

# Substrate Preferences of the EZH2 Histone Methyltransferase Complex\*

Received for publication, December 16, 2005, and in revised form, January 10, 2006. Published, JBC Papers in Press, January 23, 2006, DOI 10.1074/jbc.M513425200

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Histone methylation plays an important role in chromatin dynamics and gene expression. Methylation of histone H3-lysine 27 by the EZH2 complex has been linked to the silencing of homeotic genes and the inactivation of the X chromosome. Here we report a characterization of the substrate preferences of the enzyme complex using a reconstituted chromatin and enzyme system. We found that the linker histone H1, when incorporated into nucleosomes, stimulates the enzymatic activity toward histone H3. This stimulatory activity may be explained by protein-protein interactions between H1 and components of the EZH2 complex. In addition, we found that the EZH2 complex exhibits a dramatic preference for dinucleosomes when compared with mononucleosomes and that the stimulation of H3 methylation by H1 requires dinucleosomes or oligonucleosome substrates. Furthermore, in contrast with a recent study suggesting that Embryonic Ectoderm Development EED isoforms may affect substrate specificity, we found that EZH2 complexes reconstituted with different EED isoforms exhibit similar substrate preference and specificity. Our work supports the hypothesis that linker histone H1 and chromatin structure are important factors in determining the substrate preference of the EZH2 histone methyltransferase complex.

Covalent histone modifications play an important role in regulating chromatin dynamics and function (1). One such modification, methylation, occurs on both lysine and arginine residues and participates in a diverse range of biological processes including heterochromatin formation, Polycomb group (PcG)<sup>4</sup> gene silencing, and X chromosome inactivation (2–4).

EZH2 is one of two human homologues of the *Drosophila* protein Enhancer of Zeste (E(Z)), a member of the PcG proteins. In the fly, PcG proteins exist in at least two different protein complexes, the Polycomb repressive complex 1 (PRC1) and the Extra Sex Combs ESC-E(Z) complex (5, 6), and play a crucial role in development by maintaining the silenced state of homeotic genes in specific regions of the embryo (7). The developmental function of the PcG proteins is conserved from fly to mammals. However, in addition to regulating Homeobox (Hox) genes, PcG proteins have also been linked to the inactivation of the X chromo-

some in females (8, 9). A major advance regarding the mechanism of PcG-mediated gene silencing has been the discovery that E(Z) and EZH2 possess methyltransferase activity specific for lysine 27 of histone H3 (10–13). Methylation appears to be required for silencing as an E(Z) mutant deficient in methyltransferase activity fails to rescue the silencing defects that occur in an E(Z)-null genetic background (11). Interestingly, E(Z) and EZH2 do not possess detectable histone methyltransferase activity in isolation and appear to function only when associated with other proteins in protein complexes (14, 15). A native EZH2 complex that we purified and characterized contains EZH2, EED, SUZ12, RbAp48, and AEBP2 (10). Experiments utilizing various recombinant EZH2 subcomplexes have demonstrated that these proteins are required for optimal methyltransferase activity (14).

As part of the initial characterization of the native EZH2 complex, we found that although the enzyme complex exhibited robust histone methyltransferase (HMT) activity toward H3 in the context of oligonucleosomes, the enzyme exhibited weaker H3 methyltransferase activity when H3 is presented in other contexts including mononucleosomes, histone octamers, or histone H3 alone (10). Similar substrate preferences were also observed using a reconstituted EZH2 enzyme complex (14). There are a number of possible explanations for these results. Because the oligonucleosome substrates used in the previous studies were prepared from native chromatin, it is possible that other chromatin-associated proteins present in this substrate influence EZH2 activity. One candidate is linker histone H1, a protein present in high abundance in native chromatin. Indeed, it was recently observed that global H3 Lys-27 methylation is reduced when the levels of H1 are lowered by 50% (16). In addition to chromatin-associated proteins, however, another explanation for the oligonucleosome preference of the EZH2 complex is that the enzyme recognizes a specific feature of oligonucleosome substrates.

To further characterize the substrate preferences of the EZH2 complex, we reconstituted both the enzyme complex and various nucleosome substrates. Here we have demonstrated that both nucleosome number and linker histone H1 affect H3 methylation activity by the EZH2 complex. In addition, we also explored a potential role of the different EED isoforms in regulating EZH2 substrate preferences. In contrast to a recent study showing that EED isoforms regulate EZH2 substrate specificity (17), we found that EZH2 complexes reconstituted with different EED isoforms all displayed the same substrate preference and specificity.

