The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3

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Polycomb group (PcG) proteins are important for maintaining the silenced state of homeotic genes. Biochemical and genetic studies in *Drosophila* and mammalian cells indicate that PcG proteins function in at least two distinct protein complexes: the ESC–E(Z) or EED–EZH2 complex, and the PRC1 complex. Recent work has shown that at least part of the silencing function of the ESC–E(Z) complex is mediated by its intrinsic activity for methylating histone H3 on lysine 27. In addition to being involved in *Hox* gene silencing, the complex and its associated histone methyltransferase activity are important in other biological processes including X-inactivation, germline development, stem cell pluripotency and cancer metastasis.

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Abbreviations

EED	embryonic ectoderm development
ESC	Extra sex combs
E(Z)	Enhancer of Zeste
GFP	green fluorescent protein
HMTase	histone methyltransferase
MES	maternal effect sterile
PcG	Polycomb group
PRC1	Polycomb repressive complex 1
SAM	sterile α motif
SUZ12	Suppressor of Zeste 12
trxG	trithorax group
Xi	inactive X chromosome

Introduction

A multicellular organism is composed of various types of cells with distinct functions. These functionally distinct cell types arise during early development and are subsequently maintained during mitotic cell divisions. At the molecular level, the function of a particular cell type is determined by the set of genes that the cell expresses. Maintaining gene expression patterns after initial cell differentiation is thus crucial for maintaining cell identity.

Epigenetics, the heritable regulation of gene expression independent of DNA sequence, is emerging as an essential aspect of the mechanisms of cell lineage determination and maintenance. Studies over the past several years have shown that many epigenetic phenomena are controlled by DNA methylation and histone modifications, particularly histone methylation [1–6]. Recent demonstrations that DNA methylation requires histone methylation have provided compelling evidence that the two mechanisms are intimately connected [7,8].

Polycomb group (PcG) and trithorax group (trxG) proteins are well known to be part of the cellular memory system [9,10]. Both groups of proteins are involved in maintaining the spatial patterns of homeotic box (*Hox*) gene expression that are established early in embryonic development by the transient expression of segmentation genes. In general, PcG proteins are transcriptional repressors that maintain the 'off state', whereas trxG proteins are transcriptional activators that maintain the 'on state'. Recent demonstrations that members of PcG and trxG proteins contain intrinsic histone methyltransferase (HMTase) activity raise the possibility that PcG and trxG proteins participate in cellular memory through methylating core histones [11,12••–15••,16,17].

Biochemical and genetic studies have provided compelling evidence that *Drosophila* PcG proteins function in two distinct protein complexes: the Polycomb repressive complex 1 (PRC1) and the Extra sex combs and Enhancer of Zeste (ESC–E[Z]) complex (also known as PRC2), although the dynamic compositions of the complexes are still controversial [9,18]. Similar protein complexes have also been described in mammals [19,20], underscoring the functional conservation of the PcG proteins during evolution. The discovery that the ESC–E[Z] complex (known as the EED–EZH2 complex in mammals) contains intrinsic HMTase activity prompted the analysis of the role of histone methylation mediated by EED–EZH2 or ESC–E(Z) in PcG silencing, X-inactivation, germline and stem-cell development, and cancer. Here we review recent progress in our understanding of the biochemical and biological functions of this complex and its associated enzymatic activity in these cellular processes.

The E(Z) PcG complex is a histone H3 lysine-27-specific methyltransferase

Analysis of the *Drosophila* proteins involved in position effect variegation and PcG- and trxG-mediated epigenetic regulation identified an evolutionarily conserved 130-residue motif called the SET domain (for SU[VAR]3-9,

Enhancer of Zeste, trithorax) [21]. Since the initial demonstration that the SET domain is required for the HMTase activity of SUV39H1 [22], several SET domain HMTases have been identified [3,5].

As founding members of the family of SET domain proteins, E(Z) and its mammalian homolog EZH2 were considered to be good candidates for possessing HMTase activity. Failure to detect HMTase activity in recombinant EZH2 [22] prompted several groups to investigate potential HMTase activity in reconstituted or native E(Z) and EZH2 complexes. These studies revealed that the *Drosophila* ESC–E(Z) complex and its human counterpart, the EED–EZH2 complex, indeed contain intrinsic HMTase activity [13••–15••]. By an unbiased systematic approach to HMTase purification, a similar EZH2-containing protein complex was purified [12••]. Although different approaches and purification schemes used by different groups have resulted in the purification of protein complexes with slightly different compositions, they all share four common subunits and all have HMTase activity for histone H3. The protein compositions and characteristics of these protein complexes are summarized in Table 1.

