The role of Polycomb-mediated epigenetic regulation in embryonic stem cell differentiation

by

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Abstract

During mammalian development, a single founding cell must produce all of the different types of cells in the adult organism. What are the regulatory mechanisms required to coordinate the necessary gene expression networks for this process? Polycomb group (PcG) proteins are epigenetic regulators necessary for proper differentiation of cells and for mammalian development. Notably, faulty regulation of PRC2 has been associated with a broad range of cancers, suggesting that it has a critical role in maintaining cell identity. Polycomb Repressive Complex 2 (PRC2) catalyzes the post-translational histone modification H3K27me3, a histone modification associated with transcriptional repression. Although PRC2 has critical functions in lineage commitment and in mediating cell fate transitions, it has proved difficult to study its precise role in these processes since complete loss of H3K27me3 leads to an inability of embryonic stem cells (ESCs) to properly undergo directed differentiation *in vitro*. PRC2 functions with additional regulators and regulatory pathways, including PRC1, accessory PcG subunits, and DNA methylation, among others; however, we know little about how they work together to coordinate gene expression programs during lineage commitment. Thus, dissecting the function of PRC2 is critical to improve our understanding of mammalian development and disease.

Here, we analyzed gene expression and DNA methylation levels in several PRC2 mutant ESC lines that maintained varying levels of H3K27me3. We found that while a partial reduction of H3K27me3 levels allowed for proper temporal activation of lineage genes during directed differentiation of ESCs to spinal motor neurons (SMNs), genes that function to specify other lineages failed to be repressed, suggesting that PRC2 activity is necessary for regulating lineage fidelity. We also found that H3K27me3 is antagonistic to DNA methylation in *cis*. Thus, these data suggest a role for PRC2 in coordinating gene repression while protecting against inappropriate promoter DNA methylation during differentiation. Our work provides novel insights into the functional relationship between two distinct epigenetic regulatory mechanisms, as mediated by PRC2 and DNA methylation, in regulating lineage decisions during development.

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Chapter 1

Introduction

Polycomb group proteins set the stage for lineage commitment

Author contributions for Chapter 1:

Adapted by Seraphim Thornton in part from a review article written by Lauren Surface, Seraphim Thornton, and Laurie Boyer; figures by Seraphim Thornton.

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Summary

Precise control of gene expression patterns is critical for the specification of cellular diversity during metazoan development. Polycomb group (PcG) proteins comprise a class of chromatin modifiers that have dynamic and essential roles in the precise regulation of gene expression patterns during lineage commitment. How this is accomplished during mammalian development is incompletely understood. This chapter presents recent studies in embryonic stem cells (ESCs) that provide critical new insights into how PcG proteins may be targeted to genomic sites, as well as the mechanisms by which these regulators influence gene expression and multi-lineage differentiation in mammals.

Introduction

Mammalian development is a complex and intricately regulated process wherein a single totipotent cell must proliferate and differentiate to become all of the different cell types of the adult organism (Figure 1). This process involves loss of pluripotency, followed by the progressive specialization of cells toward a particular fate during differentiation. As such, during development, each cell must be sensitive to its environment and the signals it receives in order to differentiate into a particular lineage, and then it must restrict its fate by permanently shutting down gene networks that would specify other cell fates. Furthermore, cells must coordinate activation and repression of gene programs at the appropriate time and place during the differentiation process.

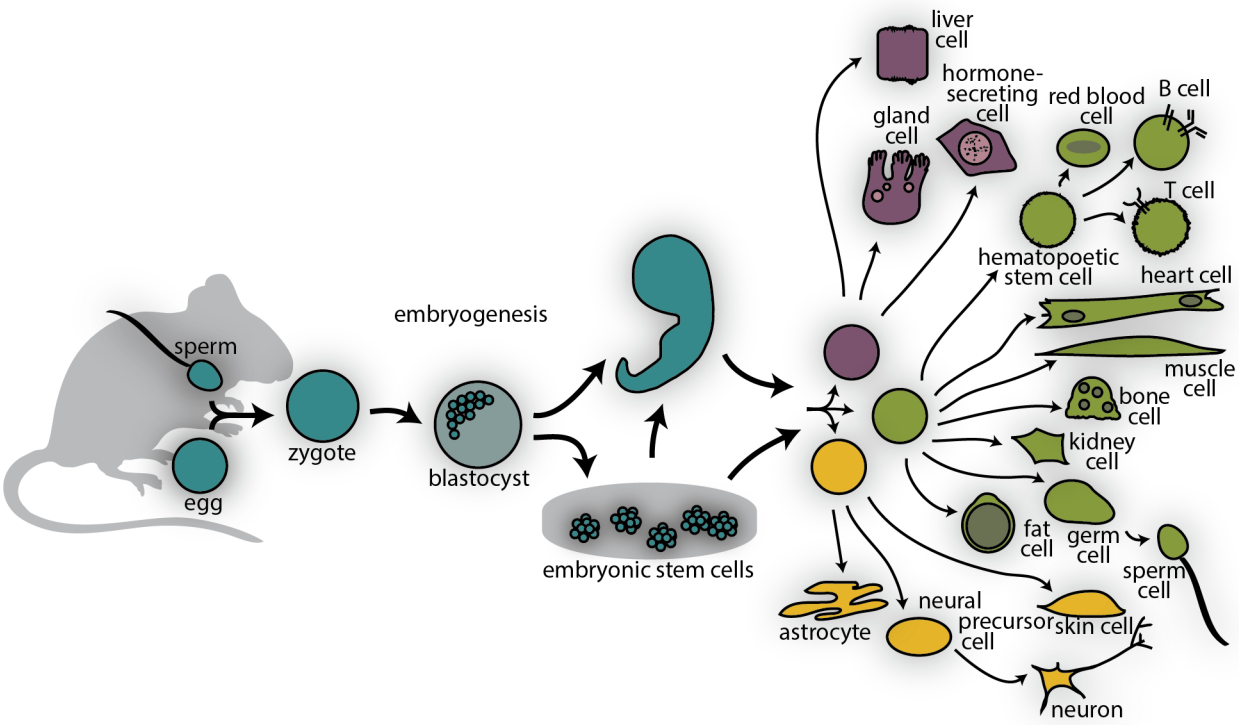


Figure 1. Pluripotent embryonic stem cells (ESCs) are derived from cells in the inner cell mass of the blastocyst. These cells can become any cell in the adult animal, some of which are represented on the right. ESCs have been widely exploited to study lineage commitment and cellular differentiation *in vitro*.

Recent work demonstrates that embryonic stem cells (ESCs) are an important model system in which to discover the core features of how cells regulate cell fate decisions in mammals (Boyer et al., 2006a; Jaenisch and Young, 2008; Loh et al., 2008; Macarthur et al., 2009; Orkin et al., 2008). ESCs, which are derived from a transient population of pluripotent cells in the blastocyst, can self-renew while maintaining the capacity to differentiate *in vivo* and *in vitro* into any cell type in the body (Keller, 2005; Murry and Keller, 2008) (Figure 1). These properties underpin the utility of ESCs as a system in which to elucidate the mechanisms that govern cell fate transitions during mammalian development.

First discovered in *Drosophila*, Polycomb group (PcG) proteins were found to be essential to the regulation of *Hox* genes and normal development (Duncan, 1982; Jürgens, 1985; Lewis, 1978). PcG proteins are now widely recognized in all metazoans for their roles in a broad range of biological processes including cell cycle control, genomic imprinting, X-inactivation, cell fate transitions, tissue homeostasis, and tumorigenesis (Martinez and Cavalli, 2006; Piunti and Pasini, 2011; Pontier and Gribnau, 2011; Prezioso and Orlando, 2011; Surface et al., 2010; Wu and Bernstein, 2008). The link between PcG proteins and cancer is notably strong; cancerous cells frequently show misregulation of PcG proteins, resulting in the altered regulation of tumor suppressors and oncogenes (Bracken and Helin, 2009; Gieni and Hendzel, 2009; Piunti and Pasini, 2011; Richly et al., 2011; Sparmann and van Lohuizen, 2006). Recently, PcG proteins have garnered much attention as modulators of stem cell differentiation in mammals (Bracken and Helin, 2009; Pietersen and van Lohuizen, 2008; Schuettengruber and Cavalli, 2009). Interestingly, PcG proteins are not necessary for the maintenance of self-renewal in ESCs, perhaps reflecting their unique cell cycle properties, including a shortened G1 and an extended S phase (White and Dalton, 2005). Rather, PcG proteins are thought to prepare ESCs for lineage commitment by temporal control of the expression of a key set of developmental gene programs (Pietersen and van Lohuizen, 2008), but we currently have an incomplete understanding of this process.

Thus, improved knowledge of how PcG proteins function in regulating gene expression has practical applications for understanding development in mammals, for designing new methods to control the differentiation of stem cells for patient-specific therapies, and for cancer therapeutics.

Polycomb Repressive Complexes catalyze histone modifications

PcG proteins comprise a class of transcriptional repressors that are found in multi-subunit complexes termed Polycomb Repressive Complexes (PRCs). Most PcG proteins are broadly associated with either PRC1 or PRC2. The composition of these complexes can vary in different cell types and among organisms; however, their core components are conserved between *Drosophila* and mammals (Kerppola, 2009; Levine et al., 2004; Schuettengruber et al., 2007). While PRCs are functionally distinct, both PRC1 and PRC2 catalyze covalent modification of histone proteins (Müller and Verrijzer, 2009; Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009). PRC2 (comprising core proteins EZH2, EED, and SUZ12) catalyzes di- and tri- methylation of lysine 27 on histone H3 (H3K27me2/3, a modification associated with transcriptional repression) (Cao and Zhang, 2004; Cao et al., 2002; Czermin et al., 2002; Kirmizis et al., 2004; Kuzmichev et al., 2002). PRC1 (canonically comprised of core protein families BMI1, RING1, CBX, and PHC)(Levine et al., 2002) mono-ubiquitylates histone H2A on lysine 119 (H2AK119ub) (de Napoles et al., 2004; Wang et al., 2004a). Biochemical and genetic studies also support the idea that PcG-mediated repression utilizes both catalytic and non-catalytic activities to perform distinct functions in gene silencing. How the post-translational histone modifications, termed chromatin marks, catalyzed by these complexes contribute to transcriptional regulation remains to be fully elucidated.

Loss of PRC2 activity results in embryonic lethality in mice (Faust et al., 1998; 1995; O'Carroll et al., 2001; Pasini et al., 2004), whereas inactivation of PRC1 generally results in less severe phenotypes that

Table 1: Loss of function phenotypes of selected discussed Polycomb subunits

Subunit	Phenotype *in vitro* (ESC)	Phenotype *in vivo* (mouse)	Key references
PRC2 components			
SUZ12	De-repression of target genes; global loss of H3K27me3 and-me2; decrease in EZH2 protein levels. Embryoid bodies lack proper structure.	Full KO lethal at early postimplantation stage. Developmental arrest ~E7.75, lethal soon thereafter. Truncated allele lethal between E8.5 and E10.5.	(Jung et al., 2010; Lee et al., 2006; Pasini et al., 2004; 2007)
EED	De-repression of target genes; global loss of H3K27me1, -me2, and –me3. Decrease in EZH2 protein levels. Fail to properly differentiate *in vitro*, but can contribute to chimeras.	Disrupted axial patterning; fail to properly gastrulate and to produce embryonic mesoderm. Die ~E8.5.	(Chamberlain et al., 2008; Faust et al., 1995; Montgomery et al., 2005; Shumacher et al., 1996)
EZH2	Fail to abolish H3K27me1/me3 at some genes. Fail to undergo mesoendoderm differentiation, but phenotype less severe than *Eed null*, as EZH1 is partially redundant, particularly at developmental genes.	Lethal at early postimplantation stage. Die ~E7.5-8.5.	(O'Carroll et al., 2001; Shen et al., 2008)
JARID2	Global H3K27 methylation unaffected; fail to properly differentiate.	Incompletely penetrant neural, cardiac, liver, and hematopoietic defects. Die ~E11.5-15.5, depending on strain background.	(Jung et al., 2005; Shen et al., 2009; Takeuchi et al., 1995)
PCL2 and PCL3	PCL2 knockdown: Upregulated pluripotency regulators; decrease in PRC2 recruitment; fail to properly differentiate. PCL3 knockdown: spontaneous differentiation.	PCL2 null mice are viable, but have growth defects. Incompletely penetrant posterior homeotic transformation.	(Casanova et al., 2011; Hunkapiller et al., 2012; Walker et al., 2010; Wang et al., 2007)
PRC1 components			
RING1B	Lose most H2AK119ub. ESC lines have propensity to differentiate; de-repression of target genes; abnormal embryoid body formation. Some lines can differentiate into all germ layers.	Developmental arrest in early gastrulation, similar to PRC2 components. Die ~E10.5.	(Leeb and Wutz, 2007; van der Stoop et al., 2008; Voncken et al., 2003)
RING1A	*Ring1A/Ring1B* double knockout ESCs lose ESC identity and fail to self-renew after several passages; lose all detectable H2AK119ub.	*Ring1A* null mice are viable. Anterior transformation and axial skeletal patterning abnormalities in both heterozygote and homozygote.	(del Mar Lorente et al., 2000; Endoh et al., 2008; 2012)
BMI1		Mice are viable, but with posterior homeotic transformation and severe immunodeficiency	(Akasaka et al., 2001; van der Lugt et al., 1996)
CBX family	CBX7 knockdown ESCs have upregulated development and differentiation genes, but normal levels of pluripotency markers. No early differentiation phenotype.	CBX2 null mice are viable; growth defects, homeotic transformation, increased sensitivity to retinoic acid during development	(Coré et al., 1997; Morey et al., 2012)
RYBP	Knockdown ESCs: cell proliferation defects, decreased H2AK19ub, fail to properly differentiate.	Embryonic lethal post-implantation. Defects in CNS and eye development.	(Gao et al., 2012; Pirity et al., 2005; 2007)
YY1		Embryonic lethal peri-implantation.	(Donohoe et al., 1999)
FBXL10 (KDM2B)	Knockdown ESCs: decrease in RING1B localization and H2AK119ub. Fail to properly differentiate.	Mice lacking full-length protein are perinatal lethal due to failure of neural tube closure. Defects in embryonic neural development.	(Fukuda et al., 2011; Wu et al., 2013)

manifest later in development (Akasaka et al., 2001; Coré et al., 1997; del Mar Lorente et al., 2000; Endoh et al., 2008; Takihara et al., 1997; van der Lugt et al., 1996) (Table 1). These phenotypic differences, however, likely reflect the degree of overlap in the function of PcG homologs and not their degree of importance during development (Kerppola, 2009; Leeb et al., 2010). Consistent with this idea, the PRC1 component RING1B, an E3 ubiquitin ligase, is essential for gastrulation during mouse development (Voncken et al., 2003). Thus, the precise mechanisms by which PcG proteins function *in vivo* have been difficult to dissect given their highly deleterious phenotypes.

This chapter highlights findings in ESCs and during their differentiation that reveal important new insights into the regulation of early lineage commitment in mammals by PRC1 and PRC2. In particular, I focus on the mechanisms by which these complexes are targeted to genomic sites and how they function to modify chromatin and interact with other regulatory elements to ensure that developmental gene expression patterns are faithfully executed during ESC differentiation.

PcG proteins silence a key set of developmental genes in ESCs

PRCs occupy and regulate the expression of a large cohort of developmental and signaling genes in ESCs, such as the Hox gene clusters as well as members of the *Dlx*, *Fox*, *Irx*, *Lhx*, *Pou*, *Pax*, *Sox*, *Tbx*, and *Wnt* gene families (Boyer et al., 2006b; Ku et al., 2008; Lee et al., 2006). This observation suggested that PcG proteins function in ESCs to prevent differentiation by repressing key developmental pathways. Notably, ESCs are able to self-renew and maintain the expression of key pluripotency genes in the absence of PcG proteins, with the possible exception of ESCs lacking both PRC1 and PRC2 components, or total loss of PRC1 (Chamberlain et al., 2008; Endoh et al., 2012; Leeb et al., 2010; Pasini et al., 2007; Shen et al., 2008; van der Stoop et al., 2008). Rather, RING1B- (PRC1 component) or PRC2-deficient ESCs fail to properly maintain the repression of lineage-specific genes, and subsequently fail to properly differentiate (Boyer et al., 2006b; Chamberlain et al., 2008; Leeb and Wutz, 2007; Leeb et al.,

2010; Pasini et al., 2007). These data led to the idea that PcG proteins are necessary for cell fate transitions, and that PcG-mediated repression must be dynamic because many of the target genes in ESCs maintain the potential to become either activated or silenced during differentiation.

Recruitment of Polycomb complexes to genomic targets in ESCs

The precise localization of PRC1 and PRC2 within the genome is necessary to facilitate the specific changes in chromatin and gene expression states that accompany lineage commitment. Because the core PcG proteins have no known DNA binding activity, they may require other factors for proper recruitment to target sites. While considerable prior knowledge exists in *Drosophila*, it is still poorly understood how PcG proteins recognize and bind specific regions within the billions of base pairs of DNA in mammals. In this section, recent work is presented that suggests roles for DNA binding elements, the local chromatin environment, accessory PRC components, and transcription factors in this process. Promising evidence for a role for non-coding RNA has also recently emerged, and is discussed in its own section later. These seemingly disparate pathways likely collaborate to regulate the targeting of Polycomb complexes and transcriptional fine-tuning of developmental programs in ESCs. Models for the recruitment of PRC2 and PRC1 are shown in Figure 2 and Figure 3, respectively.

Mammalian Polycomb Response Elements

In *Drosophila*, Polycomb complexes bind to specific sites in the genome called Polycomb Response Elements (PREs) (Müller and Kassis, 2006; Ringrose and Paro, 2007; Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009). PREs contain clusters of DNA binding sites for several transcription factors, whose binding can mediate the recruitment of Polycomb complexes. Despite the wealth of available sequence data generated from high-throughput analyses of PcG binding sites in mammals, an analogous mammalian PRE system has yet to fully emerge.

Recently, two studies have each identified a cell-type-specific putative mammalian PRE (Sing et al., 2009; Woo et al., 2010). The first potential mammalian PRE identified was an element regulating the expression of a hindbrain segmentation gene, *MafB/Kreisler,* which can recruit both PRC1 and, less strongly, PRC2, to induce silencing of an ectopically introduced transgene in both flies and mice (Sing et al., 2009). Importantly, Sing et al. also provided genetic evidence for *in vivo* PRE function in the mammalian hindbrain, suggesting this PRE is not an artifact of an *in vitro* assay. Secondly, a 1.8 kb element between *HoxD11* and *HoxD12* (D11.12) was identified as a potential PRE using a remarkable approach that analyzed nucleosome position, nuclease sensitivity, and H3K27me3 modification profiles across this locus during hESC differentiation (Woo et al., 2010). The D11.12 element displayed characteristics similar to *Drosophila* PREs in that it was able to repress the expression of an ectopic reporter gene. Moreover, this activity was moderately dependent on BMI1, a core component of PRC1, and to a lesser extent SUZ12, a PRC2 core component. RYBP, a known Yin yang 1 (YY1) binding protein that interacts with PRC1, also appears to modestly influence the targeting of PRC1 to the D11.12 region (Figure 3A). While consensus binding sites for YY1 – a homolog of PHO, a DNA binding factor that can recruit PcG proteins to *Drosophila* PREs (Brown et al., 1998; Wang et al., 2004b) – are found at both mammalian PREs, loss of YY1 binding sites had only a moderate effect on reporter repression. These data suggest that YY1 is not deterministic for PRC recruitment to potential PREs in mammals. Additionally, YY1 does not appear to associate or co-localize with PRC2 in mouse as it does in *Drosophila* (Li et al., 2010; Squazzo et al., 2006). While promising, future mammalian PRE studies will need to address the consequences of deleting D11.12 on *in vivo* function and on the regulation of *Hox* gene expression at the endogenous locus during ESC differentiation. A more general method for determining PcG binding sites genome-wide may still emerge from PRE studies such as these, as additional components involved in their recruitment are identified.

CpG islands and Polycomb complex recruitment

It has been suggested that CpG islands are involved in the recruitment of PRC2, as the vast majority of PRC2 binding sites in ESCs overlap with highly conserved CpG islands that are enriched for a variety of transcription factor binding sites (Ku et al., 2008; Tanay et al., 2007) (Figure 2). Indeed, exogenous regions of high GC content can recruit PRC2 in ESCs (Mendenhall et al., 2010). Factors that aid in this recruitment have also been identified: TET1, an enzyme capable of converting 5-methylcytosine to 5-hydroxymethylcytosine, and PCL3, homolog of *Drosophila* Polycomb-like, were found in recent studies to localize to CpG-rich promoters and to aid in recruiting PRC2 in ESCs (Hunkapiller et al., 2012; Neri et al., 2013a; Wu et al., 2011). More specifically, recent studies showed that unmethylated CpG islands recruit PcG proteins (Lynch et al., 2012); however, as discussed in a later section, the relationship between DNA methylation and PcG proteins is complex and still unfolding.

CpG islands have also been implicated in PRC1 recruitment. FBXL10, discussed below as a key mediator of H3K27me3-independent PRC1 recruitment, binds specifically to CpG islands, but its binding is blocked by DNA methylation (Farcas et al., 2012; Wu et al., 2013) suggesting a functional relationship between these two epigenetic regulatory pathways.

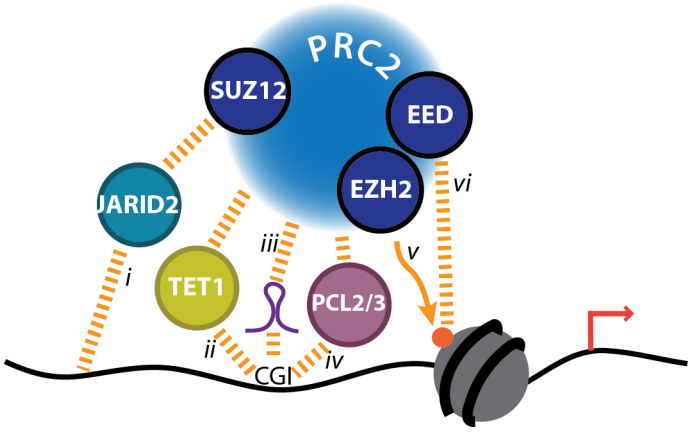


Figure 2. Model of PRC2 recruitment. i) JARID2-mediated recruitment; ii) TET1-mediated recruitment to CpG islands; iii) ncRNA-mediated recruitment to CpG islands; iv) PCL2- or PCL3-mediated recruitment to CpG islands; v) H3K27me3 catalysis; vi) H3K27me3 binding. CGI = CpG island.

Transcription Factors and Polycomb complex recruitment

In addition to GC rich sequences, transcription factors also appear to play critical roles in mediating PRC binding to genomic targets (not pictured in Figures 2 and 3 for simplicity). For example, the collective enrichment of a set of transcription factor binding sites (MYC, E2F1, ZF5, TCFCP2L1, and CTCF) predicts genomic PRC2 occupancy in ESCs with some success (Liu et al., 2010). Additionally, at the neuronal progenitor stage of differentiation, exogenous promoters with REST and SNAIL family binding sites can recruit PRC2 in a manner that is dependent upon expression of the TFs (Arnold et al., 2013). A handful of transcription factors have also been shown to aid in PRC1 recruitment, and to act in conjunction with different subunits, and thus different varieties, of PRC1. In this context, REST interaction is CBX-dependent (Ren and Kerppola, 2011); E2F6, BCL6, MAX, and MGA associate with RYBP (Gao et al., 2012; Gearhart et al., 2006; Trojer et al., 2011); and, RUNX1 associates with BMI1 (Yu et al., 2012). It is likely that PRC recruitment to specific genomic sites involves a considerably complex set of interactions. In addition to the above-mentioned factors, DNA sequence composition as well as the neighboring chromatin may also be relevant.

PRC1 recruitment to genomic targets

Studies in the past few years have shed much light on the question of how PRC1 is recruited to the genome in mammals, and provide a solid foundation for further investigation. While 6 or more varieties of PRC1 with different core components have been identified (Gao et al., 2012), for simplicity, I have grouped them into two main categories based on mechanism of recruitment: H3K27me3-dependent and –independent (Figure 3).

H3K27me3-dependent

The canonical model of PRC1 recruitment posits that the chromodomain of a CBX family component recognizes and binds to pre-existing H3K27me3 (Fischle et al., 2003; Kaustov et al., 2011; Min et al., 2003). Indeed, depletion of PRC2 does lead to a partial loss of PRC1 recruitment in mESCs (Boyer et al.,

2006b; Wu et al., 2013), and to a complete loss of recruitment of CBX7, the primary CBX family protein associated with PRC1 in ESCs. Furthermore, depletion of CBX7 leads to the derepression of a subset of developmentally relevant target genes (Morey et al., 2012). Interestingly, while H2AK119ub is catalyzed by PRC1, it is possible that the H3K27me3-dependent variety of PRC1 does not functionally contribute to this H2AK119ub (Gao et al., 2012; Morey et al., 2012; Wu et al., 2013), implicating its non-ubiquitylating functions in gene repression pathways. Notably, H2AK119ub-independent repression by PRC1 homologs has also been observed in *Drosophila* (Gutiérrez et al., 2012). Catalytic-independent functions may include the reinforcement of PRC2 recruitment, as depletion of CBX7 also leads to a reduction in SUZ12 at its genomic targets (Morey et al., 2012) and depletion of RING1B and RING1A leads to a reduction in H3K27me3 at its normal sites of enrichment (Endoh et al., 2012). Alternatively, PRC1 appears to have roles in mediating chromatin compaction independent of its catalytic activity and that this function may be mediated by its CBX subunit (Grau et al., 2011). Additionally, it is possible that H3K27me3 is responsible not for recruitment but for stabilization and maintenance of PRC1 at its target sites.