## EXPERIMENTAL PROCEDURES

**Reconstitution of EZH2 Complex**—The recombinant EZH2 complex was prepared using a baculovirus system as described previously (14). To produce EED isoforms, cDNAs encoding each isoform were cloned into a baculovirus expression vector FLAG pFASTBAC. Recombinant EZH2 complexes with different EED isoforms were prepared and purified as described previously (14).

\* This work was supported in part by National Institutes of Health Grant GM68804 (to Y. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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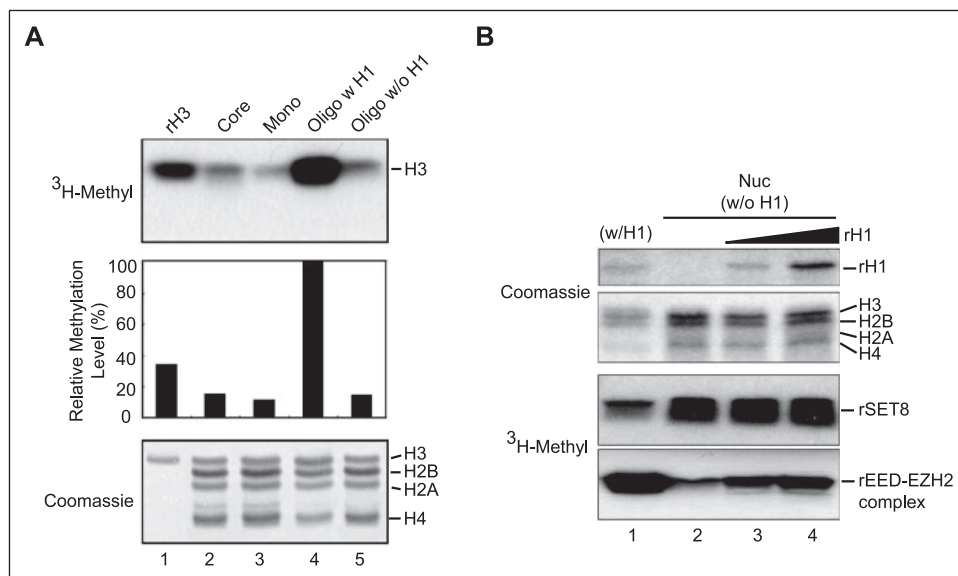
<sup>2</sup> Supported by a National Institutes of Health postdoc fellowship through the Lineberger Cancer Center training grant.

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<sup>4</sup> The abbreviations used are: PcG, Polycomb group; E(Z), Enhancer of Zeste; EED, Embryonic Ectoderm Development; HMT, histone methyltransferase; GST, glutathione S-transferase.

## The EZH2 Complex Substrate Specificity

**FIGURE 1. Linker histone H1 enhances methylation of H3 by the EZH2 complex.** *A*, various H3-containing substrates were used in methyltransferase assays with the EZH2 complex. The *top panel* is an autoradiogram of [ $^3\text{H}$ ]methyl incorporated into histone H3, and the *middle panel* shows this in quantitative form. The *bottom panel* is a Coomassie Blue-stained gel of the substrates, demonstrating that each methyltransferase reaction contains equal amounts of H3. *Mono*, mononucleosomes; *oligo*, oligonucleosomes. *B*, H1-containing (*lane 1*) and H1-depleted (*lanes 2–4*) nucleosomes (*Nuc*) were incubated with increasing amounts of H1 (*lanes 3 and 4*, 0.5:1 and 1:1 molar ratio with histone octamer, respectively) before being used in methyltransferase assays with the EZH2 complex. The *top two panels* show Coomassie Blue staining of H1 and the core histones, respectively. The *third panel* shows SET8-mediated [ $^3\text{H}$ ]methyl incorporation in histone H4, which serves as a negative control and demonstrates the specificity of the H1 effect. The *fourth panel* shows [ $^3\text{H}$ ]methyl incorporation into histone H3 mediated by the EZH2 complex.



**Preparation of Substrates**—For the experiment shown in Fig. 1, the histone substrates were prepared from chicken cord blood as described previously (18). For the assays using reconstituted substrates, the native substrates used for comparison were derived from HeLa cells and prepared using the same methods as those used to prepare substrates from chicken cord blood. Native oligonucleosomes were prepared by pooling sucrose gradient fractions containing ~5–10 nucleosomes. Native dinucleosomes were obtained by pooling the appropriate fractions derived from sucrose gradients. Recombinant histone H3 was prepared as described (10).