Notably, although all four research groups agree that both the *Drosophila* E(Z) complex and the human EZH2 complex have HMTase activity, they have found different enzymatic properties among the protein complexes (Table 1). For example, whereas two groups have shown that their enzymes have a clear preference for oligonucleosome over octomer substrates [12••,15••], the other two groups observed the opposite preference [13••,14••]. In addition, the E(Z) complex immunoprecipitated by Czermin *et al.* [13••] can methylate H3 tail peptide substrates, whereas our group [12••] has not been able to find any HMTase activity on H3 tail peptide substrates in either the native or the baculovirus-expressed reconstituted human EZH2 complex, or in immunoprecipitated MES-2–MES-3–MES-6 (MES-2/3/6) complex (the *Caenorhabditis elegans* counterpart), although all of our complexes have robust HMTase activities towards nucleosomal histone substrates ([12••]; LB Bender, R Cao, Y Zhang, S Strome, unpublished).

There is also some discrepancy among investigators about the lysine residues that the enzyme complexes methylate. Lysine 27 of histone H3 (H3-K27) is an undisputed methylation site for both the *Drosophila* and human versions of the complex. This conclusion is supported by both *in vitro* [12••–15••] and *in vivo* [12••] evidence. The discrepancy lies in whether the EZH2–E(Z) complex can also methylate H3-K9. Whereas almost no H3-K9 methylation activity was detected by two groups in wild-type EZH2 or reconstituted E(Z) complexes [12••,15••], weak [14••] and significant [13••] *in vitro* H3-K9 methylation activity has been reported by the others.

To provide *in vivo* evidence that E(Z) contributes to H3-K9 methylation, Czermin *et al.* [13••] used an antibody against trimethylated K9 (3mK9) to stain polytene chromosomes. Consistent with trimethylation of H3-K9 by the ESC–E(Z) complex, they reported the colocalization of 3mK9 and Posterior sex combs [13••]. A common

Table 1

Comparison of the various ESC–E(Z) and EED–EZH2 complexes purified by different research groups.

Approach	Composition	Substrate specificity	*In vitro* site specificity	*In vivo* site specificity	*In vitro* Pc recognition	*In vivo* Pc recognition	Reference
Affinity and conventional western blot	EZH2 SUZ12 EED RbAp46/48	Octomer >oligonucleosome	K27 major site K9 minor site	ND	Yes	ND	[14••]
Conventional and affinity HMTase activity	EZH2 SUZ12 EED RbAp48 AEBP2	Oligonucleosome >rh3 >octomer	K27	2mK27 (by ChIP)	Yes	Colocalization (by ChIP)	[12••]
Affinity and conventional techniques	E(Z) Su(Z)12 ESC NURF55	Oligonucleosome >octomer	K27	ND	ND	ND	[15••]
Affinity and conventional western blot; HMTase activity	P168 E(Z) Su(Z)12 ESC NURF55 RPD3	Octomer >oligonucleosome	K9 K27	3mK9 (by polytene staining)	Yes	ND	[13••]

ChIP, chromatin immunoprecipitation; ND, not determined.

technical problem in such studies, however, is that the antibodies may crossreact with more than one modified form of the histone. Indeed, Czermin *et al.* [13••] noted that their anti-3mK9 antibodies also recognize 3mK27. It is therefore difficult to determine which of the polytene signals represent 3mK9 or 3mK27. In addition, chromatin immunoprecipitation assays of the Polycomb-responsive element region of the well-characterized E(Z) target gene *Ubx* showed that H3-K27 methylation, but not H3-K9 methylation, depends on E(Z) binding, supporting the idea that K27, but not K9, is an *in vivo* target site [12••].

This conclusion has gained further support by the recent demonstrations that, first, mutation of mouse Ezh2 reduces H3-K27 methylation in pre-B cells [23]; second, Eed–Ezh2-mediated H3-K27 methylation, but not H3-K9 methylation, marks the inactive X chromosome (Xi) [24••,25••]; third, mutation of MES-2, the worm homolog of E(Z), results in loss of germline H3-K27 methylation but has no effect on H3-K9 methylation (LB Bender, R Cao, Y Zhang, S Strome, unpublished); and fourth, the chromodomain of Polycomb, a component of the PRC1 complex, binds specifically to methylated K27, but not to methylated K9 [12••–14••].