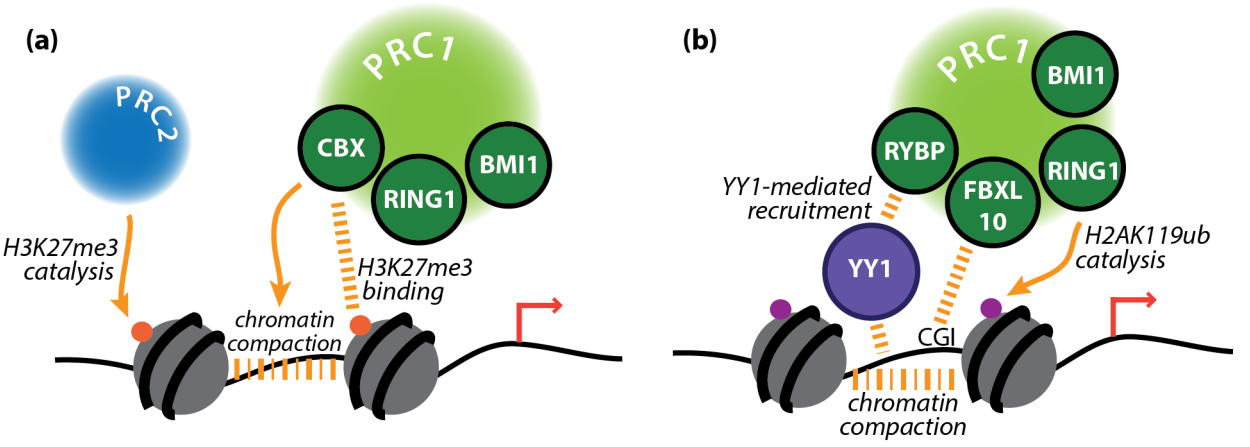


Figure 3. Models of PRC1 recruitment. a) H3K27me3-dependent PRC1 recruitment; b) H3K27me3-independent PRC1 recruitment. CGI = CpG island.

Nevertheless, the picture is probably more complex; H3K27me3 is likely not the only mechanism for PRC1 recruitment. For example, not all PRC1 co-localizes with PRC2 (Ku et al., 2008), and not all PRC1 varieties contain a CBX protein (Gao et al., 2012). Some evidence also goes as far as to suggest that H3K27me3-dependent CBX recruitment does not occur at all (Vincenz and Kerppola, 2008). Thus, additional studies will be required to dissect these critical questions.

H3K27me3-independent

Recent studies demonstrated that some types of PRC1 do not contain CBX family proteins; rather, their recruitment to genomic sites depends on subunits such as RYBP and FBXL10, independently of H3K27me3 (Gao et al., 2012; Tavares et al., 2012; Wu et al., 2013). In fact, RYBP and CBX binding to RING1B are mutually exclusive (Wang et al., 2010). Interestingly, PRC1 lacking a CBX subunit is thought to mediate the majority of H2AK119ub genome-wide (Gao et al., 2012; Tavares et al., 2012; Wu et al., 2013). Furthermore, recent findings indicate that H2AK119ub may be necessary for PRC1-mediated gene repression (Endoh et al., 2012), suggesting that H3K27me3-independent PRC1 is responsible for the majority of PRC1-mediated gene repression.

PRC2-mediated H3K27me3 is enriched at bivalent chromatin domains in ESCs

H3K27me3 is broadly associated with facultative heterochromatin and the repression of developmental programs in metazoans. An important open question is how PcG-repressed genes in ESCs maintain the potential for gene activation. A remarkable find in both mouse and human ESCs is the co-enrichment of both activating and repressive chromatin modifications at PcG target genes (Azuara et al., 2006; Bernstein et al., 2006; Pan et al., 2007; Zhao et al., 2007) (Figure 4, left). These "bivalent domains" consist of peaks of H3K4me3 enrichment (activating) that overlap with broader domains of H3K27me3 modifications (repressive) (Bernstein et al., 2006; Mikkelsen et al., 2007). This observation led to the idea that control of developmental gene expression patterns is highly

coordinated by the concerted activities of Trithorax group (TrxG) proteins, which catalyze the trimethylation of histone H3 Lysine 4

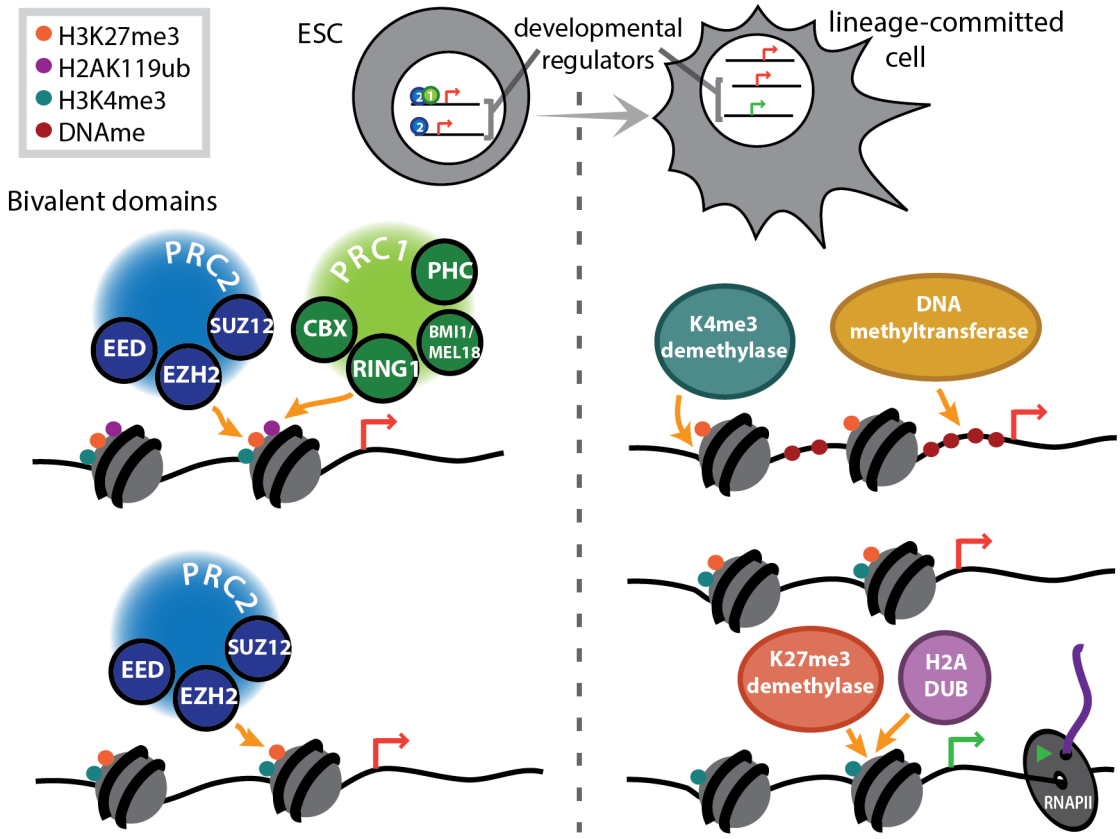


Figure 4. PcG target genes have a bivalent chromatin conformation in ESCs. Left: Bivalent domains are enriched with H3K4me3 and H3K27me3, modifications associated with TrxG and PcG activities, respectively. PRC2 consists of core subunits SUZ12, EED, and the histone methyltransferase EZH2, which catalyzes the di/trimethylation of lysine 27 on histone 3 (H3K27me2/3). PRC1 subunits comprise members of families CBX, PHC, BMI1/MEL18, and RING1. RING1B/A mono-ubiquitylates lysine 119 of H2A (H2AK119ub). In ESCs, PRC1 and PRC2 co-localize at some promoters, while only PRC2 is targeted to others. While genes are repressed in both cases, PRC1 may be primarily responsible for chromatin compaction. Right: Upon lineage commitment, many bivalent domains are resolved depending on the expression state of the gene. To stabilize the repressed state of a particular gene, DNA methyltransferases can methylate DNA near the promoter of the gene. The histone demethylases JMJD3 and UTX and possibly histone H2A deubiquitylases (H2A DUB) can allow for activation of PcG target genes by facilitating removal of the repressive H3K27me3 or H2AK119ub mark during differentiation. Some genes remain in a poised, bivalent state to allow for further lineage decisions.

(H3K4me3), and H3K27me3-catalyzing PcG proteins. Despite the overwhelming evidence that H3K4me3 by itself is associated with transcriptional initiation, bivalent genes display low expression levels. Bivalent domains are also found in other cell types, albeit seemingly less frequently (Mikkelsen et al., 2007); however, in ESCs, unlike in lineage-committed cells, the majority of H3K27me3 is associated with H3K4me3. Importantly, recent studies in Zebrafish also found co-enrichment of both H3K4me3 and H3K27me3 at a subset of genes in early embryos (Vastenhouw et al., 2010), providing strong evidence that bivalent domains are not simply an artifact of cell culture.

How do these contradictory marks in bivalent chromatin coordinate to regulate gene expression? Interestingly, PRC2 is sensitive to the local chromatin environment: its function is activated by repressive chromatin characteristics and antagonized by active chromatin characteristics. PRC2 nucleosomal binding and catalytic activity are stimulated *in vitro* (and also in *Drosophila)* by the presence of H3K27me3, to which EED binds (Margueron et al., 2009; Xu et al., 2010). The activity of the *Drosophila* PRC2-homologous complex is also augmented *in vitro* by dense chromatin (Yuan et al., 2012). This idea is supported by the observation that H3K27me3 is anti-correlated with nucleosomal spacing *in vivo* (Yuan et al., 2012). Conversely, canonically active histone marks H3K4me3 and H3K36me3 are poor substrates for PRC2 (Schmitges et al., 2011; Xu et al., 2010; Yuan et al., 2011). Collectively, these studies offer fascinating insights into the coordination and reinforcement of a changing epigenetic regulatory environment: an active chromatin environment resists becoming repressive in many ways, but once chromatin acquires some characteristics of a repressive environment, others are encouraged to follow. This balance is vital for maintaining developmental plasticity at bivalent promoters: not only are marks of both activation and repression present and performing opposite functions, but the mark of activation is actively limiting the mark of repression, thus truly keeping the gene poised for quick activation.

During differentiation, bivalent domains are generally resolved to either H3K27me3 or H3K4me3 regions, depending on the expression state of the associated gene in a given differentiated cell type (Figure 4, right). In genes that must be stably repressed upon differentiation, the binding of PcG proteins in ESCs may ultimately facilitate subsequent repression during differentiation through recruitment of a more stable silencing mechanism, such as DNA methylation (Schuettengruber et al., 2007; Simon and Kingston, 2009). Indeed, promoters associated with H3K27me3 in ESCs are more likely to become DNA methylated during differentiation (Meissner et al., 2008; Mohn et al., 2008). This transition to a more repressed state is also likely facilitated by a class of histone demethylases that selectively remove H3K4me3, consistent with their essential roles in development and differentiation (Cloos et al., 2008; Lan et al., 2008). Conversely, bivalent resolution leading to activation of genes necessary for lineage commitment may be facilitated by loss of PRCs or H3K27me3. Two histone demethylases, JMJD2 and UTX, have recently been identified as H3K27me2/me3 demethylases (Agger et al., 2007; Hong et al., 2007; Lan et al., 2007; Lee et al., 2007; Xiang et al., 2007), making them likely candidates for counteracting Polycomb-mediated gene silencing during activation of lineage specific genes. JMJD3 and UTX are necessary for proper development and differentiation in a variety of systems, including mammals, and are targeted to developmental regulators such as *Hox* genes during ESC differentiation (Swigut and Wysocka, 2007). Moreover, inactivating mutations in *Utx* have been found in multiple tumor types (van Haaften et al., 2009), suggesting that disrupting the balance in H3K27 methylation patterns can lead to changes in cell state. In support of the process of bivalent resolution, a recent study mapped the changes in four common histone marks over *in vitro* differentiation of hESCs, and noted that at a key set of pluripotency genes, promoters tended to lose H3K27ac, a mark of activation, and gain H3K27me3, while developmental gene promoters tended to undergo the opposite transition (Hawkins et al., 2011). Thus, targeted removal of H3K27me3 may be one way that cells disrupt Polycomb-mediated gene repression upon differentiation, although there

are likely other mechanisms that work in concert, mediated by signaling pathways in response to developmental cues (Cole and Young, 2008).

PRC1-mediated histone mono-ubiquitylation regulates gene expression patterns during differentiation

PRC1 is responsible for the catalysis of H2AK119ub in ESCs (Kallin et al., 2009). Deletion of *Ring1b*, an E3 ubiquitin ligase associated with PRC1, leads to widespread loss of this modification, and to gene derepression (Stock et al., 2007). Furthermore, deletion of both *Ring1b* and *Ring1a* leads to loss of all detectable H2AK119ub (Endoh et al., 2012). It should be noted that loss of RING1B also alters PRC1 integrity, suggesting that the observed gene derepression may not be a direct consequence of loss of H2AK119Ub in ESCs; however, genes normally enriched for H2AK119ub were the most derepressed upon *Ring1A/B* deletion, and these genes are enriched for functions associated with transcription and development (Endoh et al., 2012). Moreover, while a catalytically inactive RING1B seemed to function normally except for catalysis of H2AK119ub, it was not sufficient to maintain ESC identity or to repress target genes in the absence of wild-type RING1A/B (Endoh et al., 2012; Eskeland et al., 2010). Overall, H2AK119ub seems to play a pivotal role in developmental gene repression in ESCs. However, in ESCs, depletion of RYBP and FBXL10, PRC1 subunits vital to the varieties of PRC1 currently thought to catalyze the majority of H2AK119ub (Gao et al., 2012; Tavares et al., 2012; Wu et al., 2013), did not cause derepression of the handful of lineage marker genes tested (Gao et al., 2012; Wu et al., 2013). Additionally, not all PcG target genes become derepressed even upon total loss of RING1B (van der Stoop et al., 2008). This suggests the existence of additional, H2AK119ub-independent functions of PRC1 that cooperate to maintain repression of these genes.

The prevailing model posits that specific histone deubiquitylases (DUBs) have roles in counteracting PcG-mediated repression during differentiation. In support of this model, a distinct *Drosophila* Polycomb repressive complex, PR-DUB, comprising additional factors such as Additional sex combs

(Asx), has been shown to possess histone H2A de-ubiquitylase activity and to regulate *Hox* gene silencing (Scheuermann et al., 2010). Prior studies showed that Asx is necessary to maintain both homeotic gene activation and silencing (Fisher et al., 2006), indicating that additional work is needed to fully understand its role in PcG-mediated gene regulation. While *asx* homologs exist in mammals (Fisher et al., 2006), a mammalian complex similar to PR-DUB has yet to be identified. Nonetheless, there are several factors with known histone H2A DUB activity in vertebrates, including UBP-M, which is required for *Hox* gene activation and posterior development in *Xenopus laevis* (Joo et al., 2007; Weake and Workman, 2008).

In the past few years, interesting new findings have been published regarding the role of PRC1 and H2A DUBs upon differentiation of ESCs. Upon receiving signals to differentiate, embryonal carcinoma cells recruit ZRF1 in an H2AK119ub-dependent manner to activate a set of developmental genes (Richly et al., 2010). Importantly, ZRF1 both blocks the binding of PRC1 and augments the removal of the ubiquityl mark, likely by interacting with USP21, a histone DUB (Richly et al., 2010). Consistent with these findings, in ESCs, depletion of RYBP or FBXL10, and thus H2AK119ub, leads to an inability to activate key developmental regulators upon differentiation (Gao et al., 2012; Wu et al., 2013). As such, it seems likely that H2AK119ub is necessary to establish proper chromatin states for the activation of genes immediately upon loss of pluripotency.

Thus, the characterization of H2A DUBs with specific roles in counteracting PcG-mediated silencing during ESC differentiation is beginning to reveal another layer of regulation important for maintaining the balance between self-renewal and lineage commitment. Ultimately, identifying the downstream effectors of both PRC2-mediated H3K27me3 and PRC1-mediated H2AK119ub, elucidating how these modifications crosstalk in ESCs, as well as exploring histone modification-independent roles of these complexes, will be necessary to fully understand how PcG proteins function in stem cell differentiation and development.

Role of PcG proteins in regulating transcriptional machinery

Despite a growing body of knowledge regarding the roles of Polycomb-mediated gene repression, we still lack detailed mechanistic insights into how PcGs mediate this repression. Several studies have shown PcG-dependent regulation of the transcriptional machinery at various stages of the transcription process. This evidence further suggests a direct role of PcG proteins in the precise regulation of developmental genes during lineage commitment.

Several years ago, a few studies showed promising evidence for PcG-mediated "promoter-proximal polymerase pausing" – genes experiencing transcription initiation, but no productive elongation. In ESCs, most PcG target genes were shown to harbor a paused RNA polymerase II enzyme (RNAPII) (Guenther et al., 2007). These data were also consistent with the earlier finding that PcG proteins did not prevent the binding of RNAPII to promoters in *Drosophila* (Dellino et al., 2004). Interestingly, PRC1-mediated H2AK119ub was suggested to contribute to this paused state, as its loss led to RNAPII elongation and, thus, gene derepression (Stock et al., 2007). Furthermore, an analysis of different tissues in the developing *Drosophila* found that transcription was regulated through release from polymerase pausing at *Hox* genes, canonical PcG targets (Chopra et al., 2009). More recently, the H3K27-demethylase-mediated loss of H3K27me3 was shown to release RNAPII pausing, implicating PRC2 in post-initiation elongation blocking (Chen et al., 2012), and providing further support for PRC-mediated promoter pausing.

However, the emergence of new technologies such as GRO-seq (Core et al., 2008), which allows for the genome-wide mapping of RNAs in the process of being transcribed, has revealed that polymerase pausing is a more widespread occurrence, not necessarily associated with PRC-mediated repression, and that PRC1 target genes are in fact depleted of initiated RNAPII (Min et al., 2011), suggesting that

RNAPII recruitment is the key regulatory step instead of pause release. This model also has supporting evidence in *Drosophila* (Chopra et al., 2011). Regardless, whether it is PRC-mediated or not, pause escape is known to be a major point of transcriptional regulation upon loss of pluripotency and differentiation (Levine, 2011; Min et al., 2011).

Finally, PRCs may also impact transcription by binding to non-coding DNA regulatory elements such as transcriptional enhancers. Recent studies provide evidence that PRC1 blocks the binding of Mediator (Lehmann et al., 2012), which plays an important role at enhancers in addition to stabilizing the RNA polymerase pre-initiation complex (Björklund and Gustafsson, 2005; Kagey et al., 2010). A role for PRC-mediated repression at enhancers is further supported by the observation that over muscle differentiation, the *CKm* gene loses H3K27me3 at its enhancer region first, followed by gene activation and widespread loss of the histone mark (Seenundun et al., 2010).

Chromatin compaction and higher order chromatin organization do not require PRC enzymatic activity

ESCs have a characteristically open and dynamic chromatin. Upon ESC differentiation, a dramatic reorganization of chromatin structure leads to an increase in more compact and less dynamic heterochromatin and selective gene silencing (Mattout and Meshorer, 2010; Meshorer et al., 2006). Furthermore, the formation of extended H3K27me3 domains, a mark of facultative heterochromatin and gene silencing, has been observed in differentiated cells (Hawkins et al., 2010). As PcG target sites have lower histone turnover than their TrxG counterparts (associated with H3K4me3, an activating mark), PRC occupancy may stabilize chromatin (Deal et al., 2010). PcG proteins have long been thought to contribute to the formation of compact heterochromatin; however, direct evidence in favor of this model was lacking. Recent work indicates that the binding of PRCs to genomic sites might contribute to gene repression by higher order chromatin organization. For example, reconstituted PRC1 restricts chromatin-remodeling activity and can compact chromatin *in vitro* using unmodified

nucleosome templates or those that lack histone tails (Francis et al., 2001; 2004). While these studies argued that PRCs participate in the formation of higher order chromatin states in the absence of histone modifications *in vitro*, whether this phenomenon could be observed *in vivo* remained an open question.

Recent studies investigating chromatin compaction at *Hox* gene clusters in ESCs now provides *in vivo* support for the role of PcG proteins in this process. *Hox* clusters serve as a paradigm for studying the role of PcG proteins in chromatin organization and gene regulation because they are classical PcG targets and because they are temporally activated in a colinear fashion during development (Kmita and Duboule, 2003; Mallo et al., 2010). Furthermore, extensive domains of PcG proteins and H3K27me3 have been observed across all *Hox* clusters in ESCs (Boyer et al., 2006b; Ku et al., 2008; Lee et al., 2006; Mikkelsen et al., 2007), and are lost during differentiation *in vivo*, either upon lineage commitment or upon activation of transcription (Mazzoni et al., 2013; Soshnikova and Duboule, 2009). Prior observations noted a distinct nuclear reorganization of the *HoxB* cluster during ESC differentiation; however, this study did not address the role of chromatin conformation in the regulation of these genes (Chambeyron and Bickmore, 2004; Morey et al., 2007). Using DNA FISH, the same group subsequently showed striking evidence for PcG-mediated chromatin compaction at the *HoxA* and *HoxD* clusters tested in ESCs (Endoh et al., 2012; Eskeland et al., 2010) (Figure 5). Both PRC1 and PRC2 were required to maintain compact chromatin. Remarkably, the E3 ubiquitin ligase activity of Ring1b was dispensable for compaction. Moreover, an observable decompaction temporally preceded gene activation during ESC differentiation. Together, these data point to important non-catalytic roles for PRC1 in regulating higher order chromatin organization and *Hox* gene expression during early development. While the precise organization and function of this unique chromatin region is still under investigation, the role of H2AK119ub is not clear in this context, since this modification appears dispensable for chromatin compaction and gene repression. However, as

discussed earlier, in other studies, H2AK119ub appears necessary for PRC1-mediated gene repression (Wu et al., 2013). Further studies will be necessary to determine the precise roles of PRC1 in gene repression.

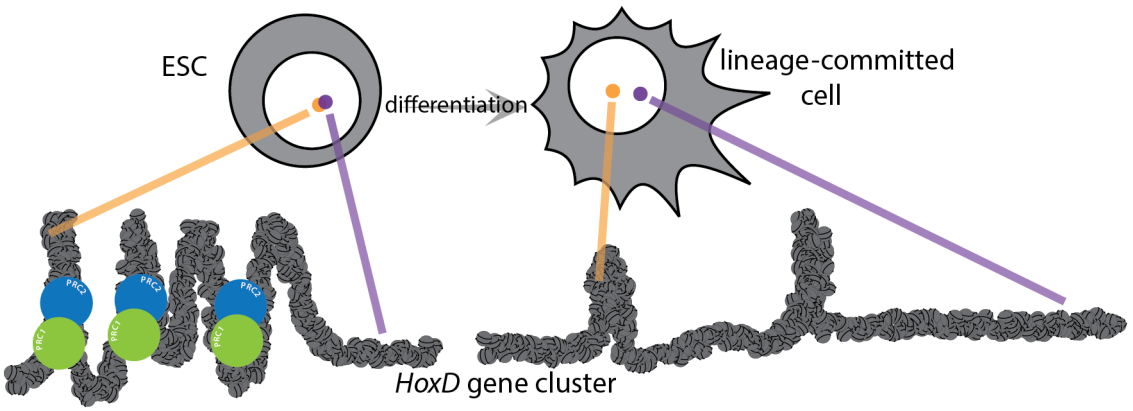


Figure 5. Polycomb group proteins mediate higher order chromatin structures. PcG proteins mediate chromatin compaction at both the *HoxB* and the *HoxD* cluster. In ESCs, the chromatin at the *HoxB* and *HoxD* clusters are compacted, while decompaction and gene activation are observed upon differentiation. Interestingly, although loss of RING1B also causes decompaction, the ubiquitin ligase activity of RING1B is dispensable.

Upon characterization of different PRC1-type complexes that perform distinct functions, chromatin compaction was subsequently found to be catalyzed by both H3K27me3-dependent, CBX-containing PRC1 (Grau et al., 2011) and H3K27me3-independent, RYBP-containing PRC1 (Gao et al., 2012), both *in vitro* and *in vivo*. In the former, the chromatin compaction was surprisingly mediated by CBX2 (Grau et al., 2011), despite it not being a homolog of *Drosophila* PSC, which is responsible for compaction in the fly (Francis et al., 2004; King et al., 2002). As compaction was not observed for either BMI1 or RING1B *in vitro* (Grau et al., 2011), it seems likely that RYBP or other non-core components mediate this activity in H3K27me3-independent PRC1. Furthermore, chromatin compaction stimulates the catalytic activity of

PRC2 (Margueron et al., 2009; Xu et al., 2010), consistent with a PRC-mediated positive feedback loop that stabilizes repression.

In addition to the role of PRC1 in chromatin compaction, EZH2, a core PRC2 component, has been implicated in the formation of higher order chromatin interactions. Long-range chromatin interactions were mapped at the PcG target gene *Gata4* in embryonic carcinoma cells (ECCs) using a high-resolution chromatin conformation capture (3C)-based assay (Tiwari et al., 2008a; 2008b). These interactions were partially lost upon knockdown of *Ezh2*, and completely lost when ECCs were induced to differentiate. However, it was unclear whether the reorganization of chromatin structure during differentiation caused the observed changes in gene expression. Given what we now know about PRC1-mediated chromatin compaction, it is possible that the changes in chromatin structure observed upon EZH2 depletion might be due to a concomitant loss of H3K27me3-dependent PRC1 at this locus. Nonetheless, these data suggested that PcG proteins may also repress gene expression in ESCs by mediating long-range chromatin interactions or DNA looping (Mateos-Langerak and Cavalli, 2008) and that this chromatin conformation may occlude access of activating factors to the DNA template.