To reconstitute nucleosomes, a DNA fragment containing two copies of the nucleosome positioning sequence from a sea urchin 5S RNA gene was used together with core histones isolated from HeLa cells. The DNA fragment was obtained by PCR and purified by non-denaturing gel electrophoresis. Briefly, two *Ava*I fragments from the 5S RNA gene were ligated together and cloned into the *EcoRV* site of pBluescriptII SK. The resulting plasmid was used as the template in a PCR reaction containing 100 pmol each of two primers specific for the vector backbone: 5'-TC-GAGGTCGACGGTATCGATAAGCTTGAT-3' and 5'-CCCCCGG-GCTGCAGGAATTCGAT-3'. Typically, 100 PCR reactions were pooled, extracted with phenol/chloroform, precipitated with ethanol, and finally resuspended in Tris-HCl-EDTA. The fragment was then purified on a 5% acrylamide tube gel using the Bio-Rad Miniprep Cell. Electrophoresis was performed at room temperature, and the running buffer was 0.5× Tris-borate-EDTA. For a single reconstitution, 500 pmol of the gel-purified fragment (1000 pmol of nucleosome positioning sequence) was mixed with 1000 pmol of core histones in a buffer containing 2 M KCl, 10 mM Tris-HCl (pH 8.0), and 1 mM dithiothreitol. This mixture was then dialyzed to 50 mM KCl in two successive 1 and 0.75 M steps. Dialysis resulted in a mixture of mononucleosomes and dinucleosomes that were purified by non-denaturing gel electrophoresis, again using the Miniprep cell. In this instance, electrophoresis was performed at 4 °C, and the running buffer was 0.2× Tris-borate-EDTA. The resulting fractions were analyzed by electrophoresis in 0.8% agarose (0.5× Tris-borate-EDTA) run at room temperature with subsequent EtBr staining. Finally, pooled fractions were concentrated with Millipore centrifugal filters.

**Histone Methyltransferase Assays**—Histone methyltransferase assays were performed as described previously (19). For experiments using reconstituted substrates containing H1, 30 pmol (by histone octamer) of

the relevant substrate was incubated with native H1 (Upstate Biotechnology) on ice for 10 min. A portion of this mixture (10 pmol) was used in a gel shift assay to verify H1 incorporation, and the remaining substrate was used for the methyltransferase assay. For the gel shift assay, the substrate was electrophoresed at room temperature in a 1% agarose gel (0.5× Tris-borate-EDTA) followed by EtBr staining.

**GST Pull-down Assays**—Full-length cDNAs for Ezh2, EED, SUZ12, RbAp48, and AEBP2 were inserted into pCITE vector for *in vitro* translation using the rabbit reticulocyte lysate kit according to the manufacturer's instructions (Promega). cDNAs encoding H1.1 and H1.2 were cloned into pGEX-KG vector for the production of GST fusion proteins. About 3 μg of GST fusion proteins were bound to 10 μl of glutathione-immobilized agarose beads (Sigma) and incubated with *in vitro*-translated products in 500 μl of 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% Nonidet P-40. After incubation at 4 °C for 2 h, the beads were washed three times with buffer A containing 300 mM KCl and 0.05% Nonidet P-40 and then washed once in buffer A containing 50 mM KCl before being subjected to SDS-PAGE and autoradiography.