On the basis of the evidence accumulated so far, it is clear that H3-K27 is a functionally relevant *in vivo* target for EZH2, E(Z) and MES-2. Whether the reported minor *in vitro* H3-K9 methylase activity is intrinsic to EZH2 and E(Z) or comes from a minor contaminating activity remains to be determined. Of particular relevance to this issue is the observation that an immunoprecipitated Flag-tagged EZH2 mutant, C588Y, corresponding to the temperature-sensitive $E(z)^{32}$ allele, which is defective for chromosome binding at restrictive temperature [26], has no H3-K27 activity but still retains the same level of H3-K9 activity as the wild type [14••]. Alternatively, the discrepancy on the H3-K9 activity may simply reflect subtle differences in complex composition or assay conditions used by the different research groups. Until *in vivo* evidence is obtained for EZH2- and E(Z)-mediated H3-K9 methylation, EZH2 and E(Z) should be regarded as a H3-K27 methyltransferase.

H3-K27 methylation and *Hox* gene silencing

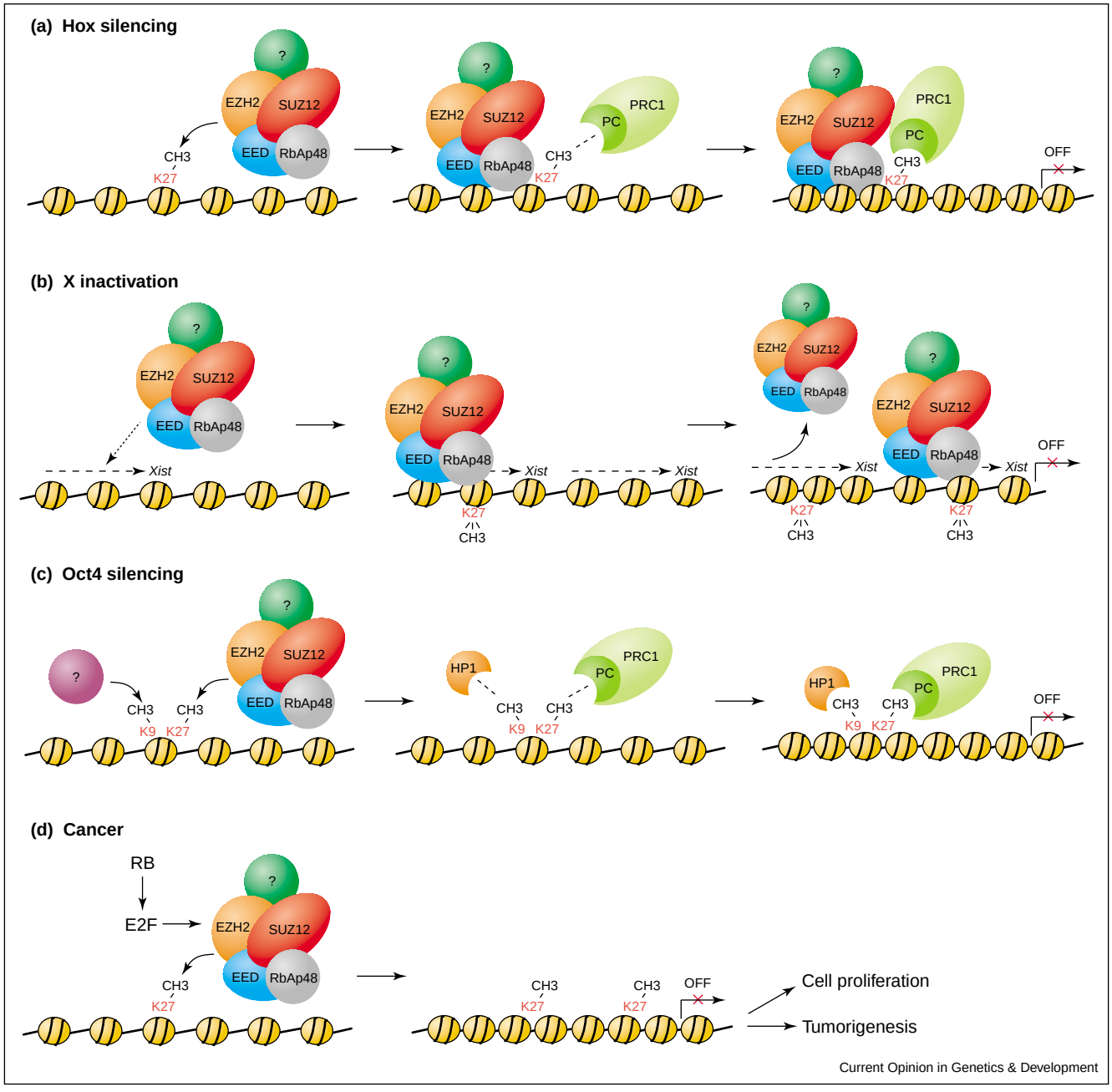
Hox genes are well-known targets for PcG proteins in *Drosophila*, vertebrates and plants. Recent studies indicate that this same regulatory system is also conserved in the nematode *C. elegans* [27,28]. The *C. elegans* counterpart of the *Drosophila* ESC–E(Z) complex, the MES-2/3/6 complex, was identified through the germline defects of mutants [29]. Recently, the MES-2/3/6 complex has been shown to participate in *Hox* gene silencing in somatic cells [28]. The lack of homologs of the PRC1 components in worms raises the question of whether proteins that are functionally analogous to those in PRC1 exist in worms. The discovery that SOP-2, a protein containing a sterile α motif (SAM) domain, is required for *Hox* gene silencing in worms, combined with the fact that the SAM domain protein Polyhomeotic is a component of the PRC1 complex, suggests that SOP-2 and its associated proteins may have functions analogous to PRC1 in *Hox* gene silencing [27]. Thus, the *Hox* gene silencing mechanism seems to be conserved in all multicellular organisms studied so far.

