Embryonic stem (ES) cells are derived from the inner cell mass of the preimplantation embryo and are pluripotent, as they are able to differentiate into all cell types of the adult organism. Once established, the pluripotent ES cells can be maintained under defined culture conditions, but can also be induced rapidly to differentiate. Maintaining this balance of stability versus plasticity is a challenge, and extensive studies in recent years have focused on understanding the contributions of transcription factors and epigenetic enzymes to the "stemness" properties of these cells. Identifying the molecular switches that regulate ES cell self-renewal versus differentiation can provide insights into the nature of the pluripotent state and enhance the potential use of these cells in therapeutic applications. Here, we review the latest models for how changes in chromatin methylation can modulate ES cell fate, focusing on two major repressive pathways, Polycomb group (PcG) repressive complexes and promoter DNA methylation.

Embryonic stem (ES) cells have the ability to self-renew, producing daughter cells with equivalent developmental potential, or to differentiate into more specialized cells. ES cells are derived from the inner cell mass of the preimplantation embryo and are pluripotent, as they are able to differentiate into cells of the three germ layers, both in vitro and in vivo (Evans and Kaufman, 1981; Thomson et al., 1998). Three transcription factors, OCT4, SOX2, and NANOG, cooperate to ensure the self-renewal and pluripotency of ES cells (Boyer et al., 2005; Loh et al., 2006). These factors are highly expressed in undifferentiated ES cells and physically interact with each other in large protein complexes (Wang et al., 2006; van den Berg et al., 2010). OCT4, SOX2, and NANOG are transcriptionally interconnected and co-occupy promoters of actively transcribed genes that promote ES cell self-renewal such as KLF4 (Boyer et al., 2005; Loh et al., 2006; Kim et al., 2008). They also occupy genes encoding a large set of developmental regulators that are silent in ES cells, but whose expression is associated with lineage commitment and cellular differentiation (Fig. 1; Boyer et al., 2005; Loh et al., 2006).

The fact that the three key regulators can activate some genes and repress others is thought to be caused by the chromatin packaging in ES cells that are regulated by epigenetic factors. Epigenetics refers to heritable changes in gene expression that are independent of nucleotide sequence. This is achieved by regulating gene activity through alterations of chromatin structure, such as posttranslational modifications of the histones and DNA methylation, which can be either permissive or restrictive for transcription. These changes are catalyzed by histone and DNA modification enzymes that work in coordination to coregulate the balance between pluripotency and lineage-specific differentiation. The question is how these multiple regulatory mechanisms are coordinated to control the transcriptional state of pluripotent versus developmental genes in ES cells and during in vitro differentiation of ES cells. In this Review, we describe recent advances in the understanding of the role of the repressive epigenetic marks deposited by Polycomb group (PcG) repressive complexes and DNA methyl transferases (DNMTs) on ES cell self-renewal and differentiation.

PcG proteins in ES cells
Genome-wide approaches revealed that the transcriptionally silent developmental genes targeted by OCT4, SOX2, and NANOG in ES cells are...
also occupied by PcG repressive complexes (Boyer et al., 2006; Lee et al., 2006). PcG proteins facilitate maintenance of cell states through gene silencing and were first identified in *Drosophila* as a result of their essential roles as repressors of body patterning genes, including *homeobox* (HOX) genes during fruit fly development (Lewis, 1978; Struhl, 1981). HOX genes are expressed in distinct domains along the body axis and act to give cells of diverse tissues their unified regional cell identities.