## RESULTS AND DISCUSSION

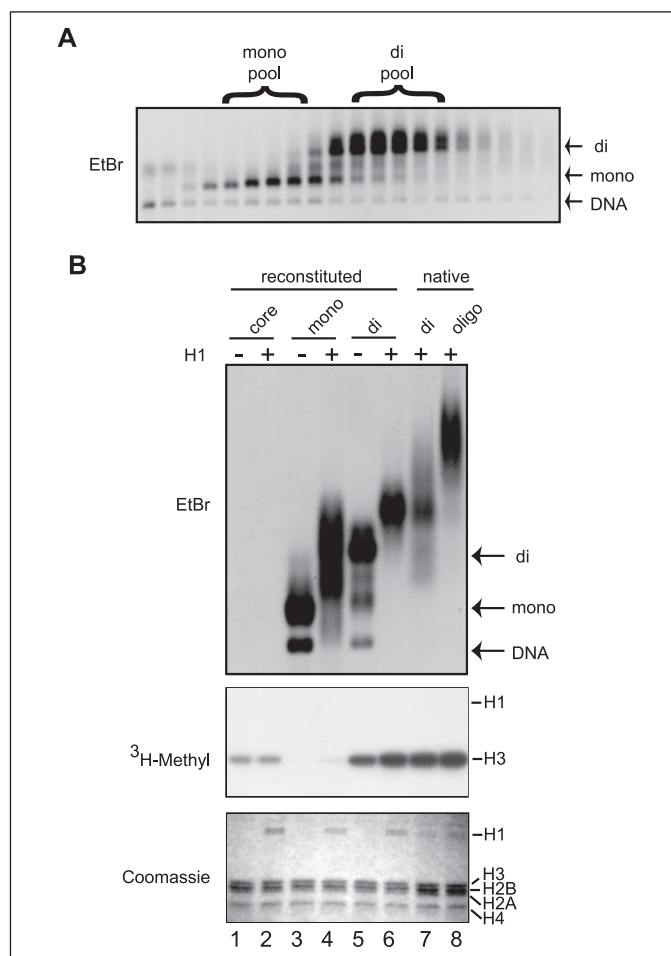
**H1-containing Nucleosomes Are Preferred Substrates of the EZH2 Complex**—Previous studies using native and reconstituted EZH2 complexes indicate that oligonucleosomes are preferred substrates when compared with mononucleosomes (10, 14). To determine whether the observed substrate preference is due to the presence of linker histone H1 in the oligonucleosomes, oligonucleosomes were extracted in high salt to remove linker histones. The resulting substrates were subjected to HMT assays using a recombinant EZH2 complex that has been described previously (14). As reported previously, oligonucleosomes containing H1 are optimal substrates when compared with mononucleosomes, octamers, and recombinant H3 alone (Fig. 1A, compare *lane 4* with *lanes 1–3*). Interestingly, removal of H1 from oligonucleosomes resulted in a relatively poor EZH2 substrate (compare *lane 5* and *lane 4*). Although these results are consistent with H1 stimulating methyltransferase activity toward H3, it was possible that other proteins that influence EZH2 substrate preferences were removed during the extraction procedure. To test the specific effect of linker histones, H1 was incorporated back into H1-depleted oligonucleosomes before the HMT

assay. Results shown in Fig. 1B demonstrate that this manipulation is sufficient to partially restore H3 methylation levels (*bottom panel*, compare lanes 3 and 4 with lane 2). Importantly, the observed H1 effect is specific to the EZH2 complex as a parallel experiment did not show an apparent H1 effect on the activity of SET8 toward histone H4 (*third panel*). These results allow us to conclude that incorporation of H1 into nucleosomes has a positive effect on the HMT activity of the EZH2 complex toward H3.

**Dinucleosomes with H1 Are Optimal EZH2 Substrates**—Although linker histone H1 can partially explain the differences in the HMT activity of the EZH2 complex toward oligonucleosome and mononucleosome substrates, it remained possible that other contributing factors were at work. In addition to the difference in H1 incorporation, another difference between mononucleosome and oligonucleosome is the number of nucleosomes present in each substrate molecule. To address the role of nucleosome number in EZH2 complex substrate preference, both mononucleosomes and dinucleosomes were reconstituted *in vitro* using purified histones and were subjected to HMT assays. For these experiments, a DNA fragment containing two copies of a nucleosome positioning sequence was mixed with core histones and subjected to salt dialysis. This procedure resulted in a mixture of both mononucleosomes and dinucleosomes that was subsequently fractionated by preparative gel electrophoresis to yield pure preparations of both species (Fig. 2A). In addition, H1 was incorporated into both the mononucleosomes and the dinucleosomes to test whether linker histones could stimulate H3 methylation in these two different contexts. To confirm the incorporation of H1 into nucleosomes, the various substrates were subjected to non-denaturing gel electrophoresis. Results shown in Fig. 2B demonstrate that the addition of H1 to nucleosomal substrates results in a shift in electrophoretic mobility, thus confirming successful incorporation of H1 into mono- and dinucleosomes (*top panel*, compare lane 3 with lane 4 and lane 5 with lane 6).