Consistent with a role for PcG proteins in higher order chromatin interactions, recent studies have identified more examples of such interactions. Interestingly, both PRC2 and PRC1 target regions are shown to be involved in higher order chromatin interactions (Choi et al., 2011; Denholtz et al., 2013). Physical associations between PcG-repressed regions have been identified in *Drosophila* as well as mouse ESCs and fibroblasts, and these associations are cell-type specific, and both exclusive to PcG-target regions, and dependent on PcG proteins (Denholtz et al., 2013; Tolhuis et al., 2011). These data suggest that PcG target genes mutually reinforce each other's repressive state; mutations in one *Hox* cluster, with concomitant depression of long-distance contacts, can partially derepress another (Bantignies et al., 2011). It is possible that only a subset of PcG-silenced loci participate in these

interactions, as some loci do not appear to interact in a PcG-dependent manner (Li et al., 2011). If this is the case, what factors determine which loci participate in these interactions? Furthermore, it is likely that these interactions are highly dynamic and that there are many interacting loci at any given time in any given nucleus, each mediated by a different regulatory protein or complex (Ferraiuolo et al., 2010). The other possibility is that, instead of physically and purposefully binding to one another, these loci are simply aggregated spatially, thus causing more associations by simple Brownian motion. Further study is required to distinguish these models from each other.

The possibility has also been raised that maintenance of PRCs at their target sites through DNA replication is accomplished by means of a chromatin looping mechanism (Lo et al., 2012). Moreover, a recent study suggested that insulator-mediated chromatin looping brings a PRE into contact with its target region, thus allowing H3K27me3 spreading. Both of these are intriguing models that address questions that have long been pursued in the field, and provide a mechanism for linking higher-order chromatin structure to regulation of developmental gene expression.

PRC2 contains accessory components that regulate recruitment and/or catalytic activity

Five groups have identified JARID2 as a new component of PRC2 in ESCs (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009) and also in *Drosophila* (Herz et al., 2012). Jarid2 is the founding member of the Jumonji C (JmjC) domain protein family of histone demethylases, which remove methyl groups from lysine residues (Klose et al., 2006). Thus, the association of JARID2 with PRC2 is predicted to be important for the balance between gene expression states. However, JARID2 lacks key residues for cofactor binding and so it is catalytically inactive (Cloos et al., 2008). Notably, JARID2 levels are abundant in undifferentiated cells and decrease during differentiation (Mikkelsen et al., 2008; Walker et al., 2007; Zhou et al., 2007) suggesting that JARID2-PRC2 may be unique to ESCs. In further support, key pluripotency factors including OCT4,

SOX2, and NANOG occupy the *Jarid2* promoter (Boyer et al., 2005; Kim et al., 2008), connecting its expression to the ESC transcriptional regulatory network. Further data indicate that the JARID2-PRC2 complex represents a significant fraction of PRC2 in ESCs.

JARID2 can directly bind DNA through its C-terminus (Li et al., 2010), suggesting that it directly recruits PRC2 to genomic sites (Figure 2). Consistent with this idea, JARID2 co-occupied the same regions of the genome as PRC2. Moreover, PRC2 binding was diminished upon depletion of *Jarid2* (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009) and JARID2 localization was dependent on PRC2 (Peng et al., 2009; Shen et al., 2009). PRC1 was also diminished upon *Jarid2* depletion (Landeira et al., 2010; Pasini et al., 2010), but this may be a consequence of loss of PRC2 at these regions. In addition to its recruitment role, JARID2 also regulates the histone methyltransferase activity of PRC2. Contrasting studies have shown that JARID2 may potentiate (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010) or attenuate (Peng et al., 2009; Shen et al., 2009) the catalytic activity of the complex. Regardless of the differences in the results of *in vitro* assays from the different studies, the reports generally agree that depletion of *Jarid2* reduces PRC2 recruitment to the target genes more substantially than H3K27me3 levels at those targets. These observations also point to JARID2-independent mechanisms that maintain basal levels of PRC2 at genomic targets.

While *Jarid2*-deficient ESCs displayed defects in their ability to differentiate (Li et al., 2010; Pasini et al., 2010; Shen et al., 2009), *Jarid2*-null embryos proceeded further in development as compared to PRC2 mutants (Takeuchi et al., 1995) (see Table 1). While these results are consistent with the idea that JARID2 is not necessary to carry out all PRC2 function, substantial evidence supports a role for JARID2-PRC2 in regulating gene expression in ESCs; however, the precise roles of JARID2 in ESCs remain to be elucidated.

Several studies, including those discussed above, reported the identification of a PCL2/MTF2 (Polycomb-like 2/ metal response element-binding transcription factor 2)-containing PRC2 complex in mESCs (Casanova et al., 2011; Landeira et al., 2010; Li et al., 2010; Shen et al., 2009; Walker et al., 2010). *Pcl2* is one of three homologs of *Drosophila Polycomb-like* (*Pcl*), suggesting that PCL2 functions as a *bona fide* PcG protein. PCL2-PRC2 occupied a subset of PcG target genes in ESCs in a similar pattern as PRC2 (Casanova et al., 2011; Li et al., 2010; Walker et al., 2010) and appeared to promote PRC2 recruitment, perhaps more strongly than H3K27 tri-methylation (Casanova et al., 2011; Walker et al., 2010). Based on the gene expression effects observed upon *Pcl2* depletion in ESCs, the authors speculate that PCL2-PRC2 may function to regulate self-renewal to enable an appropriate response to differentiation cues. PCL2 also may bind H3K36me3 and recruit demethylases to repress genes during differentiation (Brien et al., 2012); as H3K36me3 has been shown to repress PRC2 catalytic activity, this may be an alternative mechanism for stimulating its activity. Additionally, PCL2 may play a role in recruiting PRC2 to the inactive X chromosome (Casanova et al., 2011). Notably, the *Pcl2* promoter is occupied by OCT4 and NANOG in ESCs (Loh et al., 2006), and its levels decrease upon differentiation (Walker et al., 2007; 2010). Thus, similar to *Jarid2*, *Pcl2* expression may also be connected to the pluripotency transcriptional regulatory network. While this complex may also co-purify with JARID2, evidence supports the existence of biochemically distinct PRC2-like complexes (Landeira et al., 2010; Li et al., 2010; Shen et al., 2009). Interestingly, *Pcl2* mutant mice display a phenotype more consistent with PRC1 knockouts, including posterior transformation of axial skeletons (Wang et al., 2007).

Pcl3, another *Pcl* homolog, has also been shown to have an effect on PRC2. Similarly to PCL2, PCL3 physically associates with PRC2, and its knockdown phenotype in ESCs indicates that it helps to maintain self-renewal, but that it is dispensable for pluripotency (Hunkapiller et al., 2012). PCL3 binds to a subset of PRC2 target genes and aids in PRC2 recruitment to these targets (Hunkapiller et al., 2012). While PCL3 depletion does cause some changes in gene expression, these observations are

likely due to secondary effects. Interestingly, PCL2 and PCL3 appear to associate independently with PRC2; suggesting that although their target genes overlap somewhat, they are not redundant in function (Hunkapiller et al., 2012). Indeed, PCL2 may sometimes negatively effect PRC2 binding and function, while PCL3 seems to promote activity. In sum, these data support a model in which PRC2 can contain either PCL2 or PCL3, and these subunits may function in the recruitment of PRC2 to its correct targets both in ESCs and during differentiation (Figure 2). Consistent with this idea, both PCL homologs have a binding preference for CpG-rich DNA, and may play a role in PRC2 recruitment to CpG islands.

EZH2, the catalytic subunit of PRC2, is homologous to EZH1, also an H3K27-specific histone methyltransferase (Margueron et al., 2008; Shen et al., 2008). Although EZH2 plays the predominant role in ESCs, EZH1 can partially compensate for its loss, maintaining a lower level of H3K27me3 at a subset of PRC2 targets (Shen et al., 2008). However, EZH1 has distinct functions as well. Evidence points to a role for EZH1 in chromatin compaction and silencing, and EZH1 expression increases in terminally differentiated cells, suggesting that while EZH2 mediates silencing during pluripotency, EZH1 plays the important role in later stages of lineage commitment (Margueron et al., 2008). Consistent with this notion, an *Ezh1* homolog in medaka, the Japanese rice fish, is necessary for proper body patterning during development (Arai et al., 2010). Recently, however, an opposing role for EZH1-PRC2 has been proposed: one of promoting transcriptional elongation and gene activation during cellular differentiation (Mousavi et al., 2012). As such, there is a possibility that over the course of development, certain PRC2 complexes take on an activating, instead of repressing, role; this could be a mechanism for selective gene activation during the process of differentiation. Overall, it is increasingly evident that the diversity of PRC2 subunits has functional consequences on gene expression as well as biological output and that this complexity must be considered when investigating the roles of PcG proteins in pluripotent versus lineage-committed cells.

Non-coding RNA facilitates Polycomb localization during differentiation

Recent work has highlighted possible roles for non-coding RNA (ncRNA) in the recruitment and regulation of Polycomb complexes (Koziol and Rinn, 2010; Morris, 2009; Ponting et al., 2009) (Figure 6). In particular, long non-coding RNAs (lncRNA), defined by a length >200nt and by the lack of protein coding capacity (Ponting et al., 2009), have recently garnered much of the spotlight. As with protein-coding genes, lncRNAs are subject to PRC2-mediated gene regulation (Wu et al., 2010b). Additionally, many lncRNAs appear to be developmentally regulated (Dinger et al., 2008). Moreover, a knockdown screen of mESC lncRNAs identified several interesting classes of lncRNAs: those that maintain pluripotency, those that suppress lineage-specific genes, and those that bind specifically to one – or multiple – of a variety of chromatin modifying complexes (Guttman et al., 2011). These data raised the intriguing possibility that lncRNAs could coordinate PRC2 activity during cellular differentiation. This idea is further supported by new evidence showing that lncRNAs may act as a scaffold for interactions with other histone modifiers (Spitale et al., 2011; Tsai et al., 2010).

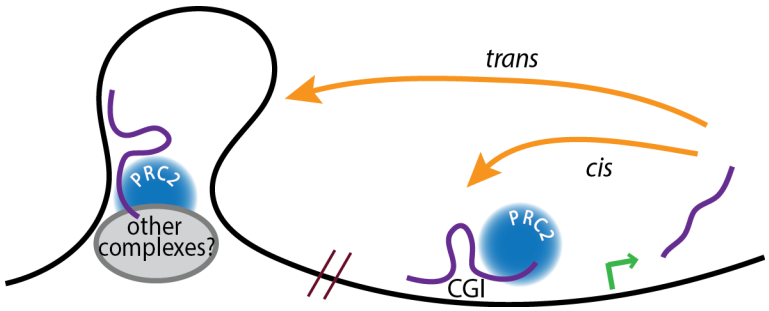


Figure 6. PRCs may interact with non-coding RNAs to regulate gene expression in cis or in trans, potentially by the non-coding RNAs acting as scaffolds that mediate interactions with chromatin modifying complexes, including PRC2. CGI = CpG island.

As PcGs have vital roles in the regulation of gene expression during pluripotency and differentiation, the observation that of the ~3,300 putative lncRNAs identified in mouse, roughly one-fifth associated

with Polycomb complexes in various cell types, including ESCs suggests that these transcripts may have very broad roles (Guttman et al., 2009; Khalil et al., 2009). Furthermore, when ESC RNAs were isolated by PcG immunoprecipitation, closer to 10,000 transcripts were shown to bind PRC2 (Zhao et al., 2010). Additionally, computational analyses have revealed a set of PcG-associated mouse lncRNAs based on the sequence and structural similarities of human PcG-binding lncRNAs (Glazko et al., 2012). Thus, while a number of candidate PRC-regulating lncRNAs have been identified, many of these interactions will require further validation. However, two recent studies show nonspecific binding of PRC2 to most RNAs, and propose a model whereby PRC2 is recruited to the promoter of all transcribed genes to determine whether they are targets (Davidovich et al., 2013; Kaneko et al., 2013). Nevertheless, certain lncRNAs have been found to have specific necessary roles in development; it is possible that lncRNA plays both specific and non-specific roles in PRC2 recruitment. An excellent review of noncoding RNA and its role in PcG protein recruitment has recently been published (Brockdorff, 2013); however, the best-characterized and most interesting examples are briefly described here.

cis-*acting PRC-associated lncRNAs*

The canonical example of this class of lncRNA is *Xist*, which is expressed from the inactive X chromosome and recruits PRC2 to that chromosome to play a role in dosage compensation (Plath et al., 2003; Silva et al., 2003; Zhao et al., 2008). Remarkably, marsupials have also independently evolved an analogous system for lncRNA-mediated X inactivation (Grant et al., 2012), suggesting an important function for lncRNAs in this process. Likewise, *Kcnq1ot1* interacts with PRC2 *in cis* to recruit PRCs to the *Kcnq1* imprinted locus in a cell-type-dependent manner, in order to initiate changes in gene expression and chromatin structure (Pandey et al., 2008; Terranova et al., 2008). Moreover, PRC-associated lncRNAs are not restricted to mammals. In *Arabidopsis,* proper flowering regulation requires PRC2-mediated silencing at the flowering control (*FLC*) gene, but only when triggered by the

vernalization process; recruitment of active PRC2 to this locus at the correct time is mediated by the lncRNA *COLDAIR* (Heo and Sung, 2011).

Finally, PRC2 is not the only PcG complex to interact with lncRNAs. In ESCs, PRC1 targeting to the INK4b-ARF-INK4a locus (reviewed in (Aguilo et al., 2011)) is mediated by CBX7 and its interactions with both H3K27me3 and ANRIL, a lncRNA encoded at this locus (Yap et al., 2010), and both are necessary for its proper transcriptional regulation. Thus, it will be interesting to determine whether other PRC1-like complexes are regulated by lncRNAs.

trans-*acting PRC-associated lncRNAs*

HOTAIR, a lncRNA transcribed from the *HOXC* locus in human fibroblasts, interacts with SUZ12 and recruits PRC2 to the *HOXD* locus *in trans* (Rinn et al., 2007) (Figures 3 and 6). Consistent with an important function, the misregulation of *HOTAIR* has consequences for the maintenance of a particular cell state (Gupta et al., 2010). More recently, *HOTAIR* binding has been mapped to over 800 genomic loci, indicating it could have more widespread PRC2-recruitment effects other than at the *HOXD* locus (Chu et al., 2011). However, surprisingly, knockout of the mouse *HOTAIR* ortholog has neither phenotype nor effect on *HOXD* expression (Schorderet and Duboule, 2011). This observation is in agreement with the fact that there is very little evolutionary conservation of lncRNA sequence (Ulitsky et al., 2011) in that, seemingly, every system must develop its own precisely tuned lncRNA system. Thus, in the lncRNA field, while broad concepts can translate from one organism to another, precise mechanistic details usually cannot.

For a handful of lncRNAs, a lineage-specific effect and mouse phenotype has been identified. *Fendrr*, for example, is expressed in the lateral plate mesoderm – which later develops into the heart and body wall – and binds both to PRC2 and to TrxG/MLL (the complex that catalyzes H3K4me3, the activating mark found at bivalent domains) (Grote et al., 2013). Interestingly, *Fendrr* seems to promote

H3K27me3 while preventing H3K4me3, leading to the down-regulation of its target genes to properly regulate heart development gene programs (Grote and Herrmann, 2013; Grote et al., 2013).

In contrast to the previously described roles for lncRNAs in PRC recruitment, these transcripts may also act to prevent PRC2 binding at specific target sites. A recent study showed that loss of *Braveheart*, a lncRNA that physically associates with the core PRC2 component SUZ12, results in a failure to activate the cardiogenic program (Klattenhoff et al., 2013). These data are consistent with a role for *Braveheart* in acting as a decoy for PRC2 at specific target genes required in the cardiac lineage during differentiation (Klattenhoff et al., 2013); however, further studies are required to determine if this effect is directly mediated by its interaction with the lncRNA.

The role of lncRNAs in regulating Polycomb-mediated cell fate transitions is still emerging; however, it is interesting to speculate that many lncRNAs could be acting to mediate crosstalk between epigenetic regulators in a cell type specific fashion. Thus, defining the set of lncRNAs that interact with PcG complexes and their modes of action during ESC differentiation will be a critical step towards understanding the role of lncRNAs in mediating cell fate transitions.

In addition to long ncRNAs, new evidence suggests that CpG-rich sequences, which are highly enriched near Polycomb target genes (Ku et al., 2008; Mikkelsen et al., 2007), produce short transcripts (~50-200 nt) (Kanhere et al., 2010). Remarkably, these transcripts can interact with Suz12, leading to recruitment of PRC2 and repression of the associated mRNA transcript *in cis*. Interestingly, this interaction is dependent on an intact double stem-loop structure within the RNA suggesting that a conserved structure rather than a defined sequence mediates the function of this class of ncRNAs. Consistent with a regulatory role, the authors found that an increase in mRNA production of PcG target genes corresponded with a decrease in the level of associated short RNAs. While this study was largely performed in T cells, production of short RNAs near PcG target genes was confirmed in ESCs,

indicating that this could be a widespread mechanism. While determining how the production of these short transcripts is regulated and how they may relate to CpG islands and CpG methylation status in ESCs requires further investigations, this study highlights the complex interplay of mechanisms that may be instrumental for the proper localization and regulation of PcG proteins in specific cell types.

Overall, ncRNAs are implicated in PRC-mediated gene repression, and the elucidation of their roles is likely to shed significant light on the issue of proper regulation of gene expression programs in lineage commitment and cell fate decisions.

DNA methylation and its relationship with PcG-mediated gene repression

Emerging evidence indicates an important functional relationship between PcG-mediated gene repression and DNA methylation-mediated gene repression. DNA can be methylated, most commonly seen in the context of CpG (Ziller et al., 2011). This modification is catalyzed by the DNA nucleotide methyltransferase (DNMT) family of proteins (Li et al., 1992; Okano et al., 1999), and globally, mammalian DNA is very highly methylated. CpG islands, however, have low levels of methylation (Bird et al., 1985), and when a CpG island in a promoter is methylated, it correlates with repression of that gene (Deaton and Bird, 2011).

PcG-mediated gene repression is thought to represent a dynamic mechanism of gene repression that allows cells to retain some plasticity over developmental time, while DNA methylation results in more permanent gene repression. Interestingly, recent models suggest that these two pathways coordinate in order to effect repression when and where necessary during the process of differentiation. In support of this model, an early study showed that at one imprinted mouse gene, DNA methylation and PRC2 are mutually antagonistic: artificial removal of either allowed the other to be recruited (Lindroth et al., 2008); however, it has not been clear whether this coordination represents a broader

paradigm for regulation. Evidence from various studies is detailed in the following paragraphs and reveals a very conflicting and complicated link between PRC2 enrichment and DNA methylation. Finally, while some studies have begun to shed light on the relationship between DNA methylation and PcG proteins during differentiation, this question has not yet been thoroughly explored, and future studies in this vein will be important for understanding the role of these two essential repressive mechanisms in mammalian development and disease.

Positional correlation

In order to determine the link between DNA methylation and H3K27me3/PRC2, localization studies have been performed to determine the relative enrichment patterns of these marks in many different cell types, both normal and diseased. Early studies suggested an antagonistic relationship between DNA methylation and H3K27me3 at promoter regions (Fouse et al., 2008), but recent studies that have examined DNA methylation at base-pair resolution have revealed a more complicated state comprising both correlation and anti-correlation.

In non-cancerous human cell lines, H3K27me3 and DNA methylation tend to anti-correlate at promoter regions and correlate elsewhere (Hawkins et al., 2010; Lister and Ecker, 2009). Indeed, when comparing somatic cells to pluripotent cells, promoter regions that have acquired H3K27me3 tend to become DNA hypomethylated (Hawkins et al., 2010).

In many different studies and many different cancer samples, both primary and cultured cells, extensive regions of hypomethylation have been found that contain intermittent peaks of hypermethylation. These regions tend to be fully methylated in ESCs, but only partially methylated in somatic cells, and correspond to an enrichment in H3K27me3 and EZH2 binding in cancer cells (Hon et al., 2012; Varley et al., 2013). Additionally, regions of H3K27me3 enrichment in normal cells are prone to lose H3K27me3 and gain DNA methylation in cancerous cells (Berman et al., 2012; Gal-Yam et al.,

2008; Varley et al., 2013). Interestingly, it is possible for the two alleles of a gene, both enriched for H3K27me3, to have different DNA methylation profiles; as such, it is unlikely that either mark directly and exclusively regulates the other (Statham et al., 2012). Although the genes contained within these domains of hypomethylation seem to be largely repressed, the mechanism for this transcriptional regulation is not clear (Hon et al., 2012; Lister and Ecker, 2009). Overall, cancer cells seem to show evidence both of correlation and of anti-correlation between H3K27me3 and DNA methylation, which could suggest a disruption in the normal regulatory pathways involving these two epigenetic marks.

Impact of DNA methylation on H3K27me3

Recent genetic experiments were performed in order to further determine the effect that DNA methylation has on H3K27me3. For example, a recent study showed that In *Dnmt1-/-* Mouse Embryonic Fibroblasts (MEFs), which are hypomethylated genomewide, H3K27me3 and PRC2 are lost from PcG target promoters; this change in status correlates with increased expression of the respective genes (Reddington et al., 2013). However, H3K27me3 enrichment increases at many non-promoter regions (Reddington et al., 2013). These data suggest that DNA methylation supports H3K27me3 at promoters, but antagonizes it in other regions of the genome.

These observations, however, are in contrast to findings that focus on CpG islands in ESCs. In *Dnmt3a/3b-/-* ESCs, CpG islands that lose DNA methylation frequently gain H3K27me3 compared to wild-type cells (Lynch et al., 2012). Another study used ChIP-BS-seq (ChIP for H3K27me3, followed by bisulfite conversion of unmethylated CpGs and adapted for high-throughput sequencing) to directly map methylated CpGs on H3K27me3-bound regions. This work showed that H3K27me3-enriched CpG islands are largely unmethylated; non-CpG-island H3K27me3-enriched regions, however, are methylated (Brinkman et al., 2012). When all three active *Dnmts* are knocked out, CpG islands that are methylated in wild-type ESCs and lose that methylation in the mutant gain H3K27me3, suggesting that DNA methylation prevents PRC2 activity (Brinkman et al., 2012). CpG islands that were not

originally methylated lose H3K27me3, perhaps as PRC2 moves to the regions it was normally excluded from by DNA methylation. Furthermore, while H3K27me3 supports PRC2 binding to the genome, addition of DNA methylation depletes its affinity, lending biochemical support for the antagonistic effect of DNA methylation on PRC2 (Bartke et al., 2010).

In contrast to promoters, DNA methylation in gene body regions appears to promote transcription. Depletion of genic DNA methylation leads to an increase in H3K27me3, and a concomitant repression of genes. Furthermore, depletion of PRC2 rescues the gene expression phenotype (Wu et al., 2010a). These data suggest that the "activating" effect of gene-body DNA methylation may be due to its antagonistic effect on PRC2-mediated repression (Wu et al., 2010a), demonstrating the complexity of this relationship.

Current models suggest that DNA methylation prevents PRC2 recruitment. However, some studies contradict this model, indicating that further detailed investigations are needed to dissect the interplay between these regulatory mechanisms.

Impact of H3K27me3/PRC2 on DNA methylation

An early study showed that both binding of DNMTs and DNA methylation at PcG target genes requires EZH2; furthermore, EZH2 physically interacts with DNMTs (Viré et al., 2006). However, it was later shown that while PRC2 does seem sufficient to recruit DNMTs, they do not catalyze DNA methylation (Rush et al., 2009). Interestingly, a recent study showed recruitment of DNMT3L, the catalytically inactive DNMT family member, by PRC2 to promoters inhibited binding of its active family members, preventing DNA methylation (Neri et al., 2013b). In support of this, recent methylation profiling studies in the context of PRC2 mutant cells show an antagonistic relationship.

Using meDIP (basically ChIP for 5-methyl-Cytosine) coupled with a promoter microarray, only about 10% of genes show a change in methylation at their promoter region upon knockout of *Eed* in ESCs,

(Hagarman et al., 2013). Among these genes is a subset of bivalent genes, indicating that PRC2 is antagonistic to DNA methylation at canonical PcG targets (but not at other, non-canonical targets). However, interestingly, this H3K27me3-dependent change in DNA methylation did not seem to be directly controlling gene expression (Hagarman et al., 2013), questioning a functional relationship at these genes. Finally, during *in vitro* differentiation, H3K27me3-enriched promoters in ESCs often lose their H3K27me3 and gain DNA methylation, suggesting a mechanism where H3K27me3-mediated repression anticipates the more permanent DNA methylation-mediated repression during development (Mohn et al., 2008). More studies that take into account developmental time are needed to better understand the effect of PRC2 on DNA methylation.

Co-regulation of downstream factors by H3K27me3 and DNA methylation

While emerging evidence indicates a complex relationship between PRC2-mediated histone methylation and DNA methylation, how this regulations impacts downstream efforts and transcriptional output has been unclear. A recent study used a proteomics-based approach to identify proteins whose binding to chromatin is modulated by DNA methylation and H3K27me3 (Bartke et al., 2010). For example, the Origin Recognition Complex binds best to regions with both marks, in agreement with its known binding preference for heterochromatin (Bartke et al., 2010). Thus, while the above studies paint a picture of mutual antagonism between DNA methylation and PRC2, there is evidence that they also have cooperative regulatory roles.