Is E(Z)-mediated H3-K27 methylation essential for *Hox* gene silencing? Using an elegant transgene rescue assay, Muller *et al.* [15••] demonstrated that enzymatically impaired E(Z) mutant proteins containing H703K or R699H failed to rescue *Ubx* gene silencing, supporting the idea that H3-K27 methylation is important in *Hox* gene silencing. How might H3-K27 methylation result in transcriptional repression? Studies of the mechanism by which SUV39H-mediated H3-K9 methylation silences genes have provided a clue: it has been shown that SUV39H-mediated H3-K9 methylation creates a binding site for the chromodomain of HP1, which mediates transcriptional silencing [30,31]. In a similar manner, ESC–E(Z)-mediated H3-K27 methylation might provide a binding site for the chromodomain of Polycomb, a core component of the PRC1 complex [32] (Figure 1a).

Consistent with this notion, Polycomb preferentially binds to the K27-methylated H3 tail peptide *in vitro* [12••–14••]. In addition, chromodomain swapping experiments have shown that an intact Polycomb chromodomain is required for the correct localization of Polycomb and other PcG proteins to PcG-regulated sites on polytene chromosomes [33••,34,35]. Furthermore, chromatin immunoprecipitation assays on the Polycomb responsive element region of the *Ubx* gene have revealed a tight colocalization of E(Z), H3-K27 methylation and Polycomb binding in both cultured S2 cells and fly wing imaginal discs [12••]. Significantly, disruption of E(Z) chromosome binding through RNA-mediated interference or through heat inactivation of an *E(z)* temperature-sensitive allele in cultured cells or *in vivo*, respectively, results in concomitant loss of H3-K27 methylation and Polycomb binding [12••]. Recruitment of PRC1 via H3-K27 methylation may inhibit chromatin remodeling, leading to transcriptional silencing [12••,36]. Although the above evidence supports a role for H3-K27 methylation in PRC1 recruitment, it does not exclude the possibility that PRC1 recruitment is also facilitated by transient protein–protein interactions between components of the ESC–E(Z) and PRC1 complexes [37], or by the sequence-specific DNA-binding proteins ZESTE or GAGA [38].

HP1 and Polycomb are localized to heterochromatin and the euchromatic arms of polytene chromosomes, respectively. The chromodomains of both proteins are important in determining their respective chromosome-binding

Figure 1



Models of the different silencing mechanisms involving the EED–EZH2 complex. The composition of the complex may vary (indicated by ?), depending on the target genes and organisms. **(a)** In Hox gene silencing, EED–EZH2-mediated H3-K27 methylation may help to recruit the PRC1 complex through binding of the Polycomb chromodomain to 3mK27. Recruitment of PRC1 may help to condense the chromatin structure by limiting the access of remodeling factors, leading to transcriptional silencing. **(b)** In X-inactivation, coding of the *Xist* RNA on the inactive chromosome (Xi) helps to recruit the EED–EZH2 complex to methylate H3-K27 on the Xi. This may help to spread *Xist* and 3mK27 throughout the whole Xi to cause X-inactivation. **(c)** In Oct3/4 silencing, the EED–EZH2 complex and an unknown H3-K9 methyltransferase methylate H3 at K27 and K9, respectively. Subsequent recruitment of PRC1 and HP1 may help to keep Oct3/4 silent permanently. **(d)** In cancer, Rb–E2F-mediated misexpression of EZH2 and SUZ12 disrupts the stoichiometry of the EED–EZH2 complex, leading to misexpression of genes involved in regulating the cell cycle and cell proliferation, resulting in tumorigenesis.