To analyze the effect of H1 on the EZH2 complex activity, the various substrates (Fig. 2B, *top panel*) were subjected to HMT assays. Similar to the native mononucleosomes purified from HeLa cells (Fig. 1A), reconstituted mononucleosomes are poor substrates for the EZH2 complex when compared with native oligonucleosomes (Fig. 2B, *middle panel*, compare lanes 3 and 4 with lane 8). In contrast, the dinucleosome substrates are strongly methylated by the EZH2 complex (*middle panel*, compare lanes 5 and 6 with lanes 3 and 4). Furthermore, incorporation of H1, although not noticeably influencing H3 methylation in mononucleosomes, results in an ~2-fold increase in methylation of H3 in dinucleosomes (*middle panel*, compare lane 5 and lane 6). Importantly, this level of methylation is equivalent to that observed for the native oligonucleosomes containing H1 (compare lane 6 and lane 8). Together, these results indicate that both the number of consecutive nucleosomes in the chromatin substrate and linker histones influence H3 methylation by the EZH2 complex.

**Histone H1 Interacts Specifically with Components of the EZH2 Complex**—There are a number of possible mechanisms that might account for the stimulatory effect of H1 observed in the above experiments. One possibility is that incorporation of H1 into nucleosomes facilitates recruitment of the EZH2 complex to chromatin through protein-protein interactions. To address this possibility, GST-H1 fusion proteins were incubated with radiolabeled components of the EZH2 complex prepared using the transcription/translation-coupled rabbit reticulocyte lysate. GST pull-down assays revealed that of the five components of the enzyme complex, EED, SUZ12, and AEBP2 are capable of specific interactions with H1 (Fig. 3, lanes 8, 9, 11, 14, 15, and 17). The same interactions are observed with two different isoforms of H1, H1.1,



**FIGURE 2. Both linker histones and nucleosome number influence methylation of H3 by the EZH2 complex.** *A*, nucleosome reconstitution resulted in a mixture of mononucleosomes (*mono*) and dinucleosomes (*di*) that were purified as described under "Experimental Procedures." Aliquots of the fractions resulting from this purification procedure were subjected to electrophoresis in agarose gel and viewed by EtBr staining as shown here. Those fractions containing pure mononucleosomes and dinucleosomes, respectively, were pooled and used as substrates for the studies described. *B*, the *top panel* shows EtBr staining of substrates subjected to non-denaturing gel electrophoresis. Incorporation of H1 (1 mole H1/mole of histone octamer) into reconstituted substrates results in slower migration, as indicated by an upward shift in the gel. The *middle panel* shows an autoradiograph of [<sup>3</sup>H]methyl incorporation into histone H3. The *bottom panel* is a Coomassie Blue-stained gel of the substrates used in the HMT assay, demonstrating equal loading of H3. For comparison, native dinucleosomes (*lane 7*) and oligonucleosomes (*oligo*, *lane 8*) containing H1 were included in these assays.