Overall, it seems likely that these two factors are mutually antagonistic at CpG islands and/or promoter regions; however, there are many contradictions to this model. It is likely that this relationship follows signals and conforms to rules that we do not yet understand. As such, the relationship between DNA methylation and PRC2/H3K27me3 merits further study.

Concluding Remarks

Current evidence indicates that PcG proteins set the stage in ESCs to allow cells to respond appropriately to developmental cues and for maintenance of specific developmental gene expression programs during development. PRCs catalyze the post-translational modification of histones to control developmental gene expression patterns. PcG proteins themselves may also contribute to chromatin compaction, chromatin interactions, and gene silencing in a catalytically independent manner. Recent developments in the field indicate that PRCs work in conjunction with a range of other factors in a cell type-specific manner. Such varied and diverse roles ascribed to PRCs are likely important for the transcriptional fine-tuning of the large number of target genes during lineage commitment.

Overwhelming evidence suggests that misregulation of PcG proteins, such as Ezh2 and Bmi1, is correlated with cancer progression. Given that transformed cells share many features with stem cells, functional analysis of PRC1 and PRC2 in ESCs may also contribute important new clues for understanding the progression from a normal to disease state and might also lead to the identification of new biomarkers or small molecule inhibitors. The potential discovery of mechanisms that govern the recruitment of PcG proteins to target sites in the genome and their subsequent repression of target genes may facilitate efforts to direct the differentiation of stem cells *in vitro* and to control disease progression *in vivo*. Therefore, continued efforts to unravel the complexities of how PcG proteins function to control gene expression in pluripotent cells has practical significance for understanding development and for treating disease.

Although the possibility exists that H3K27me3 deposition is a consequence and not a cause of gene repression (Henikoff and Shilatifard, 2011), recent evidence shows that specifically preventing H3K27me3 deposition through mutation of Histone H3 or through inhibitors causes derepression of

target genes, suggesting that this histone mark is an important component of the repressive pathway (Knutson et al., 2012; McCabe et al., 2012; Pengelly et al., 2013). In this thesis, I attempt to shed light on the important question of how PRC2 acts through H3K27me3 and its downstream effectors to mediate gene repression during pluripotency and lineage commitment.

References

Agger, K., Cloos, P.A.C., Christensen, J., Pasini, D., Rose, S., Rappsilber, J., Issaeva, I., Canaani, E., Salcini, A.E., and Helin, K. (2007). UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature *449*, 731–734.

Aguilo, F., Zhou, M.-M., and Walsh, M.J. (2011). Long noncoding RNA, polycomb, and the ghosts haunting INK4b-ARF-INK4a expression. Cancer Res *71*, 5365–5369.

Akasaka, T., van Lohuizen, M., van der Lugt, N., Mizutani-Koseki, Y., Kanno, M., Taniguchi, M., Vidal, M., Alkema, M., Berns, A., and Koseki, H. (2001). Mice doubly deficient for the Polycomb Group genes Mel18 and Bmi1 reveal synergy and requirement for maintenance but not initiation of Hox gene expression. Development *128*, 1587–1597.

Arai, D., Katsura, H., Shindo, N., Matsumoto, M., and Higashinakagawa, T. (2010). Polycomb group protein Ezh1 represses Nodal and maintains the left-right axis. Dev Biol *341*, 459–463.

Arnold, P., Scholer, A., Pachkov, M., Balwierz, P.J., Jorgensen, H., Stadler, M.B., van Nimwegen, E., and Schubeler, D. (2013). Modeling of epigenome dynamics identifies transcription factors that mediate Polycomb targeting. Genome Res *23*, 60–73.

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jørgensen, H.F., John, R.M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M., et al. (2006). Chromatin signatures of pluripotent cell lines. Nat Cell Biol *8*, 532–538.

Bantignies, F., Roure, V., Comet, I., Leblanc, B., Schuettengruber, B., Bonnet, J., Tixier, V., Mas, A., and Cavalli, G. (2011). Polycomb-dependent regulatory contacts between distant Hox loci in Drosophila. Cell *144*, 214–226.

Bartke, T., Vermeulen, M., Xhemalce, B., Robson, S.C., Mann, M., and Kouzarides, T. (2010). Nucleosome-interacting proteins regulated by DNA and histone methylation. Cell *143*, 470–484.

Berman, B.P., Weisenberger, D.J., Aman, J.F., Hinoue, T., Ramjan, Z., Liu, Y., Noushmehr, H., Lange, C.P.E., van Dijk, C.M., Tollenaar, R.A.E.M., et al. (2012). Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. Nat Genet *44*, 40–46.

Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., et al. (2006). A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. Cell *125*, 315–326.

Bird, A., Taggart, M., Frommer, M., Miller, O.J., and Macleod, D. (1985). A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. Cell *40*, 91–99.

Björklund, S., and Gustafsson, C.M. (2005). The yeast Mediator complex and its regulation. Trends Biochem Sci *30*, 240–244.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M.,

Murray, H.L., Jenner, R.G., et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947–956.

Boyer, L.A., Mathur, D., and Jaenisch, R. (2006a). Molecular control of pluripotency. Current Opinion in Genetics & Development *16*, 455–462.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., et al. (2006b). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nat Cell Biol *441*, 349–353.

Bracken, A.P., and Helin, K. (2009). Polycomb group proteins: navigators of lineage pathways led astray in cancer. Nat Rev Cancer *9*, 773–784.

Brien, G.L., Gambero, G., O'Connell, D.J., Jerman, E., Turner, S.A., Egan, C.M., Dunne, E.J., Jurgens, M.C., Wynne, K., Piao, L., et al. (2012). Polycomb PHF19 binds H3K36me3 and recruits PRC2 and demethylase NO66 to embryonic stem cell genes during differentiation. Nat Struct Mol Biol *19*, 1273–1281.

Brinkman, A.B., Gu, H., Bartels, S.J.J., Zhang, Y., Matarese, F., Simmer, F., Marks, H., Bock, C., Gnirke, A., Meissner, A., et al. (2012). Sequential ChIP-bisulfite sequencing enables direct genome-scale investigation of chromatin and DNA methylation cross-talk. Genome Res *22*, 1128–1138.

Brockdorff, N. (2013). Noncoding RNA and Polycomb recruitment. Rna *19*, 429–442.

Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.L., and Kassis, J.A. (1998). The Drosophila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. Mol Cell *1*, 1057–1064.

Cao, R., and Zhang, Y. (2004). SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell *15*, 57–67.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043.

Casanova, M., Preissner, T., Cerase, A., Poot, R., Yamada, D., Li, X., Appanah, R., Bezstarosti, K., Demmers, J., Koseki, H., et al. (2011). Polycomblike 2 facilitates the recruitment of PRC2 Polycomb group complexes to the inactive X chromosome and to target loci in embryonic stem cells. Development *138*, 1471–1482.

Chamberlain, S.J., Yee, D., and Magnuson, T. (2008). Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells *26*, 1496–1505.

Chambeyron, S., and Bickmore, W.A. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. Genes Dev *18*, 1119–1130.

Chen, S., Ma, J., Wu, F., Xiong, L.-J., Ma, H., Xu, W., Lv, R., Li, X., Villen, J., Gygi, S.P., et al. (2012). The histone H3 Lys 27 demethylase JMJD3 regulates gene expression by impacting transcriptional elongation. Genes Dev *26*, 1364–1375.

Choi, D., Goo, H.G., Yoo, J., and Kang, S. (2011). Identification of RNF2-responding loci in long-range chromatin interactions using the novel 4C-ChIP-Cloning technology. J. Biotechnol. *151*, 312–318.

Chopra, V.S., Hendrix, D.A., Core, L.J., Tsui, C., Lis, J.T., and Levine, M. (2011). The polycomb group mutant esc leads to augmented levels of paused Pol II in the Drosophila embryo. Mol Cell *42*, 837–844.

Chopra, V.S., Hong, J.-W., and Levine, M. (2009). Regulation of Hox gene activity by transcriptional elongation in Drosophila. Curr Biol *19*, 688–693.

Chu, C., Qu, K., Zhong, F.L., Artandi, S.E., and Chang, H.Y. (2011). Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. Mol Cell *44*, 667–678.

Cloos, P.A.C., Christensen, J., Agger, K., and Helin, K. (2008). Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes Dev *22*, 1115–1140.

Cole, M.F., and Young, R.A. (2008). Mapping key features of transcriptional regulatory circuitry in embryonic stem cells. Cold Spring Harb. Symp. Quant. Biol. *73*, 183–193.

Core, L.J., Waterfall, J.J., and Lis, J.T. (2008). Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science *322*, 1845–1848.

Coré, N., Bel, S., Gaunt, S.J., Aurrand-Lions, M., Pearce, J., Fisher, A., and Djabali, M. (1997). Altered cellular proliferation and mesoderm patterning in Polycomb-M33-deficient mice. Development *124*, 721–729.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185–196.

Davidovich, C., Zheng, L., Goodrich, K.J., and Cech, T.R. (2013). Promiscuous RNA binding by Polycomb repressive complex 2. Nat Struct Mol Biol *20*, 1250–1257.

de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M., et al. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell *7*, 663–676.

Deal, R.B., Henikoff, J.G., and Henikoff, S. (2010). Genome-wide kinetics of nucleosome turnover determined by metabolic labeling of histones. Science *328*, 1161–1164.

Deaton, A.M., and Bird, A. (2011). CpG islands and the regulation of transcription. Genes Dev *25*, 1010–1022.

del Mar Lorente, M., Marcos-Gutiérrez, C., Pérez, C., Schoorlemmer, J., Ramírez, A., Magin, T., and Vidal, M. (2000). Loss- and gain-of-function mutations show a polycomb group function for Ring1A in mice. Development *127*, 5093–5100.

Dellino, G.I., Schwartz, Y.B., Farkas, G., McCabe, D., Elgin, S.C.R., and Pirrotta, V. (2004). Polycomb silencing blocks transcription initiation. Mol Cell *13*, 887–893.

Denholtz, M., Bonora, G., Chronis, C., Splinter, E., de Laat, W., Ernst, J., Pellegrini, M., and Plath, K. (2013). Long-Range Chromatin Contacts in Embryonic Stem Cells Reveal a Role for Pluripotency Factors and Polycomb Proteins in Genome Organization. Cell Stem Cell.

Dinger, M.E., Amaral, P.P., Mercer, T.R., Pang, K.C., Bruce, S.J., Gardiner, B.B., Askarian-Amiri, M.E., Ru, K., Soldà, G., Simons, C., et al. (2008). Long noncoding RNAs in mouse embryonic stem cell pluripotency and differentiation. Genome Res *18*, 1433–1445.

Donohoe, M.E., Zhang, X., McGinnis, L., Biggers, J., Li, E., and Shi, Y. (1999). Targeted disruption of mouse Yin Yang 1 transcription factor results in peri-implantation lethality. Mol Cell Biol *19*, 7237–7244.

Duncan, I.M. (1982). Polycomblike: a gene that appears to be required for the normal expression of the bithorax and antennapedia gene complexes of Drosophila melanogaster. Genetics *102*, 49–70.

Endoh, M., Endo, T.A., Endoh, T., Fujimura, Y.-I., Ohara, O., Toyoda, T., Otte, A.P., Okano, M., Brockdorff, N., Vidal, M., et al. (2008). Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity. Development *135*, 1513–1524.

Endoh, M., Endo, T.A., Endoh, T., Isono, K.-I., Sharif, J., Ohara, O., Toyoda, T., Ito, T., Eskeland, R., Bickmore, W.A., et al. (2012). Histone H2A Mono-Ubiquitination Is a Crucial Step to Mediate PRC1-Dependent Repression of Developmental Genes to Maintain ES Cell Identity. PLoS Genet *8*, e1002774.

Eskeland, R., Leeb, M., Grimes, G.R., Kress, C., Boyle, S., Sproul, D., Gilbert, N., Fan, Y., Skoultchi, A.I., Wutz, A., et al. (2010). Ring1B compacts chromatin structure and represses gene expression independent of histone ubiquitination. Mol Cell *38*, 452–464.

Farcas, A.M., Blackledge, N.P., Sudbery, I., Long, H.K., McGouran, J.F., Rose, N.R., Lee, S., Sims, D., Cerase, A., Sheahan, T.W., et al. (2012). KDM2B links the Polycomb Repressive Complex 1 (PRC1) to recognition of CpG islands. Elife *1*, e00205.

Faust, C., Lawson, K.A., Schork, N.J., Thiel, B., and Magnuson, T. (1998). The Polycomb-group gene eed is required for normal morphogenetic movements during gastrulation in the mouse embryo. Development *125*, 4495–4506.

Faust, C., Schumacher, A., Holdener, B., and Magnuson, T. (1995). The eed mutation disrupts anterior mesoderm production in mice. Development *121*, 273–285.

Ferraiuolo, M.A., Rousseau, M., Miyamoto, C., Shenker, S., Wang, X.Q.D., Nadler, M., Blanchette, M., and Dostie, J. (2010). The three-dimensional architecture of Hox cluster silencing. Nucleic Acids Research *38*, 7472–7484.

Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev *17*, 1870–1881.

Fisher, C.L., Randazzo, F., Humphries, R.K., and Brock, H.W. (2006). Characterization of Asxl1, a murine homolog of Additional sex combs, and analysis of the Asx-like gene family. Gene *369*, 109–118.

Fouse, S.D., Shen, Y., Pellegrini, M., Cole, S., Meissner, A., van Neste, L., Jaenisch, R., and Fan, G. (2008). Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. Cell Stem Cell *2*, 160–169.

Francis, N.J., Saurin, A.J., Shao, Z., and Kingston, R.E. (2001). Reconstitution of a functional core polycomb repressive complex. Mol Cell *8*, 545–556.

Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science *306*, 1574–1577.

Fukuda, T., Tokunaga, A., Sakamoto, R., and Yoshida, N. (2011). Fbxl10/Kdm2b deficiency accelerates neural progenitor cell death and leads to exencephaly. Mol. Cell. Neurosci. *46*, 614–624.

Gal-Yam, E.N., Egger, G., Iniguez, L., Holster, H., Einarsson, S., Zhang, X., Lin, J.C., Liang, G., Jones, P.A., and Tanay, A. (2008). Frequent switching of Polycomb repressive marks and DNA hypermethylation in the PC3 prostate cancer cell line. Proc Natl Acad Sci USA *105*, 12979–12984.

Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., and Reinberg, D. (2012). PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. Mol Cell *45*, 344–356.

Gearhart, M.D., Corcoran, C.M., Wamstad, J.A., and Bardwell, V.J. (2006). Polycomb group and SCF ubiquitin ligases are found in a novel BCOR complex that is recruited to BCL6 targets. Mol Cell Biol *26*, 6880–6889.

Gieni, R.S., and Hendzel, M.J. (2009). Polycomb group protein gene silencing, non-coding RNA, stem cells, and cancer. Biochem Cell Biol *87*, 711–746.

Glazko, G.V., Zybailov, B.L., and Rogozin, I.B. (2012). Computational prediction of polycomb-associated long non-coding RNAs. PLoS ONE *7*, e44878.

Grant, J., Mahadevaiah, S.K., Khil, P., Sangrithi, M.N., Royo, H., Duckworth, J., McCarrey, J.R., VandeBerg, J.L., Renfree, M.B., Taylor, W., et al. (2012). Rsx is a metatherian RNA with Xist-like properties in X-chromosome inactivation. Nature *487*, 254–258.

Grau, D.J., Chapman, B.A., Garlick, J.D., Borowsky, M., Francis, N.J., and Kingston, R.E. (2011). Compaction of chromatin by diverse Polycomb group proteins requires localized regions of high charge. Genes Dev *25*, 2210–2221.

Grote, P., and Herrmann, B.G. (2013). The long non-coding RNA Fendrr links epigenetic control mechanisms to gene regulatory networks in mammalian embryogenesis. RNA Biol *10*.

Grote, P., Wittler, L., Hendrix, D., Koch, F., Währisch, S., Beisaw, A., Macura, K., Bläss, G., Kellis, M., Werber, M., et al. (2013). The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. Dev Cell *24*, 206–214.

Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R., and Young, R.A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. Cell *130*, 77–88.

Gupta, R.A., Shah, N., Wang, K.C., Kim, J., Horlings, H.M., Wong, D.J., Tsai, M.-C., Hung, T., Argani, P., Rinn, J.L., et al. (2010). Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature *464*, 1071–1076.

Gutiérrez, L., Oktaba, K., Scheuermann, J.C., Gambetta, M.C., Ly-Hartig, N., and Müller, J. (2012). The role of the histone H2A ubiquitinase Sce in Polycomb repression. Development *139*, 117–127.

Guttman, M., Amit, I., Garber, M., French, C., Lin, M.F., Feldser, D., Huarte, M., Zuk, O., Carey, B.W.,

Cassady, J.P., et al. (2009). Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature *458*, 223–227.

Guttman, M., Donaghey, J., Carey, B.W., Garber, M., Grenier, J.K., Munson, G., Young, G., Lucas, A.B., Ach, R., Bruhn, L., et al. (2011). lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature.

Hagarman, J.A., Motley, M.P., Kristjansdottir, K., and Soloway, P.D. (2013). Coordinate regulation of DNA methylation and H3K27me3 in mouse embryonic stem cells. PLoS ONE *8*, e53880.

Hawkins, R.D., Hon, G.C., Lee, L.K., Ngo, Q., Lister, R., Pelizzola, M., Edsall, L.E., Kuan, S., Luu, Y., Klugman, S., et al. (2010). Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell Stem Cell *6*, 479–491.

Hawkins, R.D., Hon, G.C., Yang, C., Antosiewicz-Bourget, J.E., Lee, L.K., Ngo, Q.-M., Klugman, S., Ching, K.A., Edsall, L.E., Ye, Z., et al. (2011). Dynamic chromatin states in human ES cells reveal potential regulatory sequences and genes involved in pluripotency. Cell Res *21*, 1393–1409.

Henikoff, S., and Shilatifard, A. (2011). Histone modification: cause or cog? Trends Genet *27*, 389–396.

Heo, J.B., and Sung, S. (2011). Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. Science *331*, 76–79.

Herz, H.-M., Mohan, M., Garrett, A.S., Miller, C., Casto, D., Zhang, Y., Seidel, C., Haug, J.S., Florens, L., Washburn, M.P., et al. (2012). Polycomb repressive complex 2-dependent and -independent functions of Jarid2 in transcriptional regulation in Drosophila. Mol Cell Biol.

Hon, G.C., Hawkins, R.D., Caballero, O.L., Lo, C., Lister, R., Pelizzola, M., Valsesia, A., Ye, Z., Kuan, S., Edsall, L.E., et al. (2012). Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res *22*, 246–258.

Hong, S., Cho, Y.-W., Yu, L.-R., Yu, H., Veenstra, T.D., and Ge, K. (2007). Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. Proc Natl Acad Sci USA *104*, 18439–18444.

Hunkapiller, J., Shen, Y., Diaz, A., Cagney, G., McCleary, D., Ramalho-Santos, M., Krogan, N., Ren, B., Song, J.S., and Reiter, J.F. (2012). Polycomb-like 3 promotes polycomb repressive complex 2 binding to CpG islands and embryonic stem cell self-renewal. PLoS Genet *8*, e1002576.

Jaenisch, R., and Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell *132*, 567–582.

Joo, H.-Y., Zhai, L., Yang, C., Nie, S., Erdjument-Bromage, H., Tempst, P., Chang, C., and Wang, H. (2007). Regulation of cell cycle progression and gene expression by H2A deubiquitination. Nature *449*, 1068–1072.

Jung, H.R., Pasini, D., Helin, K., and Jensen, O.N. (2010). Quantitative mass spectrometry of histones H3.2 and H3.3 in Suz12-deficient mouse embryonic stem cells reveals distinct, dynamic post-translational modifications at Lys-27 and Lys-36. Mol Cell Proteomics *9*, 838–850.

Jung, J., Kim, T.-G., Lyons, G.E., Kim, H.-R.C., and Lee, Y. (2005). Jumonji regulates cardiomyocyte proliferation via interaction with retinoblastoma protein. J Biol Chem *280*, 30916–30923.

Jürgens, G. (1985). A group of genes controlling the spatial expression of the bithorax complex in Drosophila. Nature *316*, 153–155.

Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D.A., van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S., et al. (2010). Mediator and cohesin connect gene expression and chromatin architecture. Nature *467*, 430–435.

Kallin, E.M., Cao, R., Jothi, R., Xia, K., Cui, K., Zhao, K., and Zhang, Y. (2009). Genome-wide uH2A localization analysis highlights Bmi1-dependent deposition of the mark at repressed genes. PLoS Genet *5*, e1000506.

Kaneko, S., Son, J., Shen, S.S., Reinberg, D., and Bonasio, R. (2013). PRC2 binds active promoters and contacts nascent RNAs in embryonic stem cells. Nat Struct Mol Biol *20*, 1258–1264.

Kanhere, A., Viiri, K., Araújo, C.C., Rasaiyaah, J., Bouwman, R.D., Whyte, W.A., Pereira, C.F., Brookes, E., Walker, K., Bell, G.W., et al. (2010). Short RNAs are transcribed from repressed polycomb target genes and interact with polycomb repressive complex-2. Mol Cell *38*, 675–688.

Kaustov, L., Ouyang, H., Amaya, M., Lemak, A., Nady, N., Duan, S., Wasney, G.A., Li, Z., Vedadi, M., Schapira, M., et al. (2011). Recognition and specificity determinants of the human cbx chromodomains. J Biol Chem *286*, 521–529.

Keller, G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev *19*, 1129–1155.

Kerppola, T.K. (2009). Polycomb group complexes--many combinations, many functions. Trends Cell Biol *19*, 692–704.

Khalil, A.M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., Thomas, K., Presser, A., Bernstein, B.E., van Oudenaarden, A., et al. (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci USA *106*, 11667–11672.

Kim, J., Chu, J., Shen, X., Wang, J., and Orkin, S.H. (2008). An extended transcriptional network for pluripotency of embryonic stem cells. Cell *132*, 1049–1061.

King, I.F.G., Francis, N.J., and Kingston, R.E. (2002). Native and recombinant polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro. Mol Cell Biol *22*, 7919–7928.

Kirmizis, A., Bartley, S.M., Kuzmichev, A., Margueron, R., Reinberg, D., Green, R., and Farnham, P.J. (2004). Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev *18*, 1592–1605.

Klattenhoff, C.A., Scheuermann, J.C., Surface, L.E., Bradley, R.K., Fields, P.A., Steinhauser, M.L., Ding, H., Butty, V.L., Torrey, L., Haas, S., et al. (2013). Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell *152*, 570–583.

Klose, R.J., Kallin, E.M., and Zhang, Y. (2006). JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet *7*, 715–727.

Kmita, M., and Duboule, D. (2003). Organizing axes in time and space; 25 years of colinear tinkering. Science *301*, 331–333.

Knutson, S.K., Wigle, T.J., Warholic, N.M., Sneeringer, C.J., Allain, C.J., Klaus, C.R., Sacks, J.D., Raimondi, A., Majer, C.R., Song, J., et al. (2012). A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. Nat. Chem. Biol. *8*, 890–896.

Koziol, M.J., and Rinn, J.L. (2010). RNA traffic control of chromatin complexes. Current Opinion in Genetics & Development *20*, 142–148.

Ku, M., Koche, R.P., Rheinbay, E., Mendenhall, E.M., Endoh, M., Mikkelsen, T.S., Presser, A., Nusbaum, C., Xie, X., Chi, A.S., et al. (2008). Genomewide Analysis of PRC1 and PRC2 Occupancy Identifies Two Classes of Bivalent Domains. PLoS Genet *4*, e1000242.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev *16*, 2893–2905.

Lan, F., Bayliss, P.E., Rinn, J.L., Whetstine, J.R., Wang, J.K., Chen, S., Iwase, S., Alpatov, R., Issaeva, I., Canaani, E., et al. (2007). A histone H3 lysine 27 demethylase regulates animal posterior development. Nature *449*, 689–694.

Lan, F., Nottke, A.C., and Shi, Y. (2008). Mechanisms involved in the regulation of histone lysine demethylases. Curr Opin Cell Biol *20*, 316–325.

Landeira, D., Sauer, S., Poot, R., Dvorkina, M., Mazzarella, L., Jørgensen, H.F., Pereira, C.F., Leleu, M., Piccolo, F.M., Spivakov, M., et al. (2010). Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. Nat Cell Biol *12*, 618–624.

Lee, M.G., Villa, R., Trojer, P., Norman, J., Yan, K.-P., Reinberg, D., Di Croce, L., and Shiekhattar, R. (2007). Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science *318*, 447–450.

Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K.-I., et al. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. Cell *125*, 301–313.

Leeb, M., and Wutz, A. (2007). Ring1B is crucial for the regulation of developmental control genes and PRC1 proteins but not X inactivation in embryonic cells. J Cell Biol *178*, 219–229.

Leeb, M., Pasini, D., Novatchkova, M., Jaritz, M., Helin, K., and Wutz, A. (2010). Polycomb complexes act redundantly to repress genomic repeats and genes. Genes Dev *24*, 265–276.

Lehmann, L., Ferrari, R., Vashisht, A.A., Wohlschlegel, J.A., Kurdistani, S.K., and Carey, M. (2012). Polycomb repressive complex 1 (PRC1) disassembles RNA polymerase II preinitiation complexes. J Biol Chem *287*, 35784–35794.

Levine, M. (2011). Paused RNA polymerase II as a developmental checkpoint. Cell *145*, 502–511.

Levine, S.S., King, I.F.G., and Kingston, R.E. (2004). Division of labor in polycomb group repression. Trends Biochem Sci *29*, 478–485.