patterns. Given the high similarity between the two chromodomains and the sequences adjacent to K9 and K27 of histone H3, the specificity of binding of HP1 and Polycomb to methylated H3-K9 and H3-K27, respectively, is surprising. Two recent studies have revealed insights into the structural basis of this binding specificity

[33••,39••]. The co-crystal structure of the Polycomb chromodomain in complex with an H3 peptide containing trimethylated K27 shows that Polycomb interacts not only with the H3 ARKS motif, but also with several residues preceding this motif (such as L20 and T22) [33••,39••]. Interestingly, Polycomb can dimerize via chromodomain–chromodomain interactions. Such dimerization juxtaposes two H3-binding clefts, resulting in histone–histone interactions involving L20, T22 and A24 on H3. This is thought to be an important factor that contributes to the specific recognition of methylated K27 by the Polycomb chromodomain [39••].

H3-K27 methylation and X-chromosome inactivation

X-chromosome inactivation (X-inactivation) is a developmentally regulated process involving choice of the active X chromosome, initiation of silencing on the Xi and maintenance of silencing throughout all subsequent cell divisions [40,41]. *Xist*, a noncoding RNA transcript, coats the Xi and is thought to mediate its silencing [42]. Studies over the past several years have shown that several epigenetic modifications, including histone H4 hypoacetylation, enrichment of the variant histone macroH2A and DNA methylation, are features of the Xi [43].

Histone methylation was first implicated as a feature of the Xi when an antibody against an H3 peptide dimethylated on K9 (2mK9) was found to specifically stain the Xi, beginning immediately after *Xist* coating [44–47]. But the HMTase that methylates H3-K9 of the Xi has remained elusive. The recent findings that Eed–Ezh2-mediated H3-K27 methylation is associated with the Xi, and that neither 2mK9 nor 3mK9 is enriched on the Xi on the basis of staining with antibodies highly specific to 2mK9 and 3mK9, have challenged the notion that K9 methylation is a general feature of the Xi [24••,25••] and have raised the possibility that the previously observed 2mK9 enrichment on the Xi might be a result of crossreactivity between the anti-2mK9 antibody and 3mK27. At present, whether or not 2mK9 is a general feature of the Xi remains unresolved.

An Eed–Ezh2 histone methyltransferase complex was first implicated in X-inactivation when mice homozygous for an Eed mutation were found to be defective in maintaining X-inactivation in extraembryonic, but not embryonic, tissues [48]. Consistent with a role of the Eed–Ezh2 complex in X-inactivation in cells of extraembryonic lineage, Eed and Ezh2 were found to be enriched on the *Xist*-coated Xi in trophoblast stem cells [49], an extraembryonic cell type. Two recent studies have further evaluated the role of Eed–Ezh2-mediated H3-K27 methylation in X-inactivation [24••,25••].

The main conclusions from these studies are that, first, Eed–Ezh2-mediated H3-K27 trimethylation, but not dimethylation, is specifically enriched on the *Xist*-coated Xi in both embryonic and extraembryonic cell lineages; second, enrichment of the Eed–Ezh2 complex and 3mK27 on the Xi occurs early during the initiation stage immediately after *Xist* coating of the Xi; third, *Xist* coating of the Xi is both necessary and sufficient for the recruitment of Eed–Ezh2 and subsequent H3-K27 trimethylation on the Xi; and last, enrichment of Eed–Ezh2 and 3mK27 on the Xi is independent of the *Xist* silencing function. Although most data obtained by the two groups are consistent, there are discrepancies regarding H3-K9 methylation. According to Plath *et al.* [24••], no enrichment of 2mK9 or 3mK9 is observed on the Xi in trophoblast or embryonic stem cells. By contrast, Silva *et al.* [25••] report the presence of 2mK9 or 3mK9 staining in *eed* mutant embryos; however, they do acknowledge the possibility that their 2/3mK9 antibodies crossreact with the 3mK27 epitope.

In a complementary study using *ezh2* mutant mice, the Surani group [50•] did not observe any colocalization of Eed and 2mK9 and 3mK9 on the Xi, but the 3mK27 mark on the Xi was completely eliminated by the loss of Ezh2 function [50•]. These results are consistent with the notion that the Eed–Ezh2 complex methylates H3-K27, but not H3-K9. The fact that enrichment of the Eed–Ezh2 complex and 3mK27 on the Xi immediately follows *Xist* coating suggests that the complex and its associated HMTase have a role in the initiation stage of X-inactivation [24••,25••,50•]. Although 3mK27 probably serves as a binding site for the recruitment of the PRC1 complex in *Hox* gene silencing, this same mechanism does not seem to hold true in X-inactivation, because the mouse homolog of *Drosophila* Polycomb is not enriched on the Xi [24••,25••]. Whether *Xist* recruits another protein complex that is functionally analogous to PRC1 is not known (Figure 1b). Therefore, dissecting the exact mechanism by which the Eed–Ezh2 complex and its associated HMTase participate in X-inactivation should be a priority of future work.