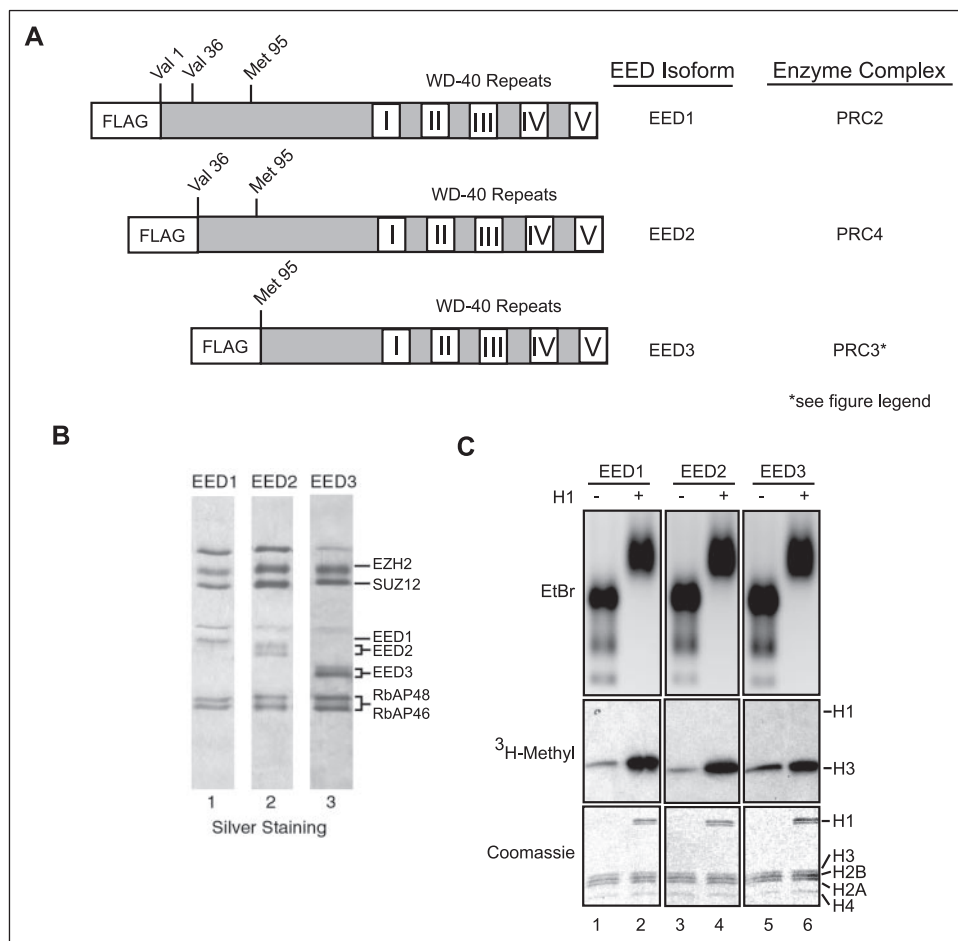
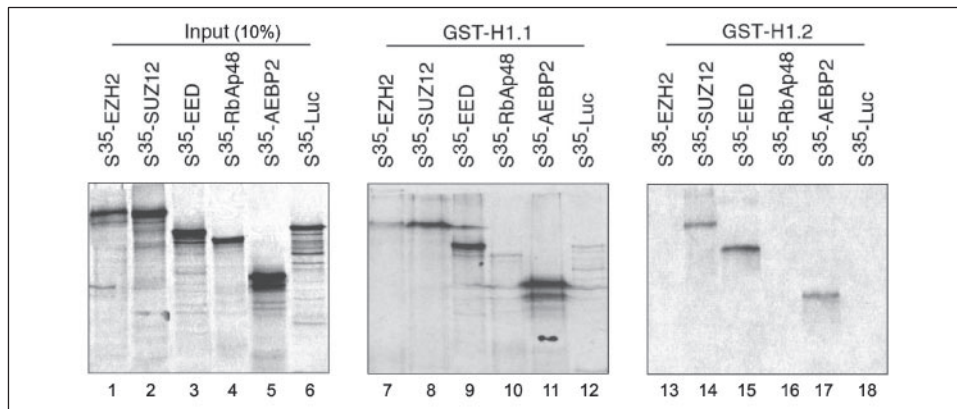
and H1.2. These observations support a model in which linker histones stimulate H3 methylation through the recruitment of the EZH2 complex.

**EED Isoforms Do Not Affect Substrate Specificity of the EZH2 Complex**—The results presented above regarding the stimulatory effect of H1 on histone H3 methylation are inconsistent with a recent report that H1 inhibits methylation of histone H3 by the EZH2 complex (17). In that study, two native EZH2 complexes, referred to as PRC2 and PRC3, were characterized. These complexes differ with respect to the specific isoform of EED present. Four EED isoforms termed EED1, EED2, EED3, and EED4 are believed to arise from different translation initiation sites (17). PRC3 corresponds to the EZH2 complex described in this study in that it contains EED3 (Fig. 4A). Although PRC3 was found to methylate oligonucleosome substrates in the absence of H1, the addition of linker histone H1 strongly inhibited H3 methylation. In contrast, PRC2, which contains EED1, methylated H3 in oligonucleosomes weakly, but upon the addition of H1, the H3 activity was inhibited, and H1 methylation



## The EZH2 Complex Substrate Specificity

**FIGURE 3. Components of the EZH2 complex interact specifically with linker histones *in vitro*.** All five components of the EZH2 complex were translated in the presence of [<sup>35</sup>S]methionine *in vitro*. The radiolabeled products were incubated with GST-H1.1 and GST-H1.2 and subjected to pull-down assays. The *left panel* is an autoradiograph of the various inputs (10%). The *middle and right panels* show the components that remain associated with the GST fusion proteins after washing. Luciferase (*Luc*) is included as a negative control.