PcG proteins act as multimers in two main protein complexes, Polycomb repressive complex 1 (PRC1) and PRC2, which are discriminated according to their biochemical functions and compositions. The three PcG proteins EED, SUZ12, and EZH2 are part of PRC2, which catalyzes di- and tri-methylation of histone H3 lysine 27 (H3K27me2/me3; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). Although the direct function of H3K27me3 is not fully understood, the modification correlates with transcriptional repression, and it can function as a docking site for PRC1, which catalyzes the monoubiquitinylation of histone H2A at lysine 119 (H2AK119Ub1; Cao et al., 2002; Min et al., 2003; de Napoles et al., 2004). In vitro studies suggested that H2AK119Ub1 blocks RNA polymerase II activity, thereby leading to transcriptional repression (Stock et al., 2007). Interestingly, the mechanism by which PRC1 represses transcription does not involve a block of RNA polymerase II recruitment to target genes, but rather attenuation of RNA polymerase II elongation (Stock et al., 2007). The H3K27me3 mark can also attract PRC2 itself, and the H3K27me3 mark is heritably transmitted to daughter cells as a self-perpetuating mark to maintain specific gene expression programs (Hansen et al., 2008; Margueron et al., 2009). Additionally, both PRC1 and PRC2 can mediate repression by direct chromatin compaction as a result of alternative subunit compositions (Francis et al., 2004; Margueron et al., 2008; Eskeland et al., 2010), and PRC1-mediated H2AK119Ub1 can occur in the absence of H3K27me3 (Leeb et al., 2010), suggesting several alternative mechanisms leading to PcG-mediated repression.

The PcG proteins are required for early mammalian embryo development (O’Carroll et al., 2001; Wang et al., 2002; Voncken et al., 2003; Pasini et al., 2004), but not for maintaining ES cell pluripotency. PcG mutant ES cells can still self-renew, maintain normal morphology, and express OCT4, SOX2, and NANOG (Pasini et al., 2007; Chamberlain et al., 2008a; Leeb et al., 2010). Moreover, although the PcG knockout ES cells do not differentiate efficiently into the three germ layers, they can still contribute to their formation, in vivo and in vitro (Pasini et al., 2007; Chamberlain et al., 2008b; Leeb et al., 2010). However, loss of individual PRC components in ES cells does lead to marginally increased expression of various lineage-affiliated genes and unscheduled differentiation (Morin-Kensicki et al., 2001; Pasini et al., 2007; Chamberlain et al., 2008b), an effect that is even more pronounced in ES cells carrying targeted deletions of both PRC1 and PRC2 genes (Leeb et al., 2010).

Genome-wide studies of PRC1 and PRC2 in ES cells have shown that they target promoters of >2,000 genes, of which a large subset overlaps with target genes of OCT4, NANOG, and SOX2 (Boyer et al., 2006; Lee et al., 2006). Further studies on the chromatin landscape in ES cells revealed that virtually all of these sites of PcG activity contain large regions of the repressive H3K27me3 modification and are strongly enriched in the activation-associated H3 lysine 4 trimethylation (H3K4me3) mark around the transcriptional start site (Bernstein et al., 2006; Guenther et al., 2007; Mikkelsen et al., 2007; Zhao et al., 2007). This mark is mediated through the SETD1 and/or MLL methyltransferase complexes (Miller et al., 2001; Rogov et al., 2001; Milne et al., 2002; Nagy et al., 2002), which are chromatin-activating factors that generally antagonize PcG silencing.

These genomic regions with opposing modifications have been termed “bivalent domains” and are proposed to silence developmental regulators while keeping them “poised.” Bivalent domains are not, however, exclusive to pluripotent cells; they can also be found in cells of restricted potency, including T cells and fibroblasts, where genes are unlikely to be poised in preparation for subsequent activation (Roh et al., 2006; Barski et al., 2007; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007; Cui et al., 2009). However, whether bivalent domains are functionally important is thus far not known. Genes carrying H3K4me3 modifications are highly common among ES cells and T cells, whereas H3K27me3 modification patterns do not overlap between ES cells and T cells (Zhao et al., 2007), indicating that PcG-mediated histone methylations show a higher level of tissue specificity than H3K4me3 marks. Interestingly, recent data indicate that
the presence of H3K4me3 mark might not itself be indicative or predictive of transcriptional activity (Guenther et al., 2007), and that the H3K4me3 mark is found on genes in the absence of sequence-specific activators in ES cells and without the stable association of RNA polymerase II (Thomson et al., 2010; Vastenhouw et al., 2010); thus, the significance of genome-wide marking of promoters by H3K4me3 is not clear at this moment. This means that the ability of the bivalent mark to silence lineage-specific gene expression is likely caused by the dominant effect of the H3K27me3 over the H3K4me3 mark, yet preserves the potential for rapid gene activation upon differentiation of stimuli-induced removal of the H3K27me3 mark.