In a follow-up study, Plath *et al.* (K Plath *et al.*, personal communication) recently reported a comprehensive analysis of the dynamic regulation of histone methylation marks on the Xi in somatic cells. Previous studies indicated that *Xist* RNA associates with the Xi from early G_1 until metaphase [51], at which time it becomes distributed throughout the nucleus and cytoplasm. Consistent with the finding that *Xist* coating is required for enriching Eed–Ezh2 and 3mK27 on the Xi [24••,25••], enrichment of H3-3mK27 on the Xi was mainly detected in G_1 and metaphase, but was lost by telophase (K Plath *et al.*, personal communication). In addition to 3mK27, Plath *et al.* (K Plath *et al.*, personal communication) also observed accumulation of 2mK9 on the Xi in 10% of mouse embryonic fibroblast cells in early S phase. Unlike the 3mK27 mark, enrichment of 2mK9 on the Xi is not dependent on *Xist* RNA.

The H3-2mK9 methyltransferase G9A is a good candidate for making the H3-2mK9 mark, because G9A tagged with enhanced green fluorescent protein (GFP) is enriched on the Xi when the H2-2mK9 mark is present. In addition to the methyl marks on H3, mono-methylated K20 in histone H4 (H4-mK20) is also enriched transiently on the Xi during early mitosis, and is no longer detected at metaphase. It is important to point out that, although the cell-cycle-dependent change in methyl marks is interesting, it raises a serious challenge to the current hypothesis that histone methylation is a mitotically heritable mark for epigenetic regulation [2,52]. Because the dynamic changes in methylation described above are based completely on immunostaining, the interpretation of these data requires caution. It is possible that other cell-cycle-regulated alterations in chromatin structure or histone modification may be masking histone methylation on the Xi.

In addition to being involved in X-inactivation, recent studies have also shown that the Eed–Ezh2 complex is important in regulating the expression of a subset of autosomal imprinted loci [53•]. In *eed*-null mutant embryos, transcription from the silent alleles of a subset of paternally repressed genes is reactivated and this allele-specific transcription reactivation correlates with changes in the DNA methylation pattern in the differentially methylated regions of the affected loci. Given the connection between histone methylation and DNA methylation [7,8], the observed change in DNA methylation may be a result of changes in H3-K27 methylation in the differentially methylated region of the affected loci. Whether or not this is the case remains to be determined (see the review by Delaval and Feil in this issue).

C. elegans MES-2/3/6-mediated H3-K27 methylation in germline development

In a genetic screen aimed at identifying genes required for early germline development in *C. elegans*, four maternal effect sterile genes, *mes-2*, *mes-3*, *mes-4* and *mes-6*, were identified [29]. Biochemical studies indicate that MES-2, MES-3 and MES-6 function in a protein complex, whereas MES-4 seems to function separately [54]. In addition, MES-3 is a novel protein, but MES-2 and MES-6 are the *C. elegans* orthologs of *Drosophila* E(Z) and ESC, respectively. The *C. elegans* germ line is subjected to transcriptional repression, most notably on the X chromosomes [55], during much of its developmental program. Recent studies indicate that the MES-2/3/6 complex is important in X-chromosome silencing in the hermaphrodite germ line, because mutation of any one of the *mes-2*, *mes*-3 and *mes-6* genes results in the X chromosomes acquiring marks of active chromatin and presumably becoming derepressed [55,56].

The recent demonstration that the *Drosophila* ESC–E(Z) complex and its human counterpart, the EED–EZH2 complex, have H3-K27 methyltransferase activity prompted Strome and colleagues (LB Bender, R Cao, Y Zhang, S Strome, unpublished) to investigate whether the MES-2/3/6 complex has similar HMTase activity and, if so, whether this enzymatic activity is important for germline development. Similar to the *Drosophila* and human complexes, the immunopurified MES-2/3/6 complex was found to have HMTase activity specific for nucleosomal histone H3. Immunostaining of wild-type and *mes* mutant worms with antibodies highly specific for 2mK9, 3mK9, 2mK27 and 3mK27 revealed that the MES-2/3/6 complex is responsible for H3-K27, but not H3-K9, methylation, because *mes* mutants showed marked alterations in H3-K27 methylation, but not in H3-K9 methylation.

