAPDH was used as control for normalization in the quantitative RT-PCR.

(B) SUZ12 knockdown results in morphological change and cell growth inhibition. Top panels show morphological changes of control and knockdown HeLa cells after 2 weeks of selection. Bottom panel shows the growth curve of control and knockdown HeLa cells. Viable cells were counted by trypan blue staining at different times after initial seeding of 4×10^4 cells.

(C) Western blot analysis of histones extracted from control and knockdown HeLa cells with antibodies specific for mono-, di-, or trimethylated K27 and trimethylated K9. Equal loading of histone H3 was verified by Coomassie staining of a parallel gel (bottom panel).

SUZ12 Is Required for the Silencing Function of the EED-EZH2 Complex

Studies in *Drosophila* have established a critical role for H3-K27 methylation in Hox gene silencing (Cao et al., 2002; Muller et al., 2002). Data presented above indicates that SUZ12 is important for H3-K27 methylation in vitro and in vivo. If H3-K27 methylation is critical for Hox gene silencing in mammalian cells, like that in *Drosophila*, we expect derepression of EED-EZH2 target genes in the SUZ12 knockdown cells. To evaluate this possibility, the expression level of several Hox genes, including HoxC6, HoxC8, and HoxA9 in the knockdown cells and the parallel control cells, were examined by real-time RT-PCR. Results shown in Figure 6A demonstrate that SUZ12 knockdown resulted in derepression of HoxC8 and HoxA9 while it has little effect on HoxC6. Since more dramatic effect on HoxA9 is observed, our further studies focus on HoxA9.

To understand the relationship between SUZ12 knockdown, H3-K27 methylation, and HoxA9 derepression, we examined SUZ12 binding and H3-K27 methylation at different locations of the human HoxA9 gene by chromatin immunoprecipitation (ChIP) using antibodies against SUZ12 and trimethyl-K27. As controls for ChIP assays and for antibody specificity, equal amounts of IgG and anti-H3-dimethyl-K4 antibodies were also included. Representative regions that cover the promoter (B), intron (C), and downstream (D) of the gene were analyzed. In addition, a region (A) about 4 kb upstream of the transcription initiation site that shows high degree of sequence homology between human and mouse genes was also analyzed. Results shown in Figure 6B indicate that SUZ12 and 3mK27 are present preferentially in regions A (lanes 4 and 5) and B (lanes 9 and 10) when compared with that present in regions C (lanes 14 and 15) and D (lanes 19 and 20) in the mock knockdown cells (top panels). Consistent with a role for SUZ12 on H3-K27 methylation, knockdown SUZ12 resulted in significant decrease of SUZ12 binding concomitant with loss of H3-3mK27 in regions A and B, particularly in region A