Cell fate specifications in mammals permit the formation of ~200 different cell types consistent with a multitude of different combinations of the epigenome in mammalian development. ES cellular differentiation entails loss of pluripotency and parallel gain of first lineage-specific and, ultimately, cell-type specific characteristics. The process of tissue fate specification is initiated by signaling molecules that drive the dynamic equilibrium of ES cells toward a particular lineage. As some results suggest that the repressive H3K27me3 mark can be heritably transmitted to daughter cells to maintain specific gene expression programs (Hansen et al., 2008; Margueron et al., 2009), the expression of developmentally regulated genes would require the removal of the H3K27me3 mark. The UTX and JMJD3 genes encode H3K27me3/me2 demethylases, suggesting a mechanism by which PcG-repressed promoters are activated (Agger et al., 2007; De Santa et al., 2007; Lan et al., 2007; Lee et al., 2007b). For example, UTX is absent from the transcriptionally silent HOXA9 gene promoter in ES cells, but is present in this region in primary fibroblasts, in which HOXA9 gene is expressed (Lan et al., 2007). In embryonic carcinoma cells undergoing differentiation, the removal of the H3K27me3 mark to permit the activation of gene expression is associated with recruitment of the UTX histone demethylase to promoters during the transcriptional activation (Agger et al., 2007). Interestingly, UTX is associated with the H3K4 methyltransferases MLL2–4, whereas JMJD3 has not been reported to bind to any of the MLL proteins or the H3K4 methyltransferase SETD1 (Agger et al., 2007; Cho et al., 2007; Issaeva et al., 2007; Lee et al., 2007a). In a similar fashion, PRC2 can interact with and recruit a histone H3K4 demethylase (Pasin et al., 2008), thereby coordinating the removal of an “active” mark with the deposition of a repressive mark. Importantly, PcG proteins are also recruited to promoters of non–ES cell–specified PcG target genes in response to differentiation signals, and this recruitment is required for the silencing of these genes during differentiation (Pasin et al., 2007; Mohn et al., 2008; Oktaba et al., 2008; Ezskhova et al., 2009). Moreover, some ES cell–specific genes such as NANTOG are marked by H3K27me3 during differentiation (Pan et al., 2007; Hawkins et al., 2010). This means that when cell lineage commitment occurs, pluripotency transcription factors are silenced, whereas the appropriate regulators of development lose PcG-mediated repression and are activated (Fig. 2). By this mechanism, which reduces the likelihood of inappropriate reactivation of stem cell–specific or lineage-unrelated genes, the PcG repressive complexes are believed to contribute to the robustness of differentiation. In support of this view, PcG-deficient ES cells can enter differentiation, but fail to maintain the differentiated phenotype (Pasin et al., 2007; Leeb et al., 2010).