Interestingly, although H3-K27 methylation is absent from the germ line and early embryos of *mes-2* mutant worms, it is present in the nuclei of adult somatic tissues, in oocytes and in late-stage embryos. These results suggest that, although MES-2 is solely responsible for H3-K27 methylation in most of the germ line, at least one additional H3-K27 methyltransferase must function in somatic cells.

Ezh2-mediated H3-K27 methylation and stem cell pluripotency

Recent studies suggest that Ezh2 and its associated H3-K27 methyltransferase activity might be important for maintaining stem cell pluripotency. Stem cells are characterized by their capacity for self-renewal and their ability to differentiate into all cell types. Characterization of stem cells has shown that several protein factors, including the POU domain homeobox transcription factor Oct3/4, are essential for stem cell pluripotency [57].

Oct3/4 is one of the earliest expressed transcription factors and is crucial for murine development at the preimplantation stage. It is expressed at high levels in stem cells such as embryonic germ cells, embryonic stem cells and embryonic carcinoma cells, but it undergoes rapid repression when these cells start to differentiate [58]. The first indication that murine Ezh2 might be linked to stem cell pluripotency came from the observation that Ezh2 is essential for the derivation of pluripotent embryonic stem cells [59]. Given the early embryonic lethal phenotype of *Ezh2* mutants, and the fact that Ezh2 is a maternally inherited protein, the function of Ezh2 in preimplantation development is difficult to address.

To overcome these difficulties, Surani and colleagues [50•] recently used a conditional knockout strategy and examined the role of Ezh2 in mouse preimplantation development. They found that Ezh2 and its associated HMTase activity differentially mark the pluripotent epiblast cells and the differentiated trophectoderm. Using a

transgenic line that expresses Oct3/4–GFP as a mark for pluripotent cells, they found that Ezh2 and 3mK27 were enriched in GFP-positive pluripotent cells. Significantly, Cre-mediated deletion of Ezh2 resulted in loss of H3-K27 methylation in pluripotent epiblast cells. These results strongly suggest that Ezh2 is important for maintaining the epigenetic modification patterns of pluripotent epiblast cells.

Consistent with Ezh2 and H3-K27 methylation having a direct role in regulating Oct3/4 expression, Ezh2, 3mK27 and mPC3, the mouse homolog of *Drosophila* Polycomb, have been found to associate with the Oct3/4 promoter in differentiating embryonic P19 cells (N Feldman *et al.*, unpublished). In addition, 3mK9 and HP1 are also found on the Oct3/4 promoter. Notably, on differentiation induced by retinoic acid, the level of Oct3/4 has been found to decrease markedly, concomitant with an increase in the association of Ezh2, 3mK27 and mPC3 with the Oct3/4 promoter (Figure 1c).

Role of the EED–EZH2 complex in cancer

In addition to the processes discussed above, another well-known biological process that PcG proteins participate in is cellular proliferation. Given the connection between dysfunction of cellular proliferation and cancer, it is not surprising that the deregulation of PcG proteins, particularly components of the human PRC1 complex, results in cancer [19,20]. In addition to PRC1 components, several recent studies have provided substantial evidence that links dysfunction of the EED–EZH2 complex to cancer.

Initial suggestions that EZH2 is involved in cell proliferation came from the observation that EZH2 is preferentially expressed in proliferating, but not resting, mantle cell lymphoma cells [60]. Significantly, forced expression of EZH2 in the B-cell-derived Ramos cell line resulted in an increase in the rate of cell proliferation, suggesting that EZH2 levels might be related to lymphoma [60]. In a cDNA microarray analysis comparing gene expression patterns in benign tumor, organ-confined and androgen-refractory metastatic prostate cancer, EZH2 was found to be overexpressed in metastatic prostate cancer [61•].

Consistent with a role of EZH2 in cell proliferation, knockdown of EZH2 expression mediated by short interfering RNA inhibited cell proliferation in prostate cells. EZH2-mediated transcriptional silencing requires the SET domain, which is crucial for its HMTase activity, as well as an associated histone deacetylase activity. Notably, clinically localized prostate cancers expressing higher levels of EZH2 were found to have a poorer prognosis, suggesting that EZH2 levels might be a potential biomarker for predicting the relative risk in individuals with prostate cancer [62].