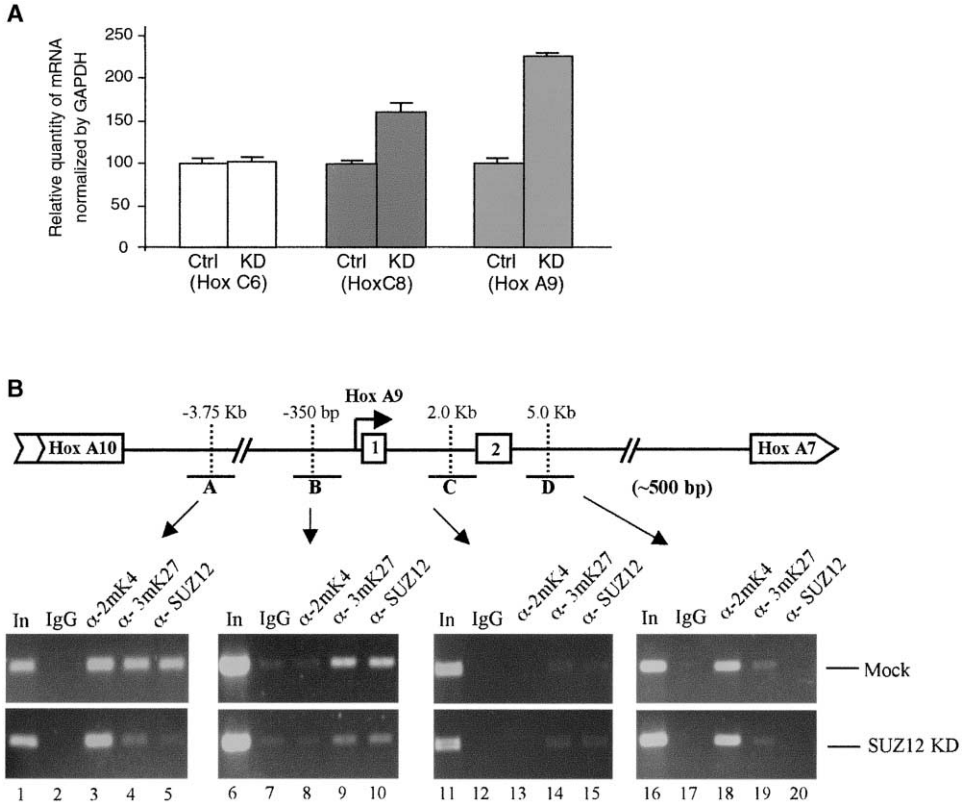

Figure 6. SUZ12 Knockdown Resulted in Derepression of Hox Gene Expression and Decreased Level of H3-K27 Methylation on the HoxA9 Gene

(A) Quantitative RT-PCR analysis of HoxC6, HoxC8, and HoxA9 expression in SUZ12 knockdown and mock knockdown cells. GAPDH was used as a control for normalization. Quantification is an average of two independent experiments with error bars.

(B) ChIP analysis of selected regions covering the HoxA9 gene. Top panel is a diagram of the HoxA9 gene where the two exons are indicated by the two boxes. The locations of the analyzed regions (A–D) relative to the transcription start site are indicated. Each region covers about 500 bps. Three antibodies (anti-2mK4, −3mK27, and −SUZ12) and an IgG control were used in the ChIP assays using SUZ12 or mock knockdown cells. ChIP results were revealed by EtBr staining of agarose gels containing PCR amplified ChIP DNA.

(Figure 6B, lanes 4 and 5, compare top and bottom panels). Interestingly, three YY1 binding sites can be identified in region A. This raises the possibility that this region may have functions similar to that of the *Drosophila* Polycomb responsive element (PRE). Therefore, loss of SUZ12 binding directly correlates with loss of H3-K27 methylation and derepression of HoxA9. Unexpectedly, very little change in H3-K4 methylation, believed to be a marker for gene activation, is noticed. Whether this is due to the limitation of partial SUZ12 knockdown remains to be determined. Collectively, our data support that SUZ12 is critical for the enzymatic activity and silencing function of the EED-EZH2 complex.

Discussion

SUZ12 Is Critical for H3-K27 Methylation and for Hox Gene Silencing

By reconstitution of the EED-EZH2 complex and subcomplexes, we demonstrate that the enzymatic activity of the EED-EZH2 complex requires a minimum of three components, including EZH2, EED, and SUZ12 (Figure 3). In addition, we demonstrate that AEBP2 is an integral component of the complex and can greatly stimulate the enzymatic activity of the four-component EED-EZH2 core complex (Figures 2 and 3). Furthermore, analysis of the substrate preference and lysine specificity of the various active complex and subcomplexes indicates that the nucleosome preference and H3-K27 specificity of the enzyme is intrinsic to the minimum three-component complex because addition of RbAp48 or AEBP2 to the minimum complex neither alters the substrate preference nor changes the lysine specificity (Figure 4). Therefore, RbAp48 and AEBP2 are likely involved in modulating the enzymatic activity or complex targeting, for example by mediating the interaction between transcription factors and one of the three core subunits. Previous studies on AEBP2 suggest that it is a transcription factor (He et al., 1999). Whether the ability of this protein to stimulate the enzymatic activity of the four-component EED-EZH2 complex is linked to its ability of recognizing a specific DNA sequence remains to be determined. Given that the nucleosome substrates used in our assay are purified from HeLa nuclei and that the DNA sequences recognized by AEBP2 are not particularly abundant in the human genome, the stimulation observed may be related to its capability of binding to nucleosomes (data not shown).