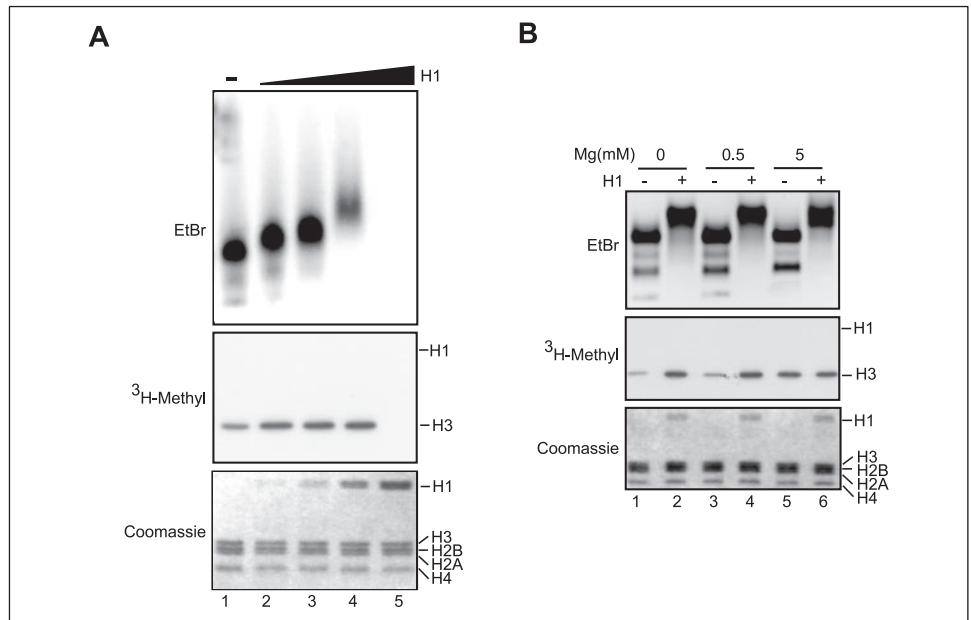


**FIGURE 4. EZH2 complexes reconstituted with different EED isoforms behave similarly with regard to the H1-stimulation effect.** *A*, a schematic representation of EED isoforms 1, 2, and 3 that were expressed as FLAG-tagged fusion proteins. Indicated are the presumed initiation amino acids of the different isoforms based on Kuzmichev *et al.* (17). Also indicated is the name of the specific EZH2 enzyme complex that each EED isoform is associated with according to Kuzmichev *et al.* (17). In addition to EED3, EED4 has also been reported to be a component of the PRC3 complex. *B*, EZH2 complexes were reconstituted with EED1, EED2, and EED3. Shown are silver-stained gels of all three complexes. For these complexes, AEBP2 was omitted, and RbAp46 was included in accordance with Kuzmichev *et al.* (17). *C*, HMT assays were performed with EZH2 complexes and reconstituted dinucleosomes with or without linker histone H1 (1 mole H1/mole of histone octamer). The *top panel* is a gel shift assay to verify H1 incorporation, whereas the *middle panel* is an autoradiograph of the Coomassie Blue-stained gel shown in the *bottom panel*.

was observed (17). To determine whether the discrepancy between our study and that mentioned above can be explained by the different EED isoforms, we reconstituted EZH2 complexes with EED isoforms 1 through 3 (Fig. 4B) and compared their substrate preference and specificity (H3 versus H1). In an attempt to replicate the aforementioned study, the EZH2 complexes were reconstituted without AEBP2, but with the RbAp46 component, in accordance with the reported composition of the enzyme complexes (13, 17). Surprisingly, results shown in Fig. 4C demonstrate that EZH2 complexes reconstituted with EED1 and EED2 behave in a similar fashion as the EZH2 complex with EED3 in terms of substrate preference. Thus, all three complexes strongly methylate histone H3, preferably in the context of H1 containing dinucleosomes, but H1 is not significantly methylated.

In an attempt to understand the basis of these discrepancies, we altered the assay conditions to determine whether this may cause a change in the substrate preferences of the EZH2 complex. First, we analyzed the effect of incubating increasing amounts of H1 with dinucleosome substrates (Fig. 5A). Interestingly, although H3 methylation peaks when H1 is present at or slightly below a 1:1 molar ratio with histone octamers, methylation is strongly inhibited when higher amounts of H1 are added (*middle panel*, compare lane 4 and lane 5). Inhibition in this case, however, most likely reflects an artifact because when these substrates are analyzed for H1 incorporation by the gel shift assay, it is clear that high molar ratios of H1 cause precipitation of the substrate (*top panel*, lane 5). In addition to varying H1 levels, the effects of magnesium were also examined. Divalent cations are known to be