Figure 2. Dynamic recruitment of PcG proteins to chromatin during lineage specification. In ES cells, differentiation and development-promoting genes (Dev. A, B, and C) are repressed by bivalent domains, whereas late differentiation genes are not marked by H3K27me3, but not expressed. Pluripotency genes such as OCT4 are methylated at H3K4 and expressed. Differentiation signals generate cells committed to various somatic lineages, and activate lineage-specific genes that lose the repressive H3K27me3 mark (Dev. A). However, many genes preserve the bivalent domains and are not expressed (Dev. B and C); a few of these genes (e.g., those that are selectively expressed in other somatic cell lineages) also gain promoter DNA methylation during lineage commitment to ensure silencing (Dev. C). Late differentiation genes become marked by H3K27 in a manner dependent on the particular committed cell type, resulting in the formation of new bivalent domains that may be resolved in more mature differentiated cells. Examples of the aforementioned dynamics during neuronal differentiation of ES cells are NEUROG1, encoding for a neurogenic transcription factor (Dev. A); GATA4, encoding for an endodermal marker (Dev. B); TPARP, encoding for a germine-specific polyadenylate polymerase (Dev. C); and SCN18, encoding for a neuronal voltage-gated sodium channel (late diff. gene; Mohn et al., 2008). The population of de novo DNA-methylated genes is also enriched in pluripotency-specific genes such as OCT4, ensuring the stable repression of transcripts that are required for ES cell maintenance.
to ensure proper PcG recruitment to and dissociation from chromatin. Because the PcG proteins themselves do not have the ability to bind DNA-specific sites, recruitment is believed to require the interaction with sequence-specific transcription factors. The availability of these transcription factors or competing transcription factors may be involved in regulating the sustained binding of PcG proteins to their target genes. In Drosophila, the PcG proteins are recruited to Polycomb repressive elements (PREs). These elements are stretches of DNA of >1,000 bp containing DNA-binding sites for different transcription factors. Several Drosophila transcription factors have been shown to bind to the PREs and to be required for the recruitment of the Drosophila PcG proteins to target genes. Importantly, however, the results obtained so far suggest that no single transcription factor is sufficient to recruit the PcG proteins, but that a combination of transcription factors is required. Moreover, only one of the PRE-associated Drosophila transcription factors is conserved in mammalian cells (YY1 in mammals, PHO in Drosophila), and YY1 binding in mammals does not overlap with PcG target genes (Squazzo et al., 2006).

PREs have not been mapped in mammalian cells, despite genome-wide PRC-binding maps (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006), though a region between two HOX genes might target PcG proteins to a reporter gene in human cells (Woo et al., 2010). In addition to YY1, it has been speculated that OCT4 could be involved in PcG recruitment (Wang et al., 2006; van den Berg et al., 2010), though these studies have not been independently confirmed.

Recent results have, however, shed new light on PcG protein recruitment in ES cells (Fig. 3). Five independent studies identified the Jumonji C (JmjC) protein JARID2 as a novel component of PRC2, displaying a significant overlap with PRC2 target sites in ES cells (Peng et al., 2009; Shen et al., 2009; Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010). JmjC domains are characteristic of lysine demethylases, but JARID2 is unusual among the JmjC proteins in that it lacks crucial residues for cofactor binding and is catalytically inactive (Cloos et al., 2008). JARID2, which can bind directly to DNA, cofractions with PRC2 in high molecular weight complexes, and localization of PRC2 to its respective target sites is dependent on JARID2. It remains unclear whether JARID2 regulates the intrinsic histone methyltransferase activity of the PRC2 complex because the five different studies reached different conclusions on this point and major global reductions in H3K27me3 levels were not detected in ES cells lacking JARID2. Importantly, however, JARID2 was shown to be required for the execution of differentiation pathways in ES cells (Shen et al., 2009; Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010). In addition to lack of recruitment of PRC2, this effect might be caused by a role of JARID2 in recruiting PRC1 and poised RNA polymerase II to PcG target genes (Landeira et al., 2010).