Levine, S.S., Weiss, A., Erdjument-Bromage, H., Shao, Z., Tempst, P., and Kingston, R.E. (2002). The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. Mol Cell Biol *22*, 6070–6078.

Lewis, E.B. (1978). A gene complex controlling segmentation in Drosophila. Nature *276*, 565–570.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915–926.

Li, G., Margueron, R., Ku, M., Chambon, P., Bernstein, B.E., and Reinberg, D. (2010). Jarid2 and PRC2, partners in regulating gene expression. Genes Dev *24*, 368–380.

Li, H.-B., Müller, M., Bahechar, I.A., Kyrchanova, O., Ohno, K., Georgiev, P., and Pirrotta, V. (2011). Insulators, Not Polycomb Response Elements, Are Required for Long-Range Interactions between Polycomb Targets in Drosophila melanogaster. Mol Cell Biol *31*, 616–625.

Lindroth, A.M., Park, Y.J., McLean, C.M., Dokshin, G.A., Persson, J.M., Herman, H., Pasini, D., Miró, X., Donohoe, M.E., Lee, J.T., et al. (2008). Antagonism between DNA and H3K27 methylation at the imprinted Rasgrf1 locus. PLoS Genet *4*, e1000145.

Lister, R., and Ecker, J.R. (2009). Finding the fifth base: genome-wide sequencing of cytosine methylation. Genome Res *19*, 959–966.

Liu, Y., Shao, Z., and Yuan, G.-C. (2010). Prediction of Polycomb target genes in mouse embryonic stem cells. Genomics *96*, 17–26.

Lo, S.M., Follmer, N.E., Lengsfeld, B.M., Madamba, E.V., Seong, S., Grau, D.J., and Francis, N.J. (2012). A bridging model for persistence of a polycomb group protein complex through DNA replication in vitro. Mol Cell *46*, 784–796.

Loh, Y.-H., Ng, J.-H., and Ng, H.-H. (2008). Molecular framework underlying pluripotency. Cell Cycle *7*, 885–891.

Loh, Y.-H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., et al. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet *38*, 431–440.

Lynch, M.D., Smith, A.J.H., de Gobbi, M., Flenley, M., Hughes, J.R., Vernimmen, D., Ayyub, H., Sharpe, J.A., Sloane-Stanley, J.A., Sutherland, L., et al. (2012). An interspecies analysis reveals a key role for unmethylated CpG dinucleotides in vertebrate Polycomb complex recruitment. Embo J *31*, 317–329.

Macarthur, B.D., Ma'ayan, A., and Lemischka, I.R. (2009). Systems biology of stem cell fate and cellular reprogramming. Nat Rev Mol Cell Biol *10*, 672–681.

Mallo, M., Wellik, D.M., and Deschamps, J. (2010). Hox genes and regional patterning of the vertebrate

body plan. Dev Biol *344*, 7–15.

Margueron, R., Justin, N., Ohno, K., Sharpe, M.L., Son, J., Drury, W.J., Voigt, P., Martin, S.R., Taylor, W.R., De Marco, V., et al. (2009). Role of the polycomb protein EED in the propagation of repressive histone marks. Nature *461*, 762–767.

Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, C.L., Dynlacht, B.D., and Reinberg, D. (2008). Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Mol Cell *32*, 503–518.

Martinez, A.-M., and Cavalli, G. (2006). The role of polycomb group proteins in cell cycle regulation during development. Cell Cycle *5*, 1189–1197.

Mateos-Langerak, J., and Cavalli, G. (2008). Polycomb group proteins and long-range gene regulation. Adv Genet *61*, 45–66.

Mattout, A., and Meshorer, E. (2010). Chromatin plasticity and genome organization in pluripotent embryonic stem cells. Curr Opin Cell Biol *22*, 334–341.

Mazzoni, E.O., Mahony, S., Peljto, M., Patel, T., Thornton, S.R., McCuine, S., Reeder, C., Boyer, L.A., Young, R.A., Gifford, D.K., et al. (2013). Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals. Nat. Neurosci. *16*, 1191–1198.

McCabe, M.T., Ott, H.M., Ganji, G., Korenchuk, S., Thompson, C., Van Aller, G.S., Liu, Y., Graves, A.P., Pietra, Della, A., Diaz, E., et al. (2012). EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature *492*, 108–112.

Meissner, A., Mikkelsen, T.S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B.E., Nusbaum, C., Jaffe, D.B., et al. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature *454*, 766–770.

Mendenhall, E.M., Koche, R.P., Truong, T., Zhou, V.W., Issac, B., Chi, A.S., Ku, M., and Bernstein, B.E. (2010). GC-rich sequence elements recruit PRC2 in mammalian ES cells. PLoS Genet *6*, e1001244.

Meshorer, E., Yellajoshula, D., George, E., Scambler, P.J., Brown, D.T., and Misteli, T. (2006). Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. Dev Cell *10*, 105–116.

Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, E.S., and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature *454*, 49–55.

Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T.-K., Koche, R.P., et al. (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553–560.

Min, I.M., Waterfall, J.J., Core, L.J., Munroe, R.J., Schimenti, J., and Lis, J.T. (2011). Regulating RNA polymerase pausing and transcription elongation in embryonic stem cells. Genes Dev *25*, 742–754.

Min, J., Zhang, Y., and Xu, R.-M. (2003). Structural basis for specific binding of Polycomb

chromodomain to histone H3 methylated at Lys 27. Genes Dev *17*, 1823–1828.

Mohn, F., Weber, M., Rebhan, M., Roloff, T.C., Richter, J., Stadler, M.B., Bibel, M., and Schübeler, D. (2008). Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell *30*, 755–766.

Montgomery, N.D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S.J., Otte, A.P., and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol *15*, 942–947.

Morey, C., Da Silva, N.R., Perry, P., and Bickmore, W.A. (2007). Nuclear reorganisation and chromatin decondensation are conserved, but distinct, mechanisms linked to Hox gene activation. Development *134*, 909–919.

Morey, L., Pascual, G., Cozzuto, L., Roma, G., Wutz, A., Benitah, S.A., and Di Croce, L. (2012). Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. Cell Stem Cell *10*, 47–62.

Morris, K.V. (2009). Long antisense non-coding RNAs function to direct epigenetic complexes that regulate transcription in human cells. Epigenetics *4*, 296–301.

Mousavi, K., Zare, H., Wang, A.H., and Sartorelli, V. (2012). Polycomb protein Ezh1 promotes RNA polymerase II elongation. Mol Cell *45*, 255–262.

Murry, C.E., and Keller, G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell *132*, 661–680.

Müller, J., and Kassis, J.A. (2006). Polycomb response elements and targeting of Polycomb group proteins in Drosophila. Current Opinion in Genetics & Development *16*, 476–484.

Müller, J., and Verrijzer, P. (2009). Biochemical mechanisms of gene regulation by polycomb group protein complexes. Current Opinion in Genetics & Development *19*, 150–158.

Neri, F., Incarnato, D., Krepelova, A., Rapelli, S., Pagnani, A., Zecchina, R., Parlato, C., and Oliviero, S. (2013a). Genome-wide analysis identifies a functional association of Tet1 and Polycomb PRC2 in mouse embryonic stem cells. Genome Biol *14*, R91.

Neri, F., Krepelova, A., Incarnato, D., Maldotti, M., Parlato, C., Galvagni, F., Matarese, F., Stunnenberg, H.G., and Oliviero, S. (2013b). Dnmt3L Antagonizes DNA Methylation at Bivalent Promoters and Favors DNA Methylation at Gene Bodies in ESCs. Cell *155*, 121–134.

O'Carroll, D., Erhardt, S., Pagani, M., Barton, S.C., Surani, M.A., and Jenuwein, T. (2001). The polycomb-group gene Ezh2 is required for early mouse development. Mol Cell Biol *21*, 4330–4336.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247–257.

Orkin, S.H., Wang, J., Kim, J., Chu, J., Rao, S., Theunissen, T.W., Shen, X., and Levasseur, D.N. (2008). The transcriptional network controlling pluripotency in ES cells. Cold Spring Harb. Symp. Quant. Biol. *73*, 195–202.

Pan, G., Tian, S., Nie, J., Yang, C., Ruotti, V., Wei, H., Jonsdottir, G.A., Stewart, R., and Thomson, J.A. (2007). Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. Cell Stem Cell *1*, 299–312.

Pandey, R.R., Mondal, T., Mohammad, F., Enroth, S., Redrup, L., Komorowski, J., Nagano, T., Mancini-Dinardo, D., and Kanduri, C. (2008). Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Mol Cell *32*, 232–246.

Pasini, D., Bracken, A.P., Hansen, J.B., Capillo, M., and Helin, K. (2007). The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol *27*, 3769–3779.

Pasini, D., Bracken, A.P., Jensen, M.R., Lazzerini Denchi, E., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J *23*, 4061–4071.

Pasini, D., Cloos, P.A.C., Walfridsson, J., Olsson, L., Bukowski, J.-P., Johansen, J.V., Bak, M., Tommerup, N., Rappsilber, J., and Helin, K. (2010). JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. Nature *464*, 306–310.

Peng, J.C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A., and Wysocka, J. (2009). Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell *139*, 1290–1302.

Pengelly, A.R., Copur, Ö., Jäckle, H., Herzig, A., and Müller, J. (2013). A histone mutant reproduces the phenotype caused by loss of histone-modifying factor Polycomb. Science *339*, 698–699.

Pietersen, A.M., and van Lohuizen, M. (2008). Stem cell regulation by polycomb repressors: postponing commitment. Curr Opin Cell Biol *20*, 201–207.

Pirity, M.K., Locker, J., and Schreiber-Agus, N. (2005). Rybp/DEDAF is required for early postimplantation and for central nervous system development. Mol Cell Biol *25*, 7193–7202.

Pirity, M.K., Wang, W.-L., Wolf, L.V., Tamm, E.R., Schreiber-Agus, N., and Cvekl, A. (2007). Rybp, a polycomb complex-associated protein, is required for mouse eye development. BMC Dev Biol *7*, 39.

Piunti, A., and Pasini, D. (2011). Epigenetic factors in cancer development: Polycomb group proteins. Future Oncology *7*, 57–75.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., La Cruz, de, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131–135.

Pontier, D.B., and Gribnau, J. (2011). Xist regulation and function explored. Hum Genet *130*, 223–236.

Ponting, C.P., Oliver, P.L., and Reik, W. (2009). Evolution and functions of long noncoding RNAs. Cell *136*, 629–641.

Prezioso, C., and Orlando, V. (2011). Polycomb proteins in mammalian cell differentiation and plasticity. FEBS Lett *585*, 2067–2077.

Reddington, J.P., Perricone, S.M., Nestor, C.E., Reichmann, J., Youngson, N.A., Suzuki, M., Reinhardt, D.,

Dunican, D.S., Prendegast, J.G., Mjoseng, H., et al. (2013). Redistribution of H3K27me3 upon DNA hypomethylation results in de-repression of Polycomb-target genes. Genome Biol *14*, R25.

Ren, X., and Kerppola, T.K. (2011). REST interacts with Cbx proteins and regulates polycomb repressive complex 1 occupancy at RE1 elements. Mol Cell Biol *31*, 2100–2110.

Richly, H., Aloia, L., and Di Croce, L. (2011). Roles of the Polycomb group proteins in stem cells and cancer. Cell Death Dis *2*, e204.

Richly, H., Rocha-Viegas, L., Ribeiro, J.D., Demajo, S., Gundem, G., Lopez-Bigas, N., Nakagawa, T., Rospert, S., Ito, T., and Di Croce, L. (2010). Transcriptional activation of polycomb-repressed genes by ZRF1. Nature *468*, 1124–1128.

Ringrose, L., and Paro, R. (2007). Polycomb/Trithorax response elements and epigenetic memory of cell identity. Development *134*, 223–232.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., et al. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311–1323.

Rush, M., Appanah, R., Lee, S., Lam, L.L., Goyal, P., and Lorincz, M.C. (2009). Targeting of EZH2 to a defined genomic site is sufficient for recruitment of Dnmt3a but not de novo DNA methylation. Epigenetics *4*, 404–414.

Scheuermann, J.C., de Ayala Alonso, A.G., Oktaba, K., Ly-Hartig, N., McGinty, R.K., Fraterman, S., Wilm, M., Muir, T.W., and Müller, J. (2010). Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB. Nature *465*, 243–247.

Schmitges, F.W., Prusty, A.B., Faty, M., Stützer, A., Lingaraju, G.M., Aiwazian, J., Sack, R., Hess, D., Li, L., Zhou, S., et al. (2011). Histone methylation by PRC2 is inhibited by active chromatin marks. Mol Cell *42*, 330–341.

Schorderet, P., and Duboule, D. (2011). Structural and functional differences in the long non-coding RNA hotair in mouse and human. PLoS Genet *7*, e1002071.

Schuettengruber, B., and Cavalli, G. (2009). Recruitment of polycomb group complexes and their role in the dynamic regulation of cell fate choice. Development *136*, 3531–3542.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. Cell *128*, 735–745.

Seenundun, S., Rampalli, S., Liu, Q.-C., Aziz, A., Palii, C., Hong, S., Blais, A., Brand, M., Ge, K., and Dilworth, F.J. (2010). UTX mediates demethylation of H3K27me3 at muscle-specific genes during myogenesis. Embo J *29*, 1401–1411.

Shen, X., Kim, W., Fujiwara, Y., Simon, M.D., Liu, Y., Mysliwiec, M.R., Yuan, G.-C., Lee, Y., and Orkin, S.H. (2009). Jumonji Modulates Polycomb Activity and Self-Renewal versus Differentiation of Stem Cells. Cell *139*, 1303–1314.

Shen, X., Liu, Y., Hsu, Y.-J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G.-C., and Orkin, S.H. (2008). EZH1

mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell *32*, 491–502.

Shumacher, A., Faust, C., and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature *383*, 250–253.

Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H.F.M., Jenuwein, T., Otte, A.P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev Cell *4*, 481–495.

Simon, J.A., and Kingston, R.E. (2009). Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol *10*, 697–708.

Sing, A., Pannell, D., Karaiskakis, A., Sturgeon, K., Djabali, M., Ellis, J., Lipshitz, H.D., and Cordes, S.P. (2009). A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. Cell *138*, 885–897.

Soshnikova, N., and Duboule, D. (2009). Epigenetic regulation of vertebrate Hox genes: a dynamic equilibrium. Epigenetics *4*, 537–540.

Sparmann, A., and van Lohuizen, M. (2006). Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer *6*, 846–856.

Spitale, R.C., Tsai, M.-C., and Chang, H.Y. (2011). RNA templating the epigenome: long noncoding RNAs as molecular scaffolds. Epigenetics *6*, 539–543.

Squazzo, S.L., O'Geen, H., Komashko, V.M., Krig, S.R., Jin, V.X., Jang, S.-W., Margueron, R., Reinberg, D., Green, R., and Farnham, P.J. (2006). Suz12 binds to silenced regions of the genome in a cell-type-specific manner. Genome Res *16*, 890–900.

Statham, A.L., Robinson, M.D., Song, J.Z., Coolen, M.W., Stirzaker, C., and Clark, S.J. (2012). Bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq) directly informs methylation status of histone-modified DNA. Genome Res *22*, 1120–1127.

Stock, J.K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A.G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat Cell Biol *9*, 1428–1435.

Surface, L.E., Thornton, S.R., and Boyer, L.A. (2010). Polycomb group proteins set the stage for early lineage commitment. Cell Stem Cell *7*, 288–298.

Swigut, T., and Wysocka, J. (2007). H3K27 demethylases, at long last. Cell *131*, 29–32.

Takeuchi, T., Yamazaki, Y., Katoh-Fukui, Y., Tsuchiya, R., Kondo, S., Motoyama, J., and Higashinakagawa, T. (1995). Gene trap capture of a novel mouse gene, jumonji, required for neural tube formation. Genes Dev *9*, 1211–1222.

Takihara, Y., Tomotsune, D., Shirai, M., Katoh-Fukui, Y., Nishii, K., Motaleb, M.A., Nomura, M., Tsuchiya, R., Fujita, Y., Shibata, Y., et al. (1997). Targeted disruption of the mouse homologue of the Drosophila

polyhomeotic gene leads to altered anteroposterior patterning and neural crest defects. Development *124*, 3673–3682.

Tanay, A., O'Donnell, A.H., Damelin, M., and Bestor, T.H. (2007). Hyperconserved CpG domains underlie Polycomb-binding sites. Proc Natl Acad Sci USA *104*, 5521–5526.

Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., et al. (2012). RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. Cell *148*, 664–678.

Terranova, R., Yokobayashi, S., Stadler, M.B., Otte, A.P., van Lohuizen, M., Orkin, S.H., and Peters, A.H.F.M. (2008). Polycomb group proteins Ezh2 and Rnf2 direct genomic contraction and imprinted repression in early mouse embryos. Dev Cell *15*, 668–679.

Tiwari, V.K., Cope, L., Mcgarvey, K.M., Ohm, J.E., and Baylin, S.B. (2008a). A novel 6C assay uncovers Polycomb-mediated higher order chromatin conformations. Genome Res *18*, 1171–1179.

Tiwari, V.K., Mcgarvey, K.M., Licchesi, J.D.F., Ohm, J.E., Herman, J.G., Schübeler, D., and Baylin, S.B. (2008b). PcG proteins, DNA methylation, and gene repression by chromatin looping. PLoS Biol *6*, 2911–2927.

Tolhuis, B., Blom, M., Kerkhoven, R.M., Pagie, L., Teunissen, H., Nieuwland, M., Simonis, M., de Laat, W., van Lohuizen, M., and van Steensel, B. (2011). Interactions among Polycomb domains are guided by chromosome architecture. PLoS Genet *7*, e1001343.

Trojer, P., Cao, A.R., Gao, Z., Li, Y., Zhang, J., Xu, X., Li, G., Losson, R., Erdjument-Bromage, H., Tempst, P., et al. (2011). L3MBTL2 protein acts in concert with PcG protein-mediated monoubiquitination of H2A to establish a repressive chromatin structure. Mol Cell *42*, 438–450.

Tsai, M.-C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science *329*, 689–693.

Ulitsky, I., Shkumatava, A., Jan, C.H., Sive, H., and Bartel, D.P. (2011). Conserved function of lincRNAs in vertebrate embryonic development despite rapid sequence evolution. Cell *147*, 1537–1550.

van der Lugt, N.M., Alkema, M., Berns, A., and Deschamps, J. (1996). The Polycomb-group homolog Bmi-1 is a regulator of murine Hox gene expression. Mech Dev *58*, 153–164.

van der Stoop, P., Boutsma, E.A., Hulsman, D., Noback, S., Heimerikx, M., Kerkhoven, R.M., Voncken, J.W., Wessels, L.F.A., and van Lohuizen, M. (2008). Ubiquitin E3 ligase Ring1b/Rnf2 of polycomb repressive complex 1 contributes to stable maintenance of mouse embryonic stem cells. PLoS ONE *3*, e2235.

van Haaften, G., Dalgliesh, G.L., Davies, H., Chen, L., Bignell, G., Greenman, C., Edkins, S., Hardy, C., O'Meara, S., Teague, J., et al. (2009). Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet *41*, 521–523.

Varley, K.E., Gertz, J., Bowling, K.M., Parker, S.L., Reddy, T.E., Pauli-Behn, F., Cross, M.K., Williams, B.A., Stamatoyannopoulos, J.A., Crawford, G.E., et al. (2013). Dynamic DNA methylation across diverse human cell lines and tissues. Genome Res *23*, 555–567.

Vastenhouw, N.L., Zhang, Y., Woods, I.G., Imam, F., Regev, A., Liu, X.S., Rinn, J., and Schier, A.F. (2010). Chromatin signature of embryonic pluripotency is established during genome activation. Nature *464*, 922–926.

Vincenz, C., and Kerppola, T.K. (2008). Different polycomb group CBX family proteins associate with distinct regions of chromatin using nonhomologous protein sequences. Proc Natl Acad Sci USA *105*, 16572–16577.

Viré, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J.-M., et al. (2006). The Polycomb group protein EZH2 directly controls DNA methylation. Nature *439*, 871–874.

Voncken, J.W., Roelen, B.A.J., Roefs, M., de Vries, S., Verhoeven, E., Marino, S., Deschamps, J., and van Lohuizen, M. (2003). Rnf2 (Ring1b) deficiency causes gastrulation arrest and cell cycle inhibition. Proc Natl Acad Sci USA *100*, 2468–2473.

Walker, E., Chang, W.Y., Hunkapiller, J., Cagney, G., Garcha, K., Torchia, J., Krogan, N.J., Reiter, J.F., and Stanford, W.L. (2010). Polycomb-like 2 associates with PRC2 and regulates transcriptional networks during mouse embryonic stem cell self-renewal and differentiation. Cell Stem Cell *6*, 153–166.

Walker, E., Ohishi, M., Davey, R.E., Zhang, W., Cassar, P.A., Tanaka, T.S., Der, S.D., Morris, Q., Hughes, T.R., Zandstra, P.W., et al. (2007). Prediction and testing of novel transcriptional networks regulating embryonic stem cell self-renewal and commitment. Cell Stem Cell *1*, 71–86.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004a). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873–878.

Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004b). Hierarchical recruitment of polycomb group silencing complexes. Mol Cell *14*, 637–646.

Wang, R., Taylor, A.B., Leal, B.Z., Chadwell, L.V., Ilangovan, U., Robinson, A.K., Schirf, V., Hart, P.J., Lafer, E.M., Demeler, B., et al. (2010). Polycomb group targeting through different binding partners of RING1B C-terminal domain. Structure *18*, 966–975.

Wang, S., He, F., Xiong, W., Gu, S., Liu, H., Zhang, T., Yu, X., and Chen, Y. (2007). Polycomblike-2-deficient mice exhibit normal left-right asymmetry. Dev Dyn *236*, 853–861.

Weake, V.M., and Workman, J.L. (2008). Histone ubiquitination: triggering gene activity. Mol Cell *29*, 653–663.

White, J., and Dalton, S. (2005). Cell cycle control of embryonic stem cells. Stem Cell Rev *1*, 131–138.

Woo, C.J., Kharchenko, P.V., Daheron, L., Park, P.J., and Kingston, R.E. (2010). A region of the human HOXD cluster that confers polycomb-group responsiveness. Cell *140*, 99–110.

Wu, H., Alessio, A.C.D., Ito, S., Xia, K., Wang, Z., Cui, K., Zhao, K., Sun, Y.E., and Zhang, Y. (2011). Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. Nature 1–6.

Wu, H., Coskun, V., Tao, J., Xie, W., Ge, W., Yoshikawa, K., Li, E., Zhang, Y., and Sun, Y.E. (2010a). Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science *329*,

444–448.

Wu, H.-A., and Bernstein, E. (2008). Partners in imprinting: noncoding RNA and polycomb group proteins. Dev Cell *15*, 637–638.

Wu, S.C., Kallin, E.M., and Zhang, Y. (2010b). Role of H3K27 methylation in the regulation of lncRNA expression. Cell Res *20*, 1109–1116.

Wu, X., Johansen, J.V., and Helin, K. (2013). Fbxl10/Kdm2b recruits polycomb repressive complex 1 to CpG islands and regulates H2A ubiquitylation. Mol Cell *49*, 1134–1146.

Xiang, Y., Zhu, Z., Han, G., Lin, H., Xu, L., and Chen, C.D. (2007). JMJD3 is a histone H3K27 demethylase. Cell Res *17*, 850–857.

Xu, C., Bian, C., Yang, W., Galka, M., Ouyang, H., Chen, C., Qiu, W., Liu, H., Jones, A.E., MacKenzie, F., et al. (2010). Binding of different histone marks differentially regulates the activity and specificity of polycomb repressive complex 2 (PRC2). Proc Natl Acad Sci USA *107*, 19266–19271.

Yap, K.L., Li, S., Muñoz-Cabello, A.M., Raguz, S., Zeng, L., Mujtaba, S., Gil, J., Walsh, M.J., and Zhou, M.-M. (2010). Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell *38*, 662–674.

Yu, M., Mazor, T., Huang, H., Huang, H.-T., Kathrein, K.L., Woo, A.J., Chouinard, C.R., Labadorf, A., Akie, T.E., Moran, T.B., et al. (2012). Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. Mol Cell *45*, 330–343.

Yuan, W., Wu, T., Fu, H., Dai, C., Wu, H., Liu, N., Li, X., Xu, M., Zhang, Z., Niu, T., et al. (2012). Dense Chromatin Activates Polycomb Repressive Complex 2 to Regulate H3 Lysine 27 Methylation. Science *337*, 971–975.

Yuan, W., Xu, M., Huang, C., Liu, N., Chen, S., and Zhu, B. (2011). H3K36 methylation antagonizes PRC2-mediated H3K27 methylation. J Biol Chem *286*, 7983–7989.

Zhao, J., Ohsumi, T.K., Kung, J.T., Ogawa, Y., Grau, D.J., Sarma, K., Song, J.-J., Kingston, R.E., Borowsky, M., and Lee, J.T. (2010). Genome-wide identification of polycomb-associated RNAs by RIP-seq. Mol Cell *40*, 939–953.

Zhao, J., Sun, B.K., Erwin, J.A., Song, J.-J., and Lee, J.T. (2008). Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science *322*, 750–756.

Zhao, X.D., Han, X., Chew, J.L., Liu, J., Chiu, K.P., Choo, A., Orlov, Y.L., Sung, W.-K., Shahab, A., Kuznetsov, V.A., et al. (2007). Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell *1*, 286–298.

Zhou, Q., Chipperfield, H., Melton, D.A., and Wong, W.H. (2007). A gene regulatory network in mouse embryonic stem cells. Proc Natl Acad Sci USA *104*, 16438–16443.