**FIGURE 5. Linker histone stoichiometry and magnesium concentration affect EZH2-mediated H3 methylation.** *A*, increasing amounts of H1 were incubated with reconstituted dinucleosomes in the following molar ratios: 0.5:1 (*lane 2*), 1:1 (*lane 3*), 2:1 (*lane 4*), 4:1 (*lane 5*). Shown in the *top panel* is an EtBr-stained agarose gel after the substrates were subjected to electrophoresis. These substrates were also subjected to an HMT assay. Shown in the *middle panel* is an autoradiograph of a Coomassie Blue-stained gel (*bottom panel*). *B*, reconstituted dinucleosomes were incubated in the presence of H1 at the indicated magnesium concentration. Shown in the panels are the same assays described above.



required for chromatin to fold into higher order structures *in vitro* (20); thus, it seemed reasonable that magnesium might affect the substrate preferences of the EZH2 complex by, for example, changing the conformation of the substrate. At low concentrations of  $Mg^{2+}$  (0.5 mM), H1 stimulated H3 methylation to the same degree as that observed in the absence of divalent cations (Fig. 5*B*, *middle panel*, compare *lanes 1* and *2* with *lanes 3* and *4*). In contrast, high  $Mg^{2+}$  (5 mM) prevented stimulation of H3 methylation by H1 (Fig. 5*B*, *middle panel*, *lanes 5* and *6*), although the level of methylation in the absence of linker histones was elevated (Fig. 5*B*, *middle panel*, compare *lane 5* with *lanes 1* and *3*). Of note, 5 mM magnesium was used in the studies by Kuzmichev *et al.* (17). In summary, these results suggest that variations in the assay conditions may contribute to the different results of the two studies. However, we have never observed an inhibitory effect of H1 on H3 methylation, nor have we observed an effect of different EED isoforms on the substrate specificity of the EZH2 complex using a pure reconstituted system.

In addition to differences in assay conditions, it is possible that other differences in experimental design explain the discrepancy between the two studies. One obvious difference is the use of native *versus* recombinant enzyme complexes. There are potential pitfalls with the use of either. Data obtained with native complexes, for example, have to be analyzed with caution because of potential contaminating activities. Contamination, on the other hand, is less of an issue with highly purified recombinant complexes, such as the one described in this study. However, the use of protein tags gives rise to additional experimental caveats. For example, the tag that was fused to the amino terminus of all three EED isoforms may somehow influence the catalytic activity of the EZH2 complex. However, the fact that the native complex and the equivalent recombinant EZH2 complex reconstituted with EED3 behave in a similar fashion with regard to substrate preference (14) argues against this possibility. Although it is difficult to dismiss the possibility that H1 is a substrate of the EZH2 complex at this point, there is as yet no *in vivo* evidence that H1 methylation is dependent on EED or any other component of the EZH2 complex. This is in contrast to a number of reports that demonstrate a clear link between components of the EZH2 complex and H3 lysine 27 methylation (10, 14, 15, 21, 22). Furthermore, it has recently been reported that a reduction in H1 levels by 50% in triple knock-out ES cells leads to a 2-fold reduction in global H3 K27 methylation

levels *in vivo* (16). This observation strongly supports the results presented here regarding the stimulatory effect of H1 on H3 methylation mediated by the EZH2 complex.

Although H1 has a positive effect on H3 methylation, it is clear that incorporation of H1 into nucleosomes is not sufficient to produce an optimal substrate. This can be deduced based on the observation that mononucleosomes with incorporated H1 are poor substrates. The minimal requirement for an optimal substrate appears to be two consecutive nucleosomes. Consistent with this notion, reconstituted dinucleosomes containing H1 were methylated to the same degree as native oligonucleosomes. Although the significance of the dinucleosome is currently unclear, it is likely that some structural feature of this substrate might be recognized by the EZH2 complex. One possibility is that the enzyme recognizes a specific conformation of the H3 tail that occurs in di- and oligonucleosomes but not in mononucleosomes. Histone tails are believed to be involved in interactions between nucleosomes to facilitate folding of the chromatin fiber (23). Perhaps these interactions make the H3 tails more accessible to the enzyme or result in some unique conformation that is relevant to EZH2 substrate preferences. A clear molecular explanation of our observation can be achieved when a co-crystal structure of a dinucleosome in complex with the EZH2 complex becomes available.

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