Several groups have extended this type of study to breast cancer and found that the EZH2 level directly correlates with the aggressiveness of breast cancer [63•,64•]. An increase in EZH2 seems to be the cause of the aggressiveness, because forced EZH2 expression in immortalized human mammary epithelial cell lines promotes anchorage-independent growth and cell invasion. Similar to the findings for metastatic prostate cancer, EZH2-mediated breast cell invasion requires an intact SET domain [63•]. In addition to EZH2, another component of the EED–EZH2 complex, Suppressor of Zeste 12 (SUZ12), is overexpressed in several human tumors, including tumors of the colon, breast and liver [65].

How does overexpression of the components of the EED–EZH2 complex promote tumor progression? Although the exact mechanism is still not known, data suggest that both EZH2 and SUZ12 are under the control of the Rb–E2F pathway [64•,66]. Because many E2F target genes encode proteins that are essential for controlling cell proliferation, it is possible that upregulation of EZH2 and SUZ12 results in misregulation of the downstream targets of the EED–EZH2 complex, leading to uncontrolled cell proliferation (Figure 1d). Although H3-K27 methylation has not been analyzed directly in these studies, we predict that overexpression of EZH2 and SUZ12 may alter normal levels of H3-K27 methylation on target genes of the EED–EZH2 complex.

Our prediction is based on the following observations. First, an intact SET domain, which is required for the HMTase activity of the EZH2 complex, is also required for its ability to promote cell proliferation. Second, EZH2 is known to function in a protein complex, and EZH2 alone does not have HMTase activity. Overexpression of EZH2 may result in formation of a nonfunctional EZH2 complex, leading to the upregulation of some EZH2 target genes. Notably, whereas the upregulation of both EZH2 and SUZ12 has been linked to cancer, similar observations regarding EED have not been reported. On the contrary, studies of a null *eed* allele in mice ($eed^{3354/+}$) suggest that EED negatively regulates cell proliferation of both lymphoid and myeloid progenitor cells [67]. Furthermore, recent studies indicate that Eed regulates thymocyte differentiation and suppresses the development of carcinogen-induced T cell lymphomas [68].

The above results highlight the importance of coordinated regulation of components of the same complex for the formation of a productive functional complex. Clearly, disruption of the stoichiometry of the complex, either through overexpression of EZH2 and SUZ12 or through downregulation of EED, can result in uncontrolled cell proliferation and cancer. Although substantial evidence indicates that there is a link between the

EED–EZH2 complex and cancer, two important questions remain unanswered. First, is overexpression of EZH2 and SUZ12 the cause or a consequence of uncontrolled proliferation and cancer metastasis? Although studies in cell culture support the former possibility, it is important to demonstrate in a transgenic mouse model that overexpression of EZH2 in prostate or breast cells actually causes cancer or promotes cancer progression. Second, how does misregulation of the EED–EZH2 complex promote cancer progression? Identification of EED–EZH2 target genes that have a role in cell proliferation will help answer this second question.

Conclusions

We have summarized recent progress in our understanding of different biological processes, including PcG silencing, X-inactivation, germline development, stem cell pluripotency and cancer metastasis, in which the E(Z) or EZH2 complex and its associated HMTase activity participate. Although the relative importance, degree of participation and underlying mechanism used may vary, these processes all are mediated through transcription regulation. The HMTase activity of the related complex is an important factor for its transcriptional regulation capability, but other protein factors or enzymes, such as histone deacetylase, that the protein complexes recruit certainly contribute to the silencing function of the ESC–E(Z) or EED–EZH2 complex.

With regard to the HMTase activity of E(Z) and EZH2 complexes, much *in vivo* evidence from several laboratories supports the idea that H3-K27 is a target and that its methylation has functional consequences. Whether or not H3-K9 is also a target for E(Z) and EZH2 complexes needs to be addressed *in vivo*, along with the potential functional links, if any, that exist between 3mK9 and E(Z) and EZH2 complexes. The available data indicate that the mechanism by which E(Z)- and EZH2-mediated H3-K27 methylation leads to gene silencing may vary among gene targets and among organisms (Figure 1).

In the case of *Hox* gene silencing, the E(Z) and EZH2 complexes may cooperate with the PRC1 complex. A similar mechanism may be used to silence Oct3/4 during the regulation of stem cell pluripotency. In mammalian X-inactivation and worm germline development, however, the EZH2 and MES-2 complexes may function independently of PRC1. The exact mechanism by which the EED–EZH2 complex functions in cancer metastasis still remains to be determined. Identifying the downstream target genes and understanding how E(Z) and EZH2 complexes are recruited to these target genes, which ultimately mediate the specific biological functions of the complexes, are challenges for the future. Given the rapid progress in the field, we expect exciting discoveries to surface in the years to come.

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