Ziller, M.J., Müller, F., Liao, J., Zhang, Y., Gu, H., Bock, C., Boyle, P., Epstein, C.B., Bernstein, B.E., Lengauer, T., et al. (2011). Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. PLoS Genet *7*, e1002389.

Chapter 2

Polycomb Repressive Complex 2 mediates lineage fidelity during embryonic stem cell differentiation

Author contributions for Chapter 2:

All experiments were designed by Seraphim Thornton and Laurie Boyer, and performed by Seraphim Thornton, with exception of the following: RNA-Seq on wild-type and *Suz12GT* ESCs contributed by Joseph Wamstad; Reduced Representation Bisulfite Sequencing (RRBS) performed by the Gnirke lab at the Broad Institute; RRBS data analysis, performed by Vincent Butty and Stuart Levine of the MIT BioMicroCenter. The manuscript was prepared by Seraphim Thornton and Laurie Boyer.

Abstract

Polycomb Repressive Complex 2 (PRC2) catalyzes histone H3 lysine 27 tri-methylation (H3K27me3), an epigenetic modification associated with gene repression. H3K27me3 is enriched at the promoters of a large cohort of developmental genes in embryonic stem cells (ESCs). Loss of H3K27me3 leads to a failure of ESCs to properly differentiate, making it difficult to determine the precise roles of PRC2 during lineage commitment. Moreover, while studies suggest that PRC2 prevents DNA methylation, how these two epigenetic regulators coordinate gene expression programs during lineage commitment is poorly understood. We analyzed global gene expression by RNA-Seq and DNA methylation profiles by reduced representations bisulfite sequencing in several PRC2 mutant ESC lines that maintain varying levels of H3K27me3. We found that partial maintenance of H3K27me3 allowed for proper temporal activation of lineage genes during directed differentiation of ESCs to spinal motor neurons (SMNs). In contrast, genes that function to specify other lineages failed to be repressed in these cells, suggesting that PRC2 activity is necessary for lineage fidelity. We also found that H3K27me3 is directly antagonistic to DNA methylation as loss of this mark leads to a modest gain in DNA methylation at PRC2 target regions in both ESCs and in SMNs. Our study demonstrates a critical role for PRC2 in coordinating lineage decisions and for maintaining lineage fidelity, and may protect against inappropriate DNA methylation during differentiation.

Introduction

Regulation of chromatin structure is a key mechanism used by cells to control gene expression patterns in response to developmental and environmental cues. Polycomb Group (PcG) proteins play crucial roles in epigenetic gene regulation in all metazoans by modifying chromatin structure. PcG proteins function in a variety of biological pathways, including lineage commitment in mammals (Di Croce and Helin, 2013; Simon and Kingston, 2009; Surface et al., 2010). Ablation of any core Polycomb Repressive Complex 2 (PRC2) component, including SUZ12, EED, or EZH2 (the catalytic histone methyltransferase), leads to embryonic lethality in mice during gastrulation, a developmental time point when complex gene expression patterns are established in the embryo (Faust et al., 1995; O'Carroll et al., 2001; Pasini et al., 2004). PRC2 catalyzes trimethylation of histone H3 lysine 27 (H3K27me3), a modification associated with transcriptional repression (Cao et al., 2002). In *Drosophila*, mutations in histone H3 that disrupt K27 methylation lead to phenotypes similar to Polycomb mutants, indicating that H3K27me3 is a crucial mediator of PRC2 function (Pengelly et al., 2013). While current evidence indicates that PRC2 activity plays roles in the initiation of gene repression and temporal regulation of cell fate, how it accomplishes this task is incompletely understood.

Embryonic stem cells (ESCs) have the potential to become any type of cell in the adult organism. This property underpins their utility as a model system to study the mechanisms that drive cell differentiation. In ESCs, PRC2 occupies a large cohort of developmental genes to regulate lineage commitment (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006a; Tolhuis et al., 2006). At these genes, H3K27me3 is largely enriched at transcription start sites (TSSs) along with H3K4me3, an activating histone mark associated with Trithorax group (TrxG) proteins (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007). These “bivalent” promoters are thought to poise genes for later activation during lineage commitment. Bivalent genes in ESCs mostly resolve to either an active (H3K4me3 only) or repressed (H3K27me3 only) state during differentiation (Mikkelsen et al., 2007),

suggesting that H3K27me3 is critical for both gene repression and for the proper activation of developmental programs during lineage commitment. However, we lack a detailed understanding of how disruption of PRC2 activity in ESCs affects the regulation of genes during lineage commitment because loss of H3K27me3 leads to a global failure of these cells to properly differentiate.

Emerging evidence suggests crosstalk between PRC2 and the DNA methylation machinery to ensure proper development. For example, H3K27me3 and DNA methylation are largely exclusive at promoters across human tissues (Varley et al., 2013) and DNA hypomethylation of bivalent promoters in ESCs appears necessary for maintaining developmental plasticity (Neri et al., 2013b). Recent studies using Me-DIP showed that loss of H3K27me3 in *Eed*^{null} ESCs leads to changes in DNA methylation levels, however, the resolution of this assay was not sufficient to test a direct relationship at genomic sites between these two regulatory pathways and the role of PRC2 in lineage commitment was not investigated (Hagarman et al., 2013). Notably, PRC2 target genes tend to be DNA hypomethylated in cancer cells that show high levels of Polycomb components such as *Ezh2* (Hawkins et al., 2010; Lister and Ecker, 2009). Thus, while these data suggest that at least in some cases Polycomb activity antagonizes DNA methylation (Mohn et al., 2008), we know little about how their activities are coordinated during lineage commitment. Thus, knowledge of how PRC2 regulates lineage commitment will be critical for understanding its roles in development and how misregulation of its activity leads to diseases such as cancer.

We investigated the role of PRC2 in regulating gene expression patterns during lineage commitment by analyzing several mutant ESC lines that maintain varying levels of H3K27me3. In particular, we found that the *Suz12* gene trap (*Suz12*^{GT}) ESC line (Pasini et al., 2007) maintained intermediate levels of H3K27me3 and was able to undergo directed differentiation, unlike *Suz12* truncation (*Suz12*$^{\Delta}$) or *Eed* point mutant (*Eed*^{null}) ESC lines, albeit less efficiently compared to wild-type cells (Chamberlain et al.,

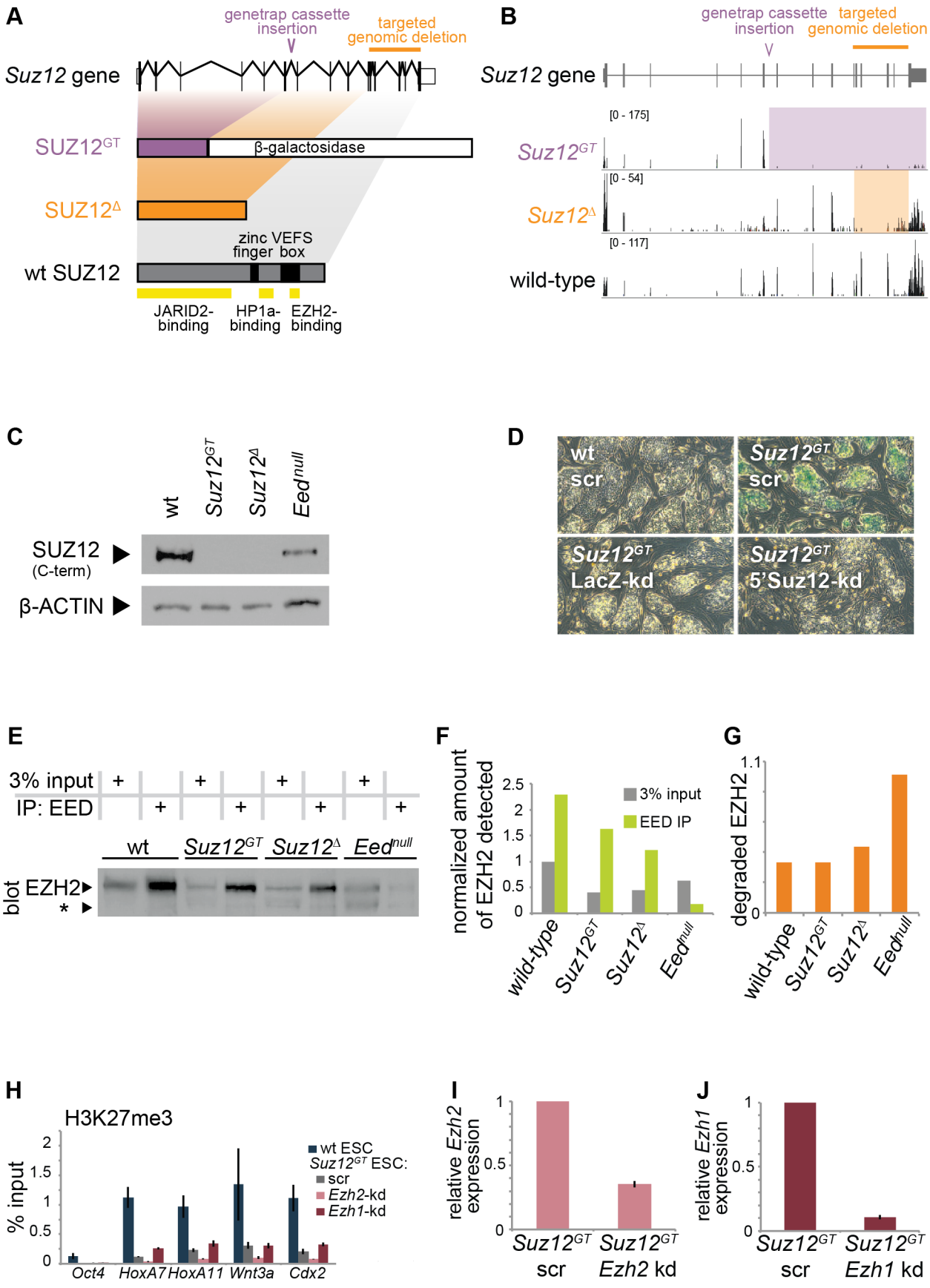
2008; Lee et al., 2006a). Using this set of genetic tools, we demonstrate that proper H3K27me3 levels are necessary for both temporal activation of lineage programs and for repression of alternate pathways to maintain lineage fidelity during directed differentiation of ESCs toward spinal motor neurons (SMNs). We next analyzed changes in DNA methylation levels in *Suz12*^{GT} cells during SMN differentiation at nucleotide resolution and found that loss of H3K27me3 directly led to a modest gain in DNA methylation at PRC2 target regions compared to regions that maintained H3K27me3 enrichment. While the modest increase of DNA methylation did not lead to apparent changes in expression PRC2 targets in *Suz12*^{GT} cells compared to wild-type cells, we propose that this low-level gain of DNA methylation at promoters may lead to further epigenetic instability of expression states during differentiation. Thus, our findings indicate that PRC2 activity is necessary to maintain cell fate plasticity and lineage fidelity during differentiation, and may safeguard developmental genes against more permanent repression.

Results

PRC2 mutant ESC lines maintain varying levels of H3K27me3

PRC2 catalyzes H3K27me3 (Cao and Zhang, 2004; Cao et al., 2002; Czermin et al., 2002; Kirmizis et al., 2004; Kuzmichev et al., 2002), and its recruitment to target promoters in ESCs suggests a critical role for PRC2 in regulating gene expression programs during mammalian development (Boyer et al., 2006; Ku et al., 2008; Lee et al., 2006a). Because PRC2-null ESCs are unable to undergo proper directed differentiation, how the activity of PRC2 regulates gene expression during lineage commitment is poorly understood. We recently showed that a *Suz12* mutant ESC line created by a genetrap insertion (denoted here as *Suz12*^{GT}) (Pasini et al., 2007) maintained H3K27me3, albeit at reduced levels compared to wild-type ESCs, as determined by ChIP-Seq (Mazzoni et al., 2013). The *Suz12*^{GT} allele contains a genetrap insertion that results in a fusion between the N-terminal 276 amino acids of SUZ12 and β-galactosidase (Figure 1A-C). The loss of H3K27me3 in *Suz12*^{GT} cells is rescued by

Figure 1. Comparison of PRC2 mutant ESC lines. (A) At top, a diagram of the structure of the wild-type (wt) *Suz12* gene. Below, the proteins encoded by the two mutant alleles used here ($SUZ12^{GT}$ and $SUZ12^{\Delta}$) and the wt allele are shown to scale, and domains of interest are noted on wt SUZ12. **(B)** RNA-seq data shows the expected *Suz12* mRNA in $Suz12^{GT}$, $Suz12^{\Delta}$, and wild-type (wt) ESCs. **(C)** Cell lysates from wt, $Suz12^{GT}$, $Suz12^{\Delta}$, and Eed^{null} ESCs were subjected to SDS-PAGE and western blotting with an antibody recognizing the C-terminal region of SUZ12. β-actin is included as a loading control. **(D)** X-gal staining was performed on wt ESCs (upper left) and $Suz12^{GT}$ ESCs (upper right) expressing either a scrambled control hairpin, a hairpin targeted to *LacZ* (encoding β-galactosidase) (lower left), or a hairpin targeted to the 5′ end of *Suz12* (lower right). **(E)** Immunoprecipitation of EED was performed in wt, $Suz12^{GT}$, $Suz12^{\Delta}$, and Eed^{null} ESCs. The samples, including 3% input, were subjected to SDS-PAGE. EZH2 immunoblot was performed as indicated by the labeled band (left). EZH2 degradation product is marked by an asterisk (*). **(F)** The immunoblot shown in (E) was quantified using QuantityOne software. Amount of EZH2 detected was normalized to the amount in the wild-type 3% input sample. **(G)** The degraded EZH2 (marked as *) in the immunoblot shown in (E) was quantified using QuantityOne software and plotted normalized to the highest amount. **(H)** ChIP-qPCR for H3K27me3 was performed on wt and $Suz12^{GT}$ ESCs expressing hairpins: scr (scrambled control), *Ezh2*-kd (targeted to *Ezh2*), and *Ezh1*-kd (targeted to *Ezh1*). All genes tested except *Oct4* are PRC2 target genes. Error bars show standard deviation of three technical replicates. **(I-J)** qRT-PCR was used to measure the depletion of **(I)** *Ezh2*, and **(J)** *Ezh1* with respect to $Suz12^{GT}$ ESCs expressing a scrambled control hairpin. Error bars represent the standard deviation of three technical replicates.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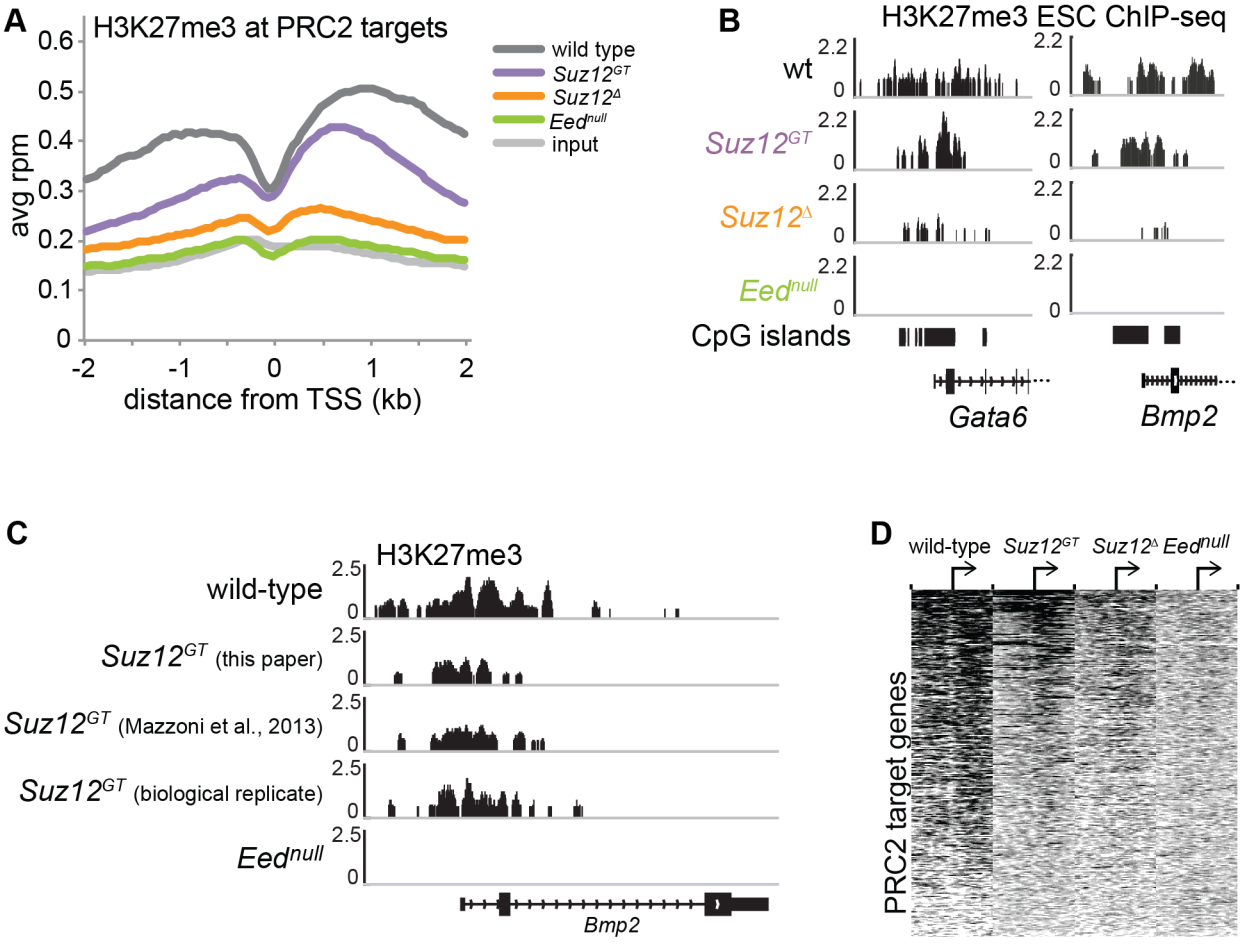
zinc VEFS finger box
wt SUZ12
JARID2-binding
HP1a-binding
EZH2-binding
B
genetrap cassette insertion
targeted genomic deletion
Suz12 gene
[0 - 175]
Suz12GT
[0 - 54]
Suz12Δ
[0 - 117]
wild-type
C
wt
Suz12GT
Suz12Δ
Eednull
SUZ12 (C-term)
β-ACTIN
D
wt scr
Suz12GT scr
Suz12GT LacZ-kd
Suz12GT 5'Suz12-kd
E
3% input
IP: EED
wt
Suz12GT
Suz12Δ
Eednull
blot EZH2
*
F
normalized amount of EZH2 detected
3% input
EED IP
wild-type
Suz12GT
Suz12Δ
Eednull
G
degraded EZH2
wild-type
Suz12GT
Suz12Δ
Eednull
H
H3K27me3
% input
wt ESC
Suz12GT ESC:
scr
Ezh2-kd
Ezh1-kd
Oct4
HoxA7
HoxA11
Wnt3a
Cdx2
I
relative Ezh2 expression
Suz12GT scr
Suz12GT Ezh2 kd
J
relative Ezh1 expression
Suz12GT scr
Suz12GT Ezh1 kd

Figure 2. *Suz12*^{GT} ESCs maintain partial H3K27me3 genome-wide. ChIP-seq for H3K27me3 was performed on wt, *Suz12*^{GT}, *Suz12*$^{\Delta}$, and *Eed*^{null} ESCs. ChIP-seq datasets are normalized to the total mapped reads. **(A)** A metagene analysis of H3K27me3 ChIP-seq enrichment is shown across the average of all PRC2 target genes +/- 2kb relative to the TSS for wt, *Suz12*^{GT}, *Suz12*$^{\Delta}$, and *Eed*^{null} ESCs, as well as input. **(B)** H3K27me3 ChIP-seq tracks in ESCs. Representative examples of PRC2 target promoters (*Gata6* and *Bmp2*) showing H3K27me3 levels in *Suz12*^{GT}, *Suz12*$^{\Delta}$, and *Eed*^{null} ESCs. **(C)** Three distinct H3K27me3 ChIP-seq experiments on *Suz12*^{GT} ESCs show a similar localization pattern with respect to wt ESCs and *Eed*^{null} ESCs, as shown here at representative PRC2 target gene *Bmp2*. **(D)** ChIP-seq signal is shown in density plots at the TSS +/- 2kb. Each horizontal line is one PRC2 target gene. Reads per million in 50bp bins is represented on a white to black scale, with black being the 95th percentile value. Genes were sorted with respect to wt H3K27me3 signal.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H3K27me3 at PRC2 targets
avg rpm
0.6
0.5
0.4
0.3
0.2
0.1
0
-2
-1
0
1
2
distance from TSS (kb)
wild type
Suz12GT
Suz12Δ
Eednull
input
B
H3K27me3 ESC ChIP-seq
wt
Suz12GT
Suz12Δ
Eednull
CpG islands
2.2
0
Gata6
Bmp2
C
H3K27me3
wild-type
Suz12GT (this paper)
Suz12GT (Mazzoni et al., 2013)
Suz12GT (biological replicate)
Eednull
2.5
0
Bmp2
D
wild-type
Suz12GT
Suz12Δ
Eednull
PRC2 target genes

exogenous expression of wild-type *Suz12*, confirming that the phenotype is caused by a loss of function in SUZ12 (Yuan et al., 2012). For comparison, we obtained a second *Suz12* mutant ESC line that harbors a targeted genomic deletion leading to truncation of SUZ12 and to complete loss of H2K27me3 (denoted here as $Suz12^{\Delta}$) (Lee et al., 2006a) (Figure 1A-C). Similarly, we used an ESC line that harbors a point mutation in *Eed* (Eed^{null}) that results in destabilization of the protein as well as the PRC2 complex, and to complete loss of H3K27me3 (Montgomery et al., 2005).

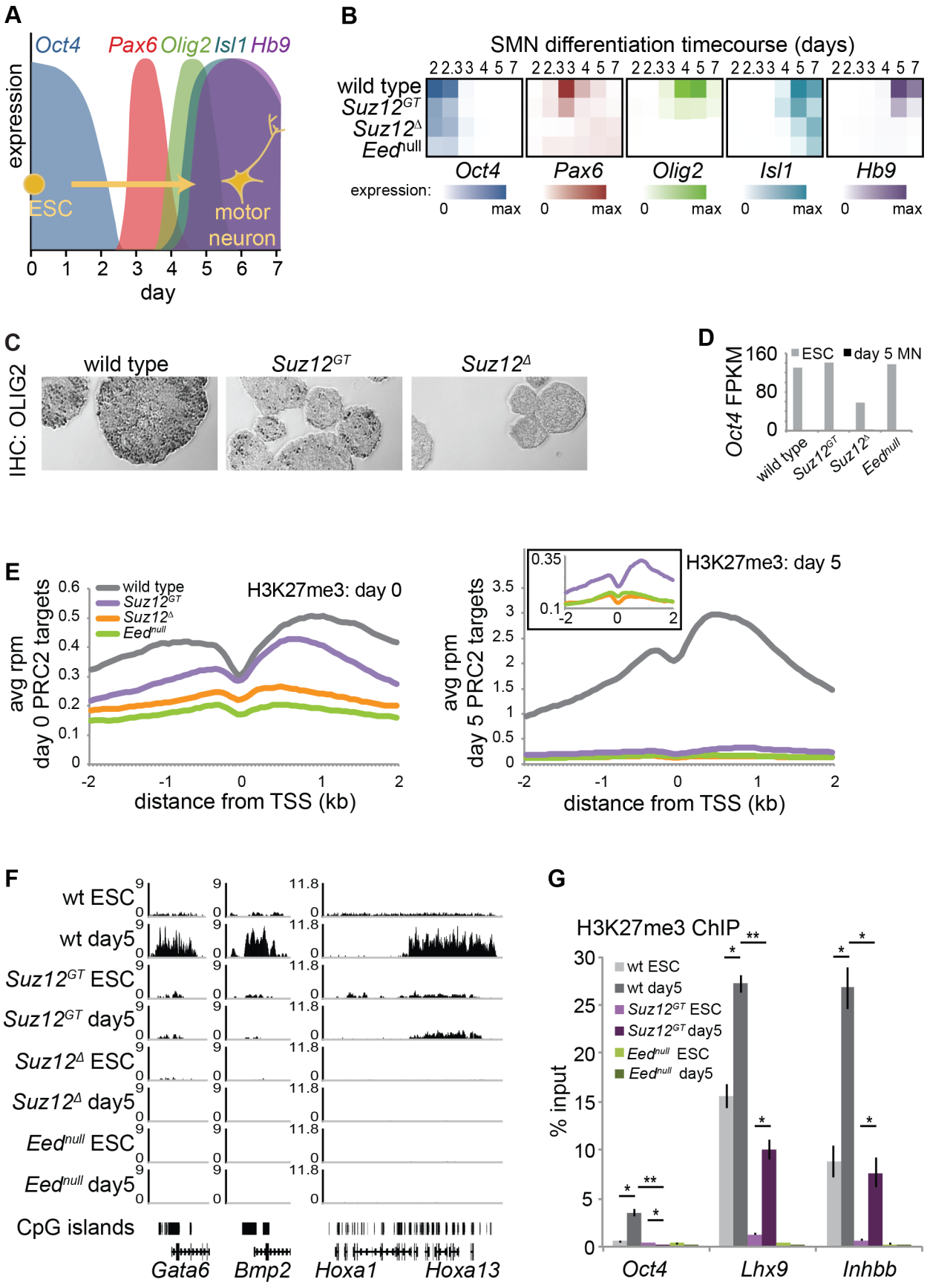
The difference in H3K27me3 levels in $Suz12^{GT}$ ESCs compared to $Suz12^{\Delta}$ lines suggested that the SUZ12-βgal fusion results in stabilization of the protein and to assembly of active PRC2 complex. Consistent with this idea, X-gal staining showed that a SUZ12-β-Galactosidase fusion protein was expressed in $Suz12^{GT}$ ESCs (Figure 1D). To test whether we could recover canonical PRC2 in mutant ESC lines, we immunoprecipitated PRC2 with an EED-specific antibody and resolved the complexes by SDS-PAGE followed by immunoblotting for EZH2. While EED interacted with EZH2 in both *Suz12* mutant ESC lines, we recovered lower levels of the complex in $Suz12^{\Delta}$ compared to $Suz12^{GT}$ cells as noted by the decrease in normalized signal observed on the immunoblot (Figure 1E-F). Moreover, EZH2 appeared more stable in $Suz12^{GT}$ ESCs compared to the $Suz12^{\Delta}$ or Eed^{null} ESC lines, as shown by the less prominent degradation product (Figure 1E,G). Prior studies have shown that EZH1, another H3K27-methyltransferase, can partially rescue loss of EZH2 in ESCs by forming an alternate form of PRC2 (Margueron et al., 2008; Shen et al., 2008). Thus, we also tested the possibility that H3K27me3 levels were maintained in $Suz12^{GT}$ ESCs through an EZH1-PRC2 complex. Whereas H3K27me3 levels were further diminished in $Suz12^{GT}$ ESCs upon shRNA-depletion of *Ezh2*, *Ezh1* suppression did not affect overall H3K27me3 levels at example target genes as measured by ChIP-qPCR (Figure 1H-J). Overall, these data suggest that the catalytic EZH2-containing PRC2 complex is partially maintained in the $Suz12^{GT}$ ESC line.