PRC2 also interacts with the protein MTF2 (also named PCL2; Shen et al., 2009; Li et al., 2010). MTF2 is one of three mammalian homologues of Drosophila Polycomb-like, which can stimulate PRC2 activity in Drosophila (Nekrasov et al., 2007). Interestingly, MTF2 was recently found in a screen for novel regulators of ES cell self-renewal and shown to be involved in recruiting PRC2 to a subset of PcG target genes in ES cells (Walker et al., 2010). Whether JARID2 and MTF2 are both found in a subset of PRC2 complexes is unclear, but coimmunoprecipitation studies indicate that JARID2 and MTF2 can reside in distinct PRC2 subcomplexes. These results highlight the importance of dissecting the composition and activities of different PRC2 complexes and their associated “recruiters” to fully understand how PcG proteins control thousands of genes in many different cellular and developmental contexts. For example, JARID2 expression declines rapidly as ES cells differentiate (Boyer et al., 2005), and other proteins must therefore regulate recruitment of PRC2 to target genes during differentiation. Strong candidates for this include Figure 3. Potential mechanisms of PRC2 recruitment to target genes. PRC2 is recruited to target genes by a combination of transcription factors and ncRNAs. A fraction of PRC2 associates with JARID2, which is required for PRC2 binding to its target genes in ES cells. JARID2 might therefore represent a core component of PRC2 in ES cells, although other PRC2 complexes exist; these contain MTF2, PHF1, and other uncharacterized factors that could represent alternative targeting mechanisms operative both during ES cell self-renewal and differentiation. Sequence-specific transcription factors (TF) and ncRNA might also recruit the PRC2 core complex to target genes during differentiation. Finally, PcG target genes are CpG-rich, and proteins binding to CpG elements such as TET1 or the histone demethylase FBXL10 might have a role in recruiting Polycomb to target genes. For additional information on the components of PRC1 and PRC2 complexes, see Morey and Helin (2010).
Recent advances suggest that noncoding RNAs (ncRNAs) also play a role in the recruitment of PcG complexes in ES cells. DNA microarray analysis showed that short ncRNAs (<200 nt) were transcribed from the 5’ end of several hundred PcG target genes in ES cells (Kanhere et al., 2010). Interestingly, these ncRNAs interact with PcR2 and are involved in stabilizing PcR2 association with chromatin. Moreover, the ncRNAs were depleted from PcR2 target genes that are derepressed during cell differentiation (Kanhere et al., 2010). This indicates that short ncRNAs might function as the interface between DNA and specific chromatin remodeling activities, though the importance of direct base pairing at specific sequence motifs is still unknown. Long ncRNAs (lincRNAs; transcripts longer than 200 nt) have also been reported to recruit PcR2 to specific targets, including the HOXD cluster and the inactive X chromosome (Rinn et al., 2007; Pandey et al., 2008; Zhao et al., 2008). In addition, lincRNAs are now emerging as a new class of noncoding transcripts, with >3,000 members of which 20% might associate with PcR2 (Guttman et al., 2009; Khalil et al., 2009). Importantly, lincRNAs show dynamic patterns of expression with suggested roles in cell fate choices, but the mechanisms by which these trans-acting ncRNAs recruit PcG complexes to specific sites is not fully understood. Interestingly, the lincRNA HOTAIR, was recently shown to target both PcR2 and the H3K4me2 demethylase LSD1 to hundreds of genes thereby acting as a modular scaffold (Tsai et al., 2010).

Collectively, these studies demonstrate that both DNA-binding factors and ncRNAs can guide de novo histone modification by PcG proteins and establish repressive chromatin domains.

DNA methylation in ES cells

A second major repressive epigenetic pathway is mediated by DNMTs. Methylation of cytosine residues in CpG dinucleotides in promoter regions catalyzed by DNMTs is an important epigenetic mark that maintains long-term repression by controlling DNA accessibility (Bird, 2002). DNMTs are essential for embryonic development (Okano et al., 1999), whereas they are not required for self-renewal or genomic integrity of ES cells (Tsutsumi et al., 2006). This probably reflects the fact that blastocysts undergo global demethylation immediately before the derivation of ES cells from blastocysts (Mayer et al., 2000).