In addition to EZH2 and EED, SUZ12 is the third subunit required for the enzymatic activity. Although we

cannot rule out the possibility that SUZ12 may assist the folding of EZH2 into an active conformation, our in vitro protein-protein and protein-nucleosome interaction studies suggest that SUZ12 mediates the interaction between EZH2 and nucleosomes (data not shown). This observation is consistent with the fact that SUZ12 and its family members contain a classical C2H2 zinc finger similar to the fingers found in sequence-specific DNA binding proteins. Whether the zinc finger is involved in nucleosome recognition remains to be determined.

Elucidation of the function of individual components of the EED-EZH2 complex in vivo has been proved to be extremely difficult as genetic manipulation of either Ezh2 or Eed in mice has resulted in early embryonic lethality, which prevents derivation of Ezh2 or Eed null cells (O'Carroll et al., 2001; Shumacher et al., 1996). Depletion of EZH2, EED, and SUZ12 by siRNA-mediated knockdown results in cell proliferation defects (Bracken et al., 2003; Varambally et al., 2002), which also prevents examination into the epigenetic effects of loss of these gene functions. After tremendous effort of selection of a large number of individual knockdown cell clones, we managed to isolate a relatively stable SUZ12 knockdown clone with 60%–70% knockdown efficiency, which can be maintained from generation to generation. Even this cell line appears to have the tendency of decreasing its SUZ12 knockdown efficiency during culturing. We note that any cell lines with higher knockdown efficiency appear to be differentiated and cease to proliferate within a few passages. These observations support that the EED-EZH2 complex plays an important role in regulating cell proliferation and differentiation.

Nevertheless, the partial SUZ12 knockdown cells that we obtained allowed us to address the function of SUZ12 in H3-K27 methylation and Hox gene silencing in mammalian cells. We demonstrate that SUZ12 knockdown resulted in a genome-wide decrease of H3-3mK27 but has no apparent effect on H3-3mK9 (Figure 5C). Importantly, H3-3mK27 decrease has direct consequence in transcription. Real-time RT-PCR and ChIP analysis demonstrate that one of the SUZ12 target genes, HoxA9, is derepressed following SUZ12 knockdown and loss of H3-K27 methylation. This indicates SUZ12 plays an important role in Hox gene silencing. Similar to our conclusion, genetic studies in *Drosophila* have demonstrated that maintenance of the repressed state of Hox genes requires functional Su(z)12 (Birve et al., 2001). The fact that SUZ12 is required for the H3-K27 methyltransferase activity in combination with a previous demonstration that H3-K27 methylation is critical for Hox gene silencing (Muller et al., 2002) allows us to connect its role in Hox gene silencing directly with its role in H3-K27 methylation. In addition to Hox gene silencing, genetic studies in *Drosophila* also suggest Su(z)12 plays an important role in heterochromatin silencing, as Su(z)12 mutations strongly suppress position-effect variegation (PEV) (Muller et al., 2002). The involvement of Su(z)12 in heterochromatin silencing is likely to be direct because the heterochromatin protein HP1α was recently reported to interact with SUZ12 (Yamamoto et al., 2004). Whether the involvement of SUZ12 in heterochromatin silencing is mediated by H3-K27 methylation is not known.

SUZ12 in Cell Proliferation and Cancer

In addition to participating in Hox gene and heterochromatin silencing, SUZ12 may also be involved in cancer. The human SUZ12 gene was reported to be frequently rearranged in endometrial stromal tumors (Koontz et al., 2001). Consistent with our observation that SUZ12 levels affect cell proliferation, SUZ12 was also found to be upregulated in a number of human tumors, including colon, breast, and liver tumors (Kirmizis et al., 2003). Interestingly, EZH2, another component of the EED-EZH2 complex, and its associated H3-K27 methyltransferase activity has also been linked to cancer, since EZH2 levels directly correlate with the invasiveness of both prostate cancer and breast cancer (Bracken et al., 2003; Kleer et al., 2003; Varambally et al., 2002). If the H3-K27 methyltransferase activity proves to be critical in cancer metastasis, identification of a small molecule inhibitor for the EED-EZH2 HMTase activity may provide us a valuable tool for the development of a therapeutic intervention for cancer. The successful reconstitution of EED-EZH2 enzymatic activity has opened the door for the production of large quantities of active enzyme, a requirement for high-throughput screening.