In wild-type ESCs, H3K27me3 is enriched at thousands of PRC2 target genes that are silent yet poised for activation. We analyzed the pattern of H3K27me3 enrichment by ChIP-Seq in *Suz12*^{GT} and wild-type ESCs. Close inspection of biological replicates showed that while H3K27me3 displayed lower average levels at PRC2 target genes in *Suz12*^{GT} ESCs, its pattern of enrichment at TSSs is similar to wild-type ESCs (Figure 2). In contrast, H3K27me3 is largely diminished in both *Suz12*$^{\Delta}$ and *Eed*^{null} ESCs. These data indicate that while PRC2 appears to be properly recruited to target sites in *Suz12*^{GT} ESCs, overall levels of H3K27me3 are not fully established or maintained in these cells.

Proper H3K27me3 levels are necessary for execution of lineage programs

We next investigated the role of PRC2 during differentiation using the various PRC2 mutant ESC lines, which has been a challenge because ESCs lacking H3K27me3 do not properly differentiate. As a model of lineage commitment, we performed directed differentiation of ESCs to Spinal Motor Neurons (SMNs) by removal of LIF and addition of retinoic acid and an agonist of the *Sonic Hedgehog* signaling pathway (Wichterle and Peljto, 2008) (Figure 3A). We found that genes normally activated in differentiating SMNs (e.g. *Pax6*, *Olig2*, *Isl1*, and *Hb9*) were expressed in a similar temporal manner in *Suz12*^{GT} cells albeit at lower levels compared to wild-type cells as detected by qRT-PCR, whereas these genes failed to activate in *Suz12*$^{\Delta}$ or *Eed*^{null} cells (Figure 3B). Consistent with this observation, immunohistochemistry showed that OLIG2, a PRC2 target and key transcription factor that directs SMN differentiation, was detected in a proportion of *Suz12*^{GT} cells at day 5 of differentiation, but not in *Suz12*$^{\Delta}$ cells (Figure 3C). In contrast, *Oct4*, a pluripotency gene that is not a PRC2 target in ESCs, was properly repressed in all cell types by day 5 of differentiation (Figure 3D). Thus, while all PRC2 mutant cell lines exited pluripotency, only *Suz12*^{GT} ESCs were able to express markers consistent with progression toward a SMN fate. Collectively, these data suggest that the genetrap allele functions as a hypomorph *in vitro* and can be used to study the role of PRC2 in lineage commitment.

Figure 3. H3K27me3 levels show differences across SMN differentiation in *Suz12^{GT}* cells compared to wt cells. (A) Cartoon showing changes in marker expression across the spinal motor neuron (SMN) differentiation time course. **(B)** Heatmap of qRT-PCR analysis of genes from (A). White: no expression; saturated color: the maximum expression level observed for that gene. Expression for each of the five genes is shown for wild-type (wt) (top), *Suz12^{GT}* (2nd), *Suz12$^{\Delta}$* (3rd), and *Eed^{null}* (bottom) cells. The time course progresses from left to right for 7 days. **(C)** IHC for OLIG2 on paraffin-embedded sectioned day 5 SMNs. OLIG2 expression is shown as darkly stained cells. **(D)** RNA-seq FPKM values for *Pou5f1* (*Oct4*) are plotted for wt, *Suz12^{GT}*, *Suz12$^{\Delta}$*, and *Eed^{null}* ESCs and day 5 SMNs. In **(E)** and **(F)**, ChIP-seq enrichment for H3K27me3 is shown for wt, *Suz12^{GT}*, *Suz12$^{\Delta}$*, and *Eed^{null}* ESCs and corresponding day 5 differentiated cells. **(E)** ChIP-seq datasets are represented as metagene plots showing average reads per million within 2kb of all TSSs for wt, *Suz12^{GT}*, *Suz12$^{\Delta}$*, and *Eed^{null}* cells. Day 0 (ESC) is at top, while day 5 SMN is shown at bottom. Inset in bottom graph with a smaller-scale y-axis is included to permit visualization of the differences between the three PRC2 mutant cell lines. **(F)** H3K27me3 ChIP-seq tracks for *Gata6* promoter (left); *Bmp2* promoter (middle); and the *HoxA* cluster (right) show that H3K27me3 levels change (increase, decrease, or stay the same, depending upon locus) in *Suz12^{GT}* cells upon differentiation whereas *Suz12$^{\Delta}$* and *Eed^{null}* cells show little to no H3K27me3 at day 5, consistent with lack of the mark in ESCs. **(G)** ChIP-qPCR data confirm that *Suz12^{GT}* cells are capable of gaining significant H3K27me3 at *Lhx9* and *Inhbb*, the two genes that gain the most H3K27me3 over differentiation in wt cells according to the ChIP-Seq data, whereas *Eed^{null}* cells show no gain in H3K27me3 at these genes. Error bars represent standard deviation of three technical replicates. P-values were calculated with a Student's two-sided *t*-test. *: p<5E-10; **: p<5E-15.


A
Oct4
Pax6
Olig2
Isl1
Hb9
expression
ESC
motor neuron
0 1 2 3 4 5 6 7
day
B
SMN differentiation timecourse (days)
2 2.3 3 4 5 7
wild type
Suz12GT
Suz12Δ
Eednull
Oct4
Pax6
Olig2
Isl1
Hb9
expression:
0 max
C
IHC: OLIG2
wild type
Suz12GT
Suz12Δ
D
Oct4 FPKM
160
80
0
ESC
day 5 MN
wild type
Suz12GT
Suz12Δ
Eednull
E
H3K27me3: day 0
avg rpm
day 0 PRC2 targets
wild type
Suz12GT
Suz12Δ
Eednull
distance from TSS (kb)
H3K27me3: day 5
day 5 PRC2 targets
F
wt ESC
wt day5
Suz12GT ESC
Suz12GT day5
Suz12Δ ESC
Suz12Δ day5
Eednull ESC
Eednull day5
CpG islands
Gata6
Bmp2
Hoxa1
Hoxa13
G
H3K27me3 ChIP
% input
wt ESC
wt day5
Suz12GT ESC
Suz12GT day5
Eednull ESC
Eednull day5
Oct4
Lhx9
Inhbb

Upon ESC differentiation, previous work has shown that a subset of PRC2 target genes gain H3K27me3 in large domains (Hawkins et al., 2010). Similarly, we observed an increase in H3K27me3 enrichment during SMN differentiation in wild-type cells (Figure 3E). Specifically, about half of PRC2 target genes in ESCs gain H3K27me3 by day 5 of SMN differentiation. Gene ontology (GO) analysis suggested that a larger than expected fraction of genes with increased H3K27me3 have functions in the regulation of transcription, neuronal differentiation (e.g. genes in non-SMN lineage), pattern specification, and embryonic morphogenesis, among other biological pathways (Appendix C, Supplemental Table ST4). While In *Suz12*^{GT} cells did not show the overall large-scale gain in H3K27me3 observed in wild-type cells (Figure 3E), there was an increased enrichment at at some of the genes that gained H3K27me3 in wild-type cells at day 5 of SMN differentiation (Figure 3F). In contrast, we did not observe H3K27me3 in the *Suz12*$^{\Delta}$ or *Eed*^{null} mutant lines at day 5 of SMN differentiation (Figure 3E-F). The gene promoters that gained the most H3K27me3 in wild-type cells during the differentiation time course also gained H3K27me3 in *Suz12*^{GT} cells, albeit at significantly lower levels (Figure 3G).

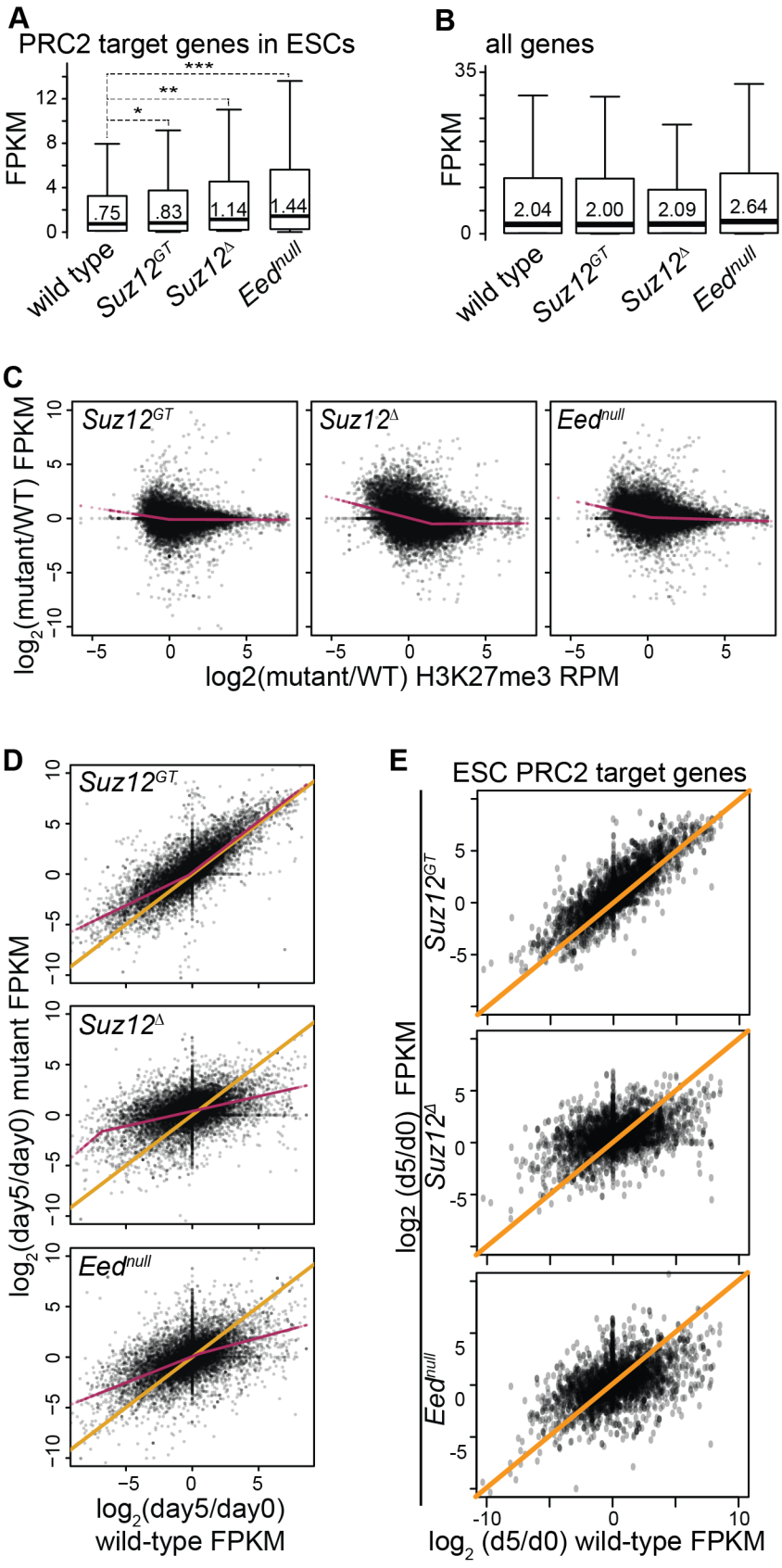
Many genes involved in neuronal differentiation also lose H3K27me3 during differentiation. ~13% of PRC2 target genes showed a two-fold or greater loss of H3K27me3 levels over the SMN differentiation time course in wild-type cells. GO analysis revealed enrichment for genes that have roles in cell adhesion (e.g. *cadherins* and *protocadherins*), neuron differentiation (e.g. *HoxA1*, *HoxA2*, *Sox1*), and axon guidance (e.g. *Gap43*, *Sema6c*), consistent with the progressive activation of the spinal motor neuron pathway (Appendix C, Supplemental Table ST4). In contrast, about a third of all ESC PRC2 targets in wild-type cells lose at least two-fold H3K27me3 over differentiation in *Suz12*^{GT} cells, GO analysis revealed a broad spectrum of functions for these genes (Appendix C, Supplemental Table ST4), suggesting that additional pathways are disrupted in the mutant cells.

Proper H3K27me3 levels are necessary for lineage fidelity during SMN differentiation

While studies have shown that PRC2 target genes are de-repressed in Polycomb mutant ESC lines, it has remained a challenge to determine how changes in H3K27me3 levels impact gene expression over differentiation. Thus, we performed RNA-Seq on *Suz12*^{GT}, *Suz12*$^{\Delta}$, *Eed*^{null}, and wild-type ESCs and compared differences in expression patterns with changes in H3K27me3 levels. As expected, PRC2 target genes are expressed at a higher level in *Eed*^{null} (median=1.44 fpkm; p<5E-7) and *Suz12*$^{\Delta}$ ESCs (median=1.14 fpkm; p<5E-5) compared to wild-type ESCs (median=0.75 fpkm) (Figure 4A). Expression of genes in *Suz12*^{GT} ESCs is more similar to wild-type, albeit slightly higher (median=.83 fpkm; p<5E-2). Differences in overall gene expression profiles between the mutant and wild-type cells were largely due to altered regulation of PRC2 target genes, as demonstrated by the nominal changes observed when considering all genes (Figure 4B). We also found that genes that displayed the most significant loss of H3K27me3 in PRC2 mutants relative to wild-type ESCs correlated with the highest increase in expression levels (Figure 4C).

We next analyzed the relationship between gene expression and H3K27me3 levels in wild-type and in all three PRC2 mutant cell lines after 5 days of directed SMN differentiation. As expected, the expression profiles of *Eed*^{null} and *Suz12*$^{\Delta}$ cells did not show global activation of the SMN gene expression program, consistent with their inability to undergo directed differentiation (Figure 4D, middle and bottom). The same trend was observed when examining only PRC2 target genes (Figure 4E, middle and bottom). In contrast, while *Suz12*^{GT} cells showed overall global activation of the appropriate genes during differentiation, as shown by the clustering of the data points around the x=y line on the right side of the plot, many of the genes down-regulated in wild-type cells failed to be properly repressed in *Suz12*^{GT} cells by day 5 of SMN differentiation, as shown by the upward shift in the left-hand side of the scatter plot (Figure 4D, top panel).

Figure 4. *Suz12GT* cells are able to activate gene expression programs upon differentiation, while *Suz12Δ* and *Eed*^{null} cells are not. (A) RNA-seq of wild-type (wt), *Suz12*^{GT}, *Suz12*$^{\Delta}$, and *Eed*^{null} ESCs. Distributions of FPKMs of PRC2 target genes are shown as box and whisker plots that extend from the 25th to 75th percentile; whiskers represent 1.5x the length of the box. P-values were calculated with Student's two-sided *t*-test. *: p<5E-2; **: p<5E-5; ***: p<5E-7. **(B)** The same plot as (A), except calculated for all genes. **(C)** RNA-seq and H3K27me3 ChIP-seq are shown as scatterplots for *Suz12*^{GT} (left panel), *Suz12*$^{\Delta}$ (middle panel), and *Eed*^{null} (right panel) ESCs with respect to wt. A segmented regression method was used to calculate localized best-fit and is plotted in red. **(D)** RNA-seq of wt, *Suz12*^{GT}, *Suz12*$^{\Delta}$, and *Eed*^{null} ESCs and day 5 SMNs. y-axis shows $\log_2$ of the ratio of FPKM in differentiated:ESC in mutant lines as indicated; x-axis represents this ratio in wt cells. A segmented regression method was used to calculate localized best-fit and is plotted in red. The y=x line is plotted in orange. **(E)** A similar plot to (D), except that only ESC PRC2 target genes were used, to better visualize how the expression of this set of genes changes over differentiation in PRC2 mutant cells versus wild type cells. The y=x line is also plotted in orange for visual reference; if the change in expression for a given gene does not change between the mutant and wt cell lines, that gene will fall on the orange line. As a large number of genes are represented here, data points were rendered transparent such that the density of points plotted in one place can be approximated by the opacity of the signal.


A
PRC2 target genes in ESCs
FPKM
12
8
4
0
***
**
*
.75
.83
1.14
1.44
wild type
Suz12GT
Suz12Δ
Eednull
B
all genes
FPKM
35
0
2.04
2.00
2.09
2.64
wild type
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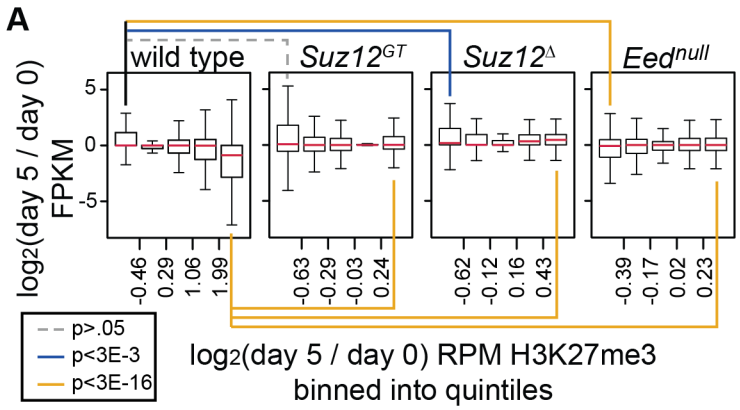
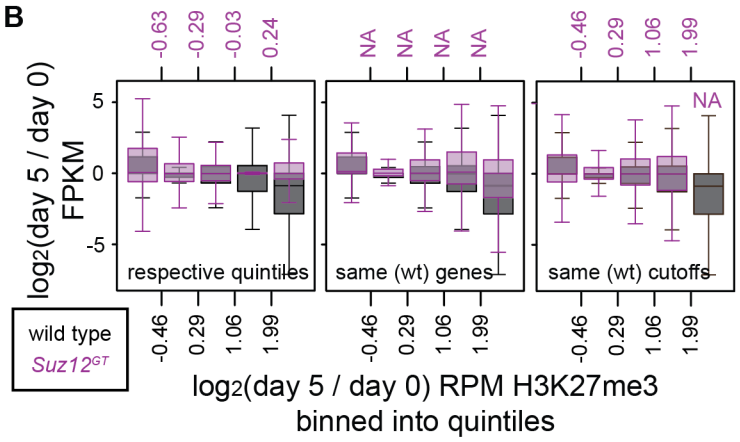
Suz12Δ
Eednull
C
Suz12GT
Suz12Δ
Eednull
log2(mutant/WT) FPKM
log2(mutant/WT) H3K27me3 RPM
D
Suz12GT
Suz12Δ
Eednull
log2(day5/day0) mutant FPKM
log2(day5/day0) wild-type FPKM
E
ESC PRC2 target genes
Suz12GT
Suz12Δ
Eednull
log2 (d5/d0) FPKM
log2 (d5/d0) wild-type FPKM

Figure 5. Proper H3K27me3 levels are necessary for repression of developmental gene programs. (A) Relationship between change in H3K27me3 and expression over differentiation is shown as box plots. All genes were binned by change in H3K27me3 levels over differentiation in each respective cell types (log_2 of H3K27me3 (day 5 / day 0)). y-axis shows distribution of change in expression (log_2 of FPKM (day 5/day 0)). P-values were calculated using a Student's t-test and are represented by colored lines between bins. **(B)** Alternate representation of (A). (Left) wt and *Suz12*^{GT} box plots from (A), superimposed. (Center) Gene list from wt quintiles was used to generate box plots with *Suz12*^{GT} expression data. (Right) Threshold H3K27me3 values from wt quintiles were used to generate box plots for *Suz12*^{GT} cells. **(C)** Example genes that show changes in expression and H3K27me3 levels over SMN differentiation in wt and *Suz12*^{GT} cells are depicted. *Sox3* and *Nes* are expressed in neurectoderm. *Sox17* and *Gata4* are expressed in endoderm. *T* and *Bmp4* are expressed in mesoderm. **(D)** Cartoon representation of (C). Change in H3K27me3 and expression over differentiation for representative genes. Gain or loss in H3K27me3 or expression is represented by upward or downward arrow, respectively, whereas magnitude is represented by size of arrow.


A
wild type
Suz12GT
Suz12Δ
Eednull
log2(day 5 / day 0) FPKM
5
0
-5
-0.46 0.29 1.06 1.99
-0.63 -0.29 -0.03 0.24
-0.62 -0.12 0.16 0.43
-0.39 -0.17 0.02 0.23
p>.05
p<3E-3
p<3E-16
log2(day 5 / day 0) RPM H3K27me3
binned into quintiles


B
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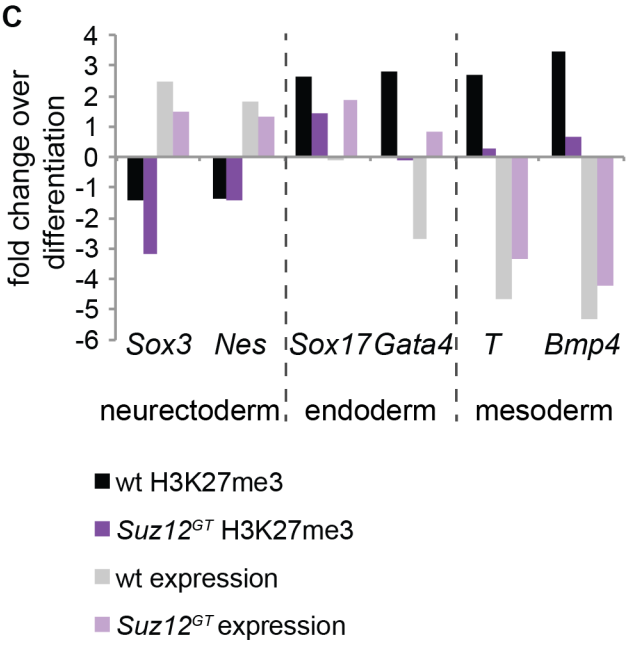
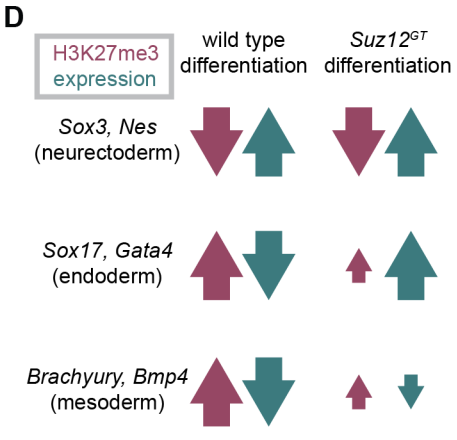
NA NA NA NA
-0.46 0.29 1.06 1.99
NA
log2(day 5 / day 0) FPKM
5
0
-5
respective quintiles
same (wt) genes
same (wt) cutoffs
-0.46 0.29 1.06 1.99
wild type
Suz12GT
log2(day 5 / day 0) RPM H3K27me3
binned into quintiles


C
fold change over differentiation
4
3
2
1
0
-1
-2
-3
-4
-5
-6
Sox3 Nes Sox17 Gata4 T Bmp4
neurectoderm
endoderm
mesoderm
wt H3K27me3
Suz12GT H3K27me3
wt expression
Suz12GT expression


D
H3K27me3 expression
wild type differentiation
Suz12GT differentiation
Sox3, Nes
(neurectoderm)
Sox17, Gata4
(endoderm)
Brachyury, Bmp4
(mesoderm)

To more carefully quantify the relationship between changes in H3K27 methylation levels and expression of PRC2 target genes during SMN differentiation, all genes were binned into quintiles based on fold change in H3K27me3 levels over differentiation and the change in expression for each quintile was then plotted for each cell type (Figure 5A). The set of genes that gained the most H3K27me3 over differentiation in wild-type cells displayed the largest expression changes. Consistent with PRC2's repressive role, these genes were expressed at significantly lower levels compared to genes that did not gain H3K27me3 (Figure 5A, left panel). GO analysis indicates that PRC2 target genes in this category have roles in transcription regulation, pattern specification, embryonic morphogenesis, neuronal differentiation (which largely include genes of non-SMN neuronal lineages), and cell fate commitment, suggesting that increased H3K27me3 is necessary for lineage restriction during normal differentiation by directly suppressing gene expression (Appendix C, Supplemental Table ST4).