Promoters can be classified according to whether they contain high or low CpG content within a certain region around the transcriptional start sites. These distinct promoter classes show differences in their methylation levels and in the mechanisms through which they are regulated. In ES cells, high CpG promoters have low DNA methylation levels, whereas low CpG promoters have relatively high DNA methylation levels (Fouse et al., 2008; Meissner et al., 2008; Mohn et al., 2008). CpG-rich promoters—almost by default—appear to associate with nucleosomes carrying the H3K4me3 mark. Some of these regulate constitutively expressed housekeeping genes, but others corresponding to developmental regulators also contain the H3K27me3 mark. This observation raises the question of why these poised, yet inactive, CpG-rich promoters are protected from DNA methylation. Recent data showed that the CPF1 protein has affinity for nonmethylated CpG islands, and because CPF1 associates with the H3K4 methyltransferase SETD1 it leads to H3K4 trimethylation of CpG-rich promoters (Thomson et al., 2010). As some DNMT domain–containing proteins bind to unmethylated, but not methylated, H3K4 and recruit DNMTs (Ooi et al., 2007), this SETD1-induced H3K4 methylation may protect both active and inactive CpG-rich promoters from DNA methylation. Methylated low CpG promoters are marked neither by H3K4me3 nor by H3K27me3 and are mostly repressed in ES cells (Fouse et al., 2008; Meissner et al., 2008; Mohn et al., 2008).

The conversion of ES cells into somatic cells only leads to modest changes in DNA methylation at promoter regions and the majority of promoters maintain their methylation levels during differentiation (Lagarkova et al., 2006; Fouse et al., 2008; Meissner et al., 2008; Mohn et al., 2008; Hawkins et al., 2010). These observations question the importance of DNA methylation as a controlling element of cellular differentiation. However, a small number of genes do become methylated during development, an event that is accompanied by silencing of the associated promoters (Lagarkova et al., 2006; Fouse et al., 2008; Meissner et al., 2008; Mohn et al., 2008; Hawkins et al., 2010). DNA methylation is thought to keep such genes silent in differentiated cells and prevent their aberrant reactivation and the risk of de-differentiation (Fig. 2). In line with this hypothesis, in vitro differentiation of DNMT mutant ES cells leads to rapid apoptotic cell death (Panning and Jaenisch, 1996), reflecting the importance of DNA methylation in differentiated cells, which is also described for other cell types (Jackson-Grusby et al., 2001).

As a consequence it is a prerequisite that some promoters of stem cell genes are demethylated during reprogramming to enable reacquisition of pluripotency (Simonsson and Gurdon, 2004; Takahashi and Yamanaka, 2006). This demethylation may occur through a DNA repair process (Bhutani et al., 2010; Hajkova et al., 2010), or involve the recently isolated enzymes of the TET family that can convert methylated cytosine to hydroxymethylated cytosine (Tahiliani et al., 2009).

Concluding remarks

Progress in the past few years has greatly enhanced our understanding of epigenetic control of ES cells. Epigenetic factors appear to be essential for regulating cell fate decisions and maintaining the cellular state of differentiated cells. Surprisingly,
however, the results so far have suggested that epigenetic regulation may be dispensable for maintaining ES cell identity. Epigenetic mechanisms of gene silencing contribute to the overall stability of pluripotency, but are downstream in this setting. Thus, ES cell identity might primarily be regulated by transcription factors, whereas epigenetic chromatin-based repressive and activating modifiers may serve transcriptional corepressor and co-activating functions in this process. Nevertheless, the stability of a given cell state relies on the silencing of genes encoding inducers of other cell states. For example, expression of only a few transcription factors that produce cell type–specific gene expression programs can cause cells to adopt new states (Vierbuchen et al., 2010). By setting thresholds, PcG proteins may help prevent inappropriate gene expression induced by weak activating signals and thereby limit noise-induced errors and neutralize extrinsic perturbations. DNA methylation is used to lower the chance of spurious activation, which is more likely to occur in a bivalent chromatin environment.

Our understanding of how different regulatory networks are activated to guide lineage commitment is still limited. For example, the identity of differentiation signals that lead to selective activation of genes encoding specific developmental regulators by turning off PcG repression and recruiting UTx or JMD3 remains unknown. Moreover, mechanisms of recruiting PcG proteins to PcG targets in a developmental stage-specific context by transcription factors remain to be dissected. Further characterization of epigenetic regulation will help to provide a more comprehensive view of the molecular mechanisms that govern the balance between self-renewal and lineage commitment. This advance may lead to improved protocols for directing the differentiation of ES cells toward particular lineages and for the generation of ES-like cells from somatic cells.

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