Polycomb Responsive Element and Hox Gene Silencing in Mammals

In *Drosophila*, Hox gene silencing requires both the *trans* PcG proteins and *cis* DNA elements termed PREs. *Drosophila* PREs are usually several hundred base pairs to several kilobases long and can be located several to hundreds kbs away from the gene promoter. They have been traditionally defined based on their ability to recruit PcG proteins and to silence adjacent reporter genes in a PcG-dependent manner. Although several short motifs required for PRE function have been identified (Bloyer et al., 2003; Mihaly et al., 1998), alignment of several PREs revealed little sequence conservation among the PREs. A computer program for the prediction of the *Drosophila* PREs has been recently developed (Ringrose et al., 2003). However, similar programs cannot be developed for the prediction of PREs in mammals because not a single PRE has been identified in the mammalian system. In this regard, our identification of a 500 bp region located 4 kb upstream of the HoxA9 transcription initiation site has the potential to be the first mammalian PRE identified. The fact that its sequence is conserved in human and mouse and that three YY1 binding sites have been identified in this region coupled with the fact that SUZ12 is bound to this region and that removal of SUZ12 by RNAi correlates with HoxA9 upregulation is consistent with the notion that it serves as a PRE-like element. However, conclusive demonstration that this element is a true PRE requires functional analysis when taken out of its endogenous context and inserted elsewhere into the genome.

Experimental Procedures

Purification of Recombinant EED-EZH2 Complex and Subcomblexes

Ezh2 cDNA was cloned into BamHI and XhoI sites pFASTBAC (GIBCO) without a Flag. EED cDNA was cloned into EcoRI and XhoI sites of a N-terminal Flag-tagged vector pFASTBAC; SUZ12 and AEBP2 cDNAs were inserted into EcoRI and NotI sites of the same

vector. Baculovirus for RbAp48 has been previously described (Zhang et al., 1999). Each baculovirus expressing a different component was generated and amplified following the manufacturer's protocol. To purify the recombinant EED-EZH2 complex, different baculoviruses were used to coinfect SF9 cells. After 2 days of infection, cells were collected and resuspended in F lysis buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 4 mM $MgCl_2$, 0.4 mM EDTA, 2 mM DTT, 20% glycerol, 0.1% NP40) with proteinase inhibitors. Then cells were homogenized with pestle A three times (10 strokes each) in a period of 30 min. The supernatant was recovered by centrifuging at 11,000 rpm for 10 min. The supernatant was adjusted to 300 mM NaCl by adding dilution buffer (20 mM Tris [pH 7.9], 10% glycerol), then incubated with the M2 α-Flag agarose (Sigma) equilibrated with F lysis buffer for 4 hr at 4°C. After being washed with F washing buffer (20 mM Tris [pH 7.9], 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 15% glycerol, 0.01% NP40) until no protein came out, bound proteins were eluted with Flag peptide (0.2 mg/ml) for 20 min each time at RT, then the eluted complexes were further purified through a gel-filtration S200 or Superose 6 column.

HMTase Assay, Substrate Preparation, and Antibodies

Histone methylation assay was performed essentially as previously described (Wang et al., 2001). Substrates for HMTase assay, including oligonucleosomes, mononucleosomes, and core histone, were purified from chicken blood as previously described (Fang et al., 2003). Wild-type and mutant recombinant H3 were expressed and purified as described (Cao et al., 2002). Antibodies against EZH2, SUZ12, 2mK27, 3mK27, 3mK9, and 1mK27 have been previously described (Cao et al., 2002; Peters et al., 2003; Plath et al., 2003).

Plasmids and GST Pull-Down Assay

Full-length AEBP2 cDNA was cloned by PCR amplification from a HeLa cDNA library and the sequence was verified by DNA sequencing. Full-length cDNAs for Ezh2, EED, SUZ12, RbAp48, AEBP2, and their deletions were inserted into pCITE vector for in vitro translation using the rabbit reticulocyte lysate kit according to the manufacturer's instructions (Promega). Full-length cDNAs for SUZ12, EED, RbAp48, and AEBP2 were also cloned into pGEX-KG vector for the production of GST-fusion proteins. About 3 μg of GST or GST fusion proteins were bound to 10 μl of glutathione-immobilized agarose beads (Sigma) and incubated with in vitro-translated products in 500 μl buffer A (50 mM Tris-HCl [pH 7.9], 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol) containing 150 mM KCl and 0.05% NP-40. After incubation at 4°C for 2 hr, the beads were washed three times with buffer A containing 300 mM KCl and 0.05% NP-40 and then washed once in buffer A containing 50 mM KCl before being subjected to SDS-PAGE and autoradiogram.

Generation of a Stable SUZ12 Knockdown Cell Line and Cell Growth Analyses

HeLa cells were cultured in DMEM media supplied with 10% FBS. A desired 64 bp oligonucleotide that targets SUZ12 was cloned into pHTPsiRNA vector as previously described (Wang et al., 2003). The two oligonucleotides that were used in generating the stem-loop RNA that target SUZ12 are 5′-GATCCCCGTCGCAACGGACCAGTTAATTCAAGAGATTAACTGGTCCGTTGCGACTTTTTGGAAA-3′ and 5′-TCGATTTCCAAAAAGTCGCAACGGACCAGTTAATCTCTTGAATTAACTGGTCCGTTGCGACGGG-3′. The SUZ12 siRNA vector was transfected into HeLa cells by effectene (Invitrogen). Stable transfectants were selected in the presence of 2 μg/ml puromycin. Cells derived from these transfectants were used for Western blotting and real-time RT-PCR analysis. For cell growth analyses, 4×10^4 cells were seeded in 12-well plates. After 24, 48, and 96 hr in culture, cells were trypsinized, collected in triplicate, and counted by trypan-blue staining.

Real-Time PCR and ChIP Assays

Real-time PCR was performed in triplicate using SYBR Green PCR Master Mix and the ABI prism 7900 sequence detection system. Quantitative PCR reactions were performed under conditions standardized for each primer. Standard curves were generated using 10-fold dilutions of standard plasmids. To compare the relative amount of target in different samples, all values were normalized to the appropriately quantified GAPDH control. The primers used in quantitative PCR were: SUZ12 cDNA primers—AAACGAAATCGT GAGGATGG and CCATTTCCTGCATGGCTACT, HoxC6 cDNA primers—CCAGGACCAGAAAGCCAGTA and GGTCTGGTACCGCGAG TAGA, HoxC8 cDNA primers—CTCAG GCTACCAGCAGAACC and GAGCCCCATAAAGGGACTGT, HoxA9 cDNA primers—TGCAGCTT CCAGTCCAAGG and GTAGGGGTGGTGGTGATGGT.

For ChIP assays, approximately 5×10^6 HeLa cells in 150 mm dishes were first treated with DMEM containing 1% formaldehyde for 10 min. The crosslink was stopped by the addition of 0.125 M glycine for 10 min. After washing twice with PBS, the cells were resuspended in 300 μl of cell lysis buffer (10 mM HEPES [pH 7.9], 0.5% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) by pipetting and kept on ice for 10 min. After centrifuge at 4000 rpm for 5 min, the cell pellets were resuspended in nuclear lysis buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) containing protease inhibitors to extract nuclear proteins at 4°C for 20 min and then the chromatin were sonicated into fragments with an average length of 0.5–3 kb. After centrifugation at 13,000 rpm for 10 min, the supernatants were diluted in equal volume of dilution buffer containing 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 7.9], 50 mM NaCl, and protease inhibitors. ChIP assays were then performed with indicated antibodies. ChIP DNA was detected using standard PCR with the following primer pairs for the four different regions in HoxA9: A-TCCACCTTTCTCTC GACAGCAC and GTGGGAGGCTCAGGATGGAAG, B-TCGCCAAC CAAACACAACAGTC and AAAGGGATCGTGCCGCTCTAC, C-CTCACC GAGAGGCAGGTCAAG and AGCCTACCATCAACAGTTGTGC, D-GAA CGGCCACAACTTCGGAGG and CCGGGGAGTCTGCGTGGAG.

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