In contrast, even the genes that gained some H3K27me3 in *Suz12*^{GT} cells failed to show a similar repression to wild-type cells during differentiation (Figure 5A, 2nd panel). On the other hand, the top 20% of genes that lose H3K27me3 over the course of differentiation in wild-type cells showed a similar relative increase in expression in both wild-type and *Suz12*^{GT} cells (Figure 5A, 2nd panel, and Figure 5B). This set of genes comprises many PRC2 targets that function in cell adhesion, regionalization, axon guidance, and neuron differentiation; genes important for SMN differentiation and function (Appendix C, Supplemental Table ST4). *Eed*^{null} and *Suz12*$^{\Delta}$ cells showed no strong directional change in expression in any of the quintiles, in agreement with their failure to undergo proper directed differentiation (Figure 5A, two right panels). While *Suz12GT* cells are able to activate SMN genes during differentiation, these cells are unable to repress genes expressed in other germ layers such as *Sox17*, *Gata4*, *T*, and *Bmp4* that were normally silenced during lineage commitment in wild-type cells (Figure

5C-D). Thus, PRC2 activity is necessary for proper activation of lineage programs and to maintain lineage fidelity by repressing inappropriate pathways in response to developmental cues.

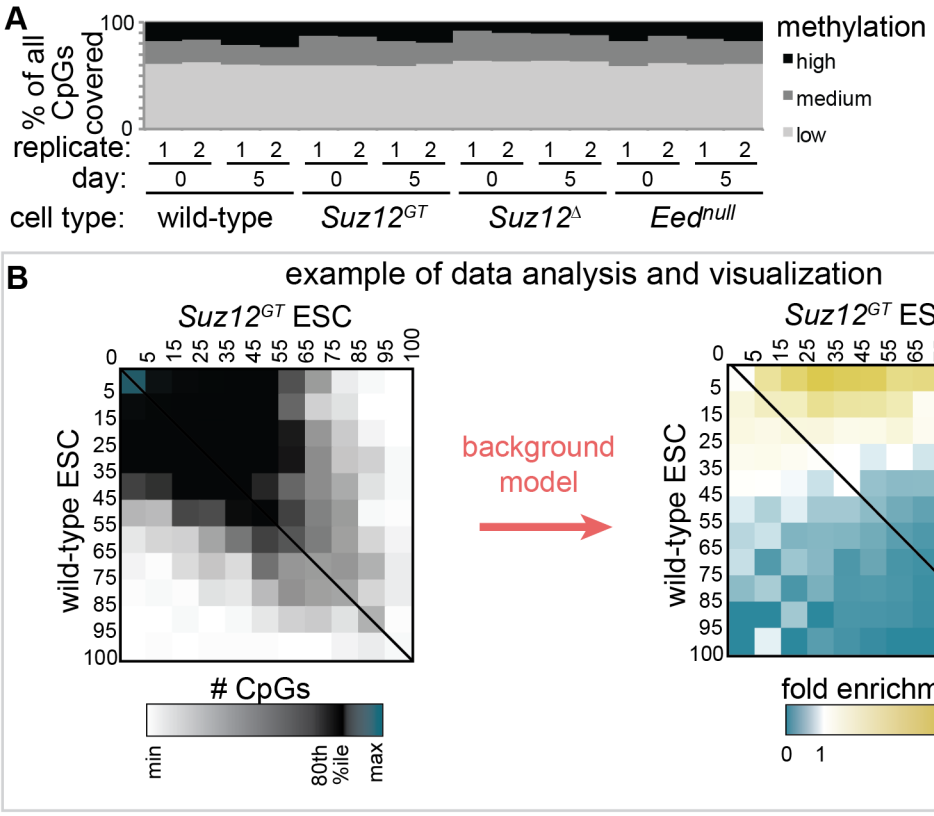
PRC2 activity antagonizes DNA methylation during lineage commitment

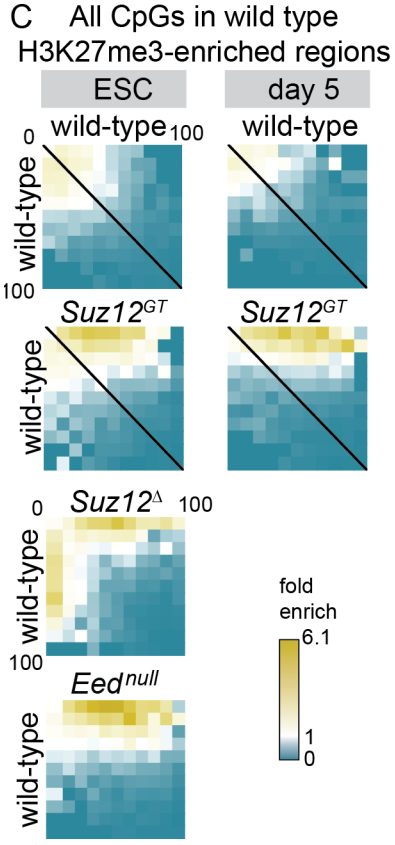
Emerging evidence indicates that PRC2 functions with other epigenetic modifiers to regulate differentiation. For example, a recent study using meDIP-chip (ChIP for 5-methyl-cytosine coupled with a promoter microarray) suggested that DNA methylation levels were modestly affected in *Eed*^{null} ESCs compared to wild-type cells (Hagarman et al., 2013), however, this method measures DNA methylation levels over hundreds of base pairs, making it difficult to determine a direct relationship between the marks catalyzed by these complexes. Moreover, how these two pathways are coordinated has not been examined during lineage commitment. Thus, we performed reduced representation bisulfite sequencing (RRBS) during SMN differentiation as a method to analyze DNA methylation at individual CpG sites across the genome in wild-type and PRC2 mutant cells (Gu et al., 2011; Meissner et al., 2005). While cell lines lacking all H3K27me3 were slightly hypomethylated (Figure 6A), we did not observe dramatic changes in global DNA methylation either over differentiation or upon loss of H3K27me3 in PRC2 mutants. However, by focusing on individual CpG sites in the genome, we were able to detect significant changes in DNA methylation at some sites.

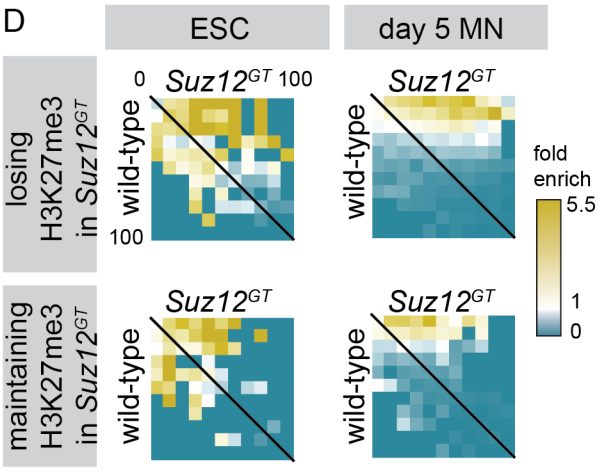
To further analyze the relationship between H3K27me3 and DNA methylation, we limited our analysis to those CpGs with ≥10x coverage and within H3K27me3-enriched regions defined in wild-type cells in either ESC or day 5 SMNs. Figure 6B is a diagram of the data analysis and visualization methods we used for comparison. First, each CpG was binned according to its percent DNA methylation in wild-type ESCs on the y-axis, and according to its percent DNA methylation in *Suz12*^{GT} ESCs on the x-axis (Figure 6B, left). The results can be displayed as a heatmap showing the number of CpGs in each 2-D bin. Fold enrichment over the distribution of the data was then determined for each bin using a

replicate-based background model (see Methods) (Figure 6B, right). Overall, we found that CpG sites associated with PRC2 target regions showed very low levels of DNA methylation. However, in PRC2 mutant ESCs and at day 5 of SMN differentiation, a significantly larger-than-expected number of CpGs

Figure 6. PRC2 is antagonistic to DNA methylation in *cis*. Through RRBS, percent methylation at each CpG with ≥10-fold coverage was calculated in wild-type (wt), *Suz12*^{GT}, *Suz12*$^{\Delta}$, and *Eed*^{null} ESCs and day 5 differentiated cells. **(A)** Distribution of methylation at all CpGs is shown. Low: ≤15% methylated; high: ≥80%. **(B)** This panel is an explanatory example of the data analysis and visualizations used in Figures 6C-D, using the lower-left heatmap of 6C as an example. **(left)** CpGs were binned according to % methylation in wt (y-axis) and *Suz12*^{GT} (x-axis) ESCs. Thus, the matrix displays the number of CpGs in each 2-D bin. The data is largely along the x=y line (CpGs with the same % methylation in *Suz12*^{GT} as wild-type), shifting towards the top right (more methylation in *Suz12*^{GT}). **(right)** Fold enrichment over the overall distribution of the data was determined for each bin using a replicate-based background model (see Methods). In this example, high statistical enrichment over background in *Suz12*^{GT} cells (yellow) is visible for CpGs with little methylation in wt.cells **(C)** CpGs in wt H3K27me3-enriched regions are used to analyze changes in DNA methylation in ESCs (left panel) and day 5 SMN (right panel). The enrichment in the heatmaps in the 2nd row shows CpGs with low methylation in wt (y-axis) gaining methylation in *Suz12*^{GT} (x-axis). **(D)** (Top) H3K27me3-enriched regions in wt ESCs that lose enrichment in *Suz12*^{GT}. (Bottom) Regions maintaining H3K27me3 enrichment in *Suz12*^{GT} ESCs. Regions losing H3K27me3 in *Suz12*^{GT} cells gain overall more DNA methylation than those maintaining significant H3K27me3.


A
% of all CpGs covered
100
0
methylation
high
medium
low
replicate: 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
day: 0 5 0 5 0 5 0 5
cell type: wild-type Suz12GT Suz12Δ Eednull
B
example of data analysis and visualization
Suz12GT ESC
wild-type ESC
0 5 15 25 35 45 55 65 75 85 95 100
CpGs
min
80th %ile
max
background model
Suz12GT ESC
wild-type ESC
fold enrichment
0 1 6.1


C All CpGs in wild type H3K27me3-enriched regions
ESC
day 5
wild-type
wild-type
Suz12GT
Suz12GT
Suz12Δ
Eednull
0
100
fold enrich
6.1
1
0


D
ESC
day 5 MN
losing H3K27me3 in Suz12GT
maintaining H3K27me3 in Suz12GT
Suz12GT
wild-type
0
100
fold enrich
5.5
1
0

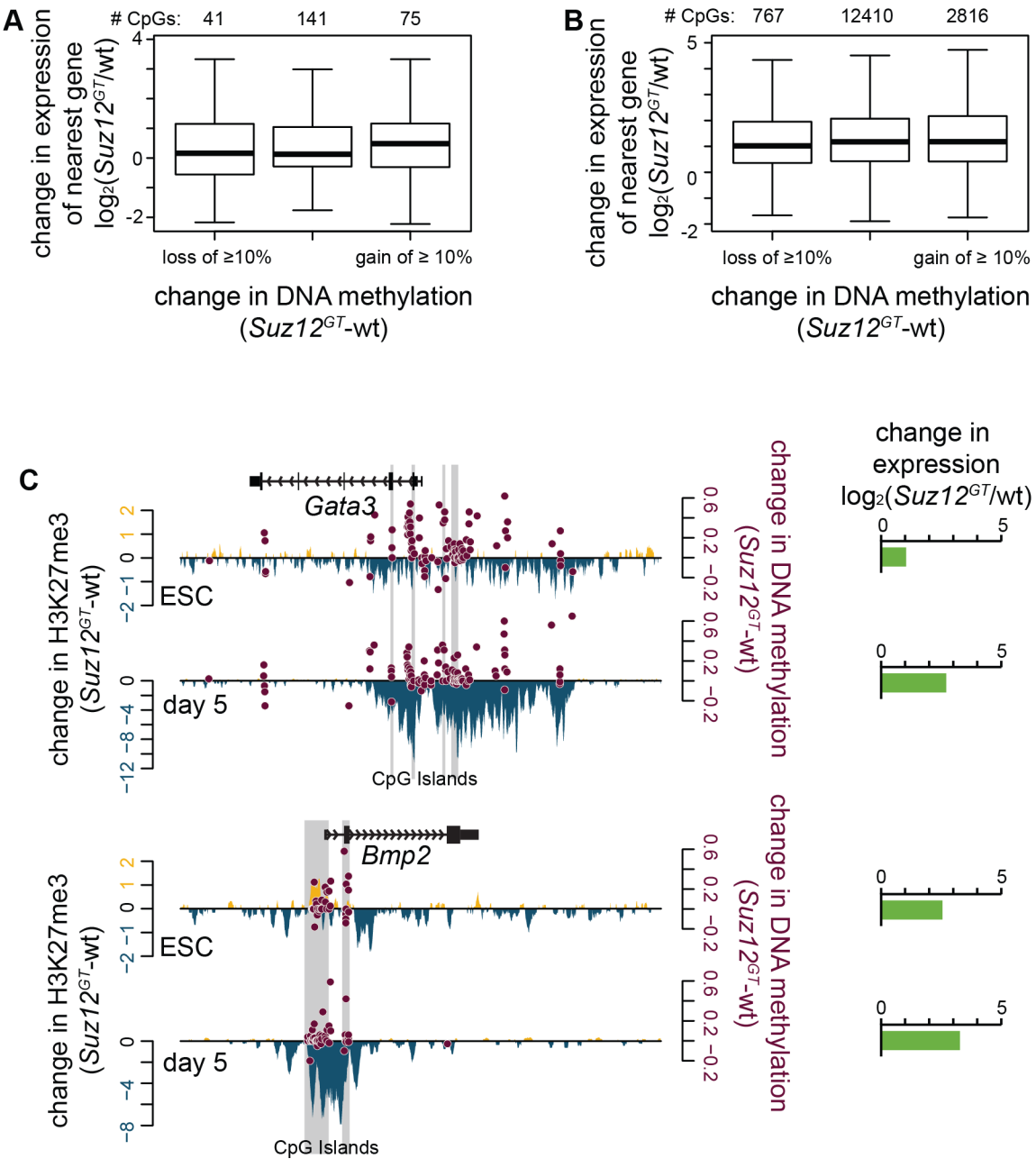
that were unmethylated in wild-type cells displayed increased levels of DNA methylation, as shown by the signal at the top-right corner of the heatmaps in all but the first row of Figure 6C. Furthermore, we also found that CpG sites that are within regions that lose H3K27me3 in *Suz12*^{GT} cells gained more DNA methylation as compared to regions that maintained H3K27me3, both in ESCs (Figure 6D, left panel) and in day 5 SMNs (Figure 6D, right panel). These results indicate that PRC2 (or H3K27me3) directly antagonizes DNA methylation, and loss of the mark permits increased DNA methylation levels.

Increase in DNA methylation in PRC2 mutants is insufficient to mediate gene repression

Developmental promoters do not appear to be regulated by DNA methylation but instead are dynamically repressed by PRC2 activity. Thus, we next wanted to test the consequence of the increase in DNA methylation on expression of PRC2 target genes in *Suz12*^{GT} cells. To address this question, CpG dinucleotides in regions losing H3K27me3 enrichment in *Suz12*^{GT} cells were first assigned to a gene based on distance and position relative to the nearest transcription start site (see Methods), and the change in expression at those genes compared to wild-type cells was then determined using our RNA-seq data. In ESCs, about 25% of CpG sites in H3K27me3-depleted regions gained ≥10% methylation in *Suz12*^{GT} ESCs, and mapped to genes including *Bmp2*, *Gata3*, and *Fgf8*, as well as a number of homeobox genes. In day 5 differentiated cells, about 18% of CpG sites in H3K27me3-depleted regions gained ≥10% DNA methylation in *Suz12*^{GT} cells, and are associated with genes like *En1* and *Wnt6*. We also observed that a smaller proportion of CpG sites displayed a ≥10% decrease in DNA methylation. While some of these CpG sites map to different genes than the CpGs gaining DNA methylation, some map to the same genes. Thus, while we observed a trend toward an overall increase in DNA methylation at CpG sites within PRC2 target regions, individual CpGs in proximity to a given gene can either gain or lose DNA methylation in *Suz12*^{GT} cells. By comparison of RNA-Seq data sets in wild-type and *Suz12*^{GT} ESCs or day 5 differentiated cells, we did not observe a significant change in expression for genes that showed either an overall increase or decrease in DNA methylation at these sites (Figure

7A-B). Figure 7C shows representative examples of PRC2 target genes and their observed changes in H3K27me3, DNA methylation, and gene expression in *Suz12*^{GT} cells. On the left-hand y-axis, change in H3K27me3 signal between wild-type and *Suz12*^{GT} cells is plotted; regions where H3K27me3 is lost in *Suz12*^{GT} cells are shown in blue, while regions where that display a gain in H3K27me3 are shown in yellow. On the right-hand y-axis, the change in DNA methylation at every CpG with sufficient coverage is plotted as a maroon data point. Concomitantly, the change in expression between wild-type and *Suz12*^{GT} cells is plotted on the right side of the panel. For example, we observe overall loss of H3K27me3, gain of DNA methylation, and increased expression in *Suz12*^{GT} compared to wild-type cells, both in ESCs and over the differentiation time course for *Gata3* and *Bmp2*. Notably, the increase in expression at PRC2 target genes in *Suz12*^{GT} compared to wild-type cells is similar in magnitude to genes that do not display changes in DNA methylation, suggesting that the modest increase in DNA methylation does not suppress the effects of loss of PRC2 activity. Collectively, our data suggest that PRC2 plays a role in preventing inappropriate DNA methylation at developmental and lineage-specific genes, and that the activity of these two pathways may be coordinated to allow for proper development and differentiation.

Figure 7. Increased DNA methylation upon loss of PRC2 does not lead to target gene repression. (A) Regions with significantly enriched H3K27me3 in wild-type (wt) ESCs were considered. All CpGs in these regions with 10x coverage via RRBS in both wt and *Suz12*^{GT} ESCs, and ≥.1 FPKM in at least one of these cell types, were used in the analysis. Of these 257 CpGs, 41 lose ≥10% DNA methylation, 75 gain ≥10% DNA methylation, and 141 do not change. The distribution of change in expression in *Suz12*^{GT} ESCs with respect to wt ESCs of the genes associated with these CpGs is plotted on the y-axis. No association between change in DNA methylation and gene expression is observed. **(B)** Same as in (A) except in day 5 SMNs. Of these 15993 CpGs, 767 lose ≥10% DNA methylation, 2816 gain ≥10% DNA methylation, and 12410 do not change. The distribution of change in expression in *Suz12*^{GT} cells with respect to wt cells of the genes associated with these CpGs is plotted on the y-axis. No association between change in DNA methylation and gene expression is observed. **(C)** Two example genes, *Gata3* and *Bmp2*, are shown here. Change in H3K27me3 signal between wt and *Suz12*^{GT} cells is plotted on the left y-axis; gain in *Suz12*^{GT} is shown in yellow, and loss is shown in blue. Change in DNA methylation for each CpG with 10x coverage in both cell types is plotted in maroon on the right y-axis. Change in gene expression ($\log_2$ of the ratio of the FPKMs (*Suz12*^{GT}/wt)) is shown in horizontal bar graphs to the right of the panel.


A
CpGs: 41 141 75
change in expression of nearest gene log2(Suz12GT/wt)
loss of ≥10%
gain of ≥ 10%
change in DNA methylation (Suz12GT-wt)
B
CpGs: 767 12410 2816
change in expression of nearest gene log2(Suz12GT/wt)
loss of ≥10%
gain of ≥ 10%
change in DNA methylation (Suz12GT-wt)
C
Gata3
change in H3K27me3 (Suz12GT-wt)
ESC
day 5
CpG Islands
change in DNA methylation (Suz12GT-wt)
change in expression log2(Suz12GT/wt)
Bmp2
ESC
day 5
CpG Islands
change in DNA methylation (Suz12GT-wt)

Discussion

Regulation of PRC2 activity is essential to mammalian development and differentiation. Loss of PRC2 and its catalyzed mark, H3K27me3, leads to lethality during gastrulation, a period of development when complex gene expression patterns are established in the embryo (Montgomery et al., 2005; O'Carroll et al., 2001; Pasini et al., 2004). While studies have shown that PRC2 silences developmental programs in ESCs, its roles during differentiation and lineage commitment have not been extensively studied due to the inability of PRC2 mutant ESCs to properly differentiate. In order to gain new insights, we exploited a mutant genetrap allele of *Suz12* that acts as a hypomorph *in vitro* in that it maintains partial H3K27me3 levels and allows for low-efficiency directed differentiation of ESCs to SMNs. We show that the proper lineage programs can be activated during differentiation, however, genes that normally gain H3K27me3 over differentiation and become repressed in wild-type cells failed to be fully repressed in $Suz12^{GT}$ cells. Comparatively, Eed^{null} and $Suz12^{\Delta}$ ESCs lack H3K27me3 and fail to properly induce differentiation programs. Together, these data indicate that regulation of H3K27me3 levels is necessary for proper activation of gene programs in response to developmental cues and for repression of alternate pathways to restrict cell fate and to maintain lineage fidelity.

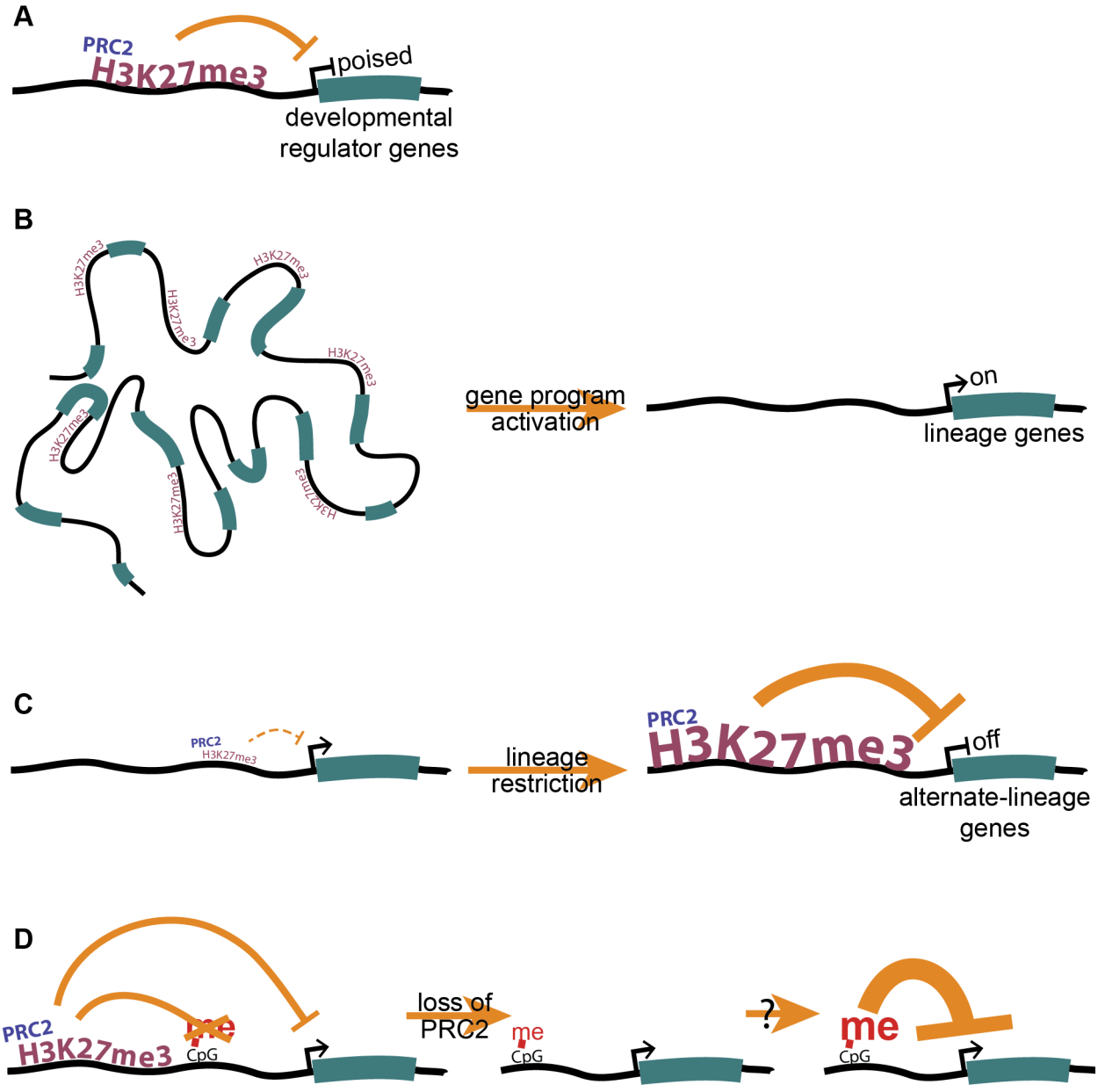
CpG dinucleotides at the promoters of developmental genes are primarily unmethylated in the genome. While transcription factor binding appears to be a major mechanism for preventing DNA methylation, our data also support a role for PRC2 in antagonizing DNA methylation during lineage commitment. Consistent with this idea, accumulating evidence indicates that repression of developmental genes is largely regulated by H3K27 methylation and not DNA methylation (Smith and Meissner, 2013). Our findings that $Suz12^{GT}$ ESCs can differentiate, albeit less efficiently, and that these cells harbor regions of variable H3K27me3 maintenance in the genome compared to wild-type cells, make them an important tool to investigate this relationship. As such, we used this system to examine PRC2-dependent changes in DNA methylation at nucleotide resolution using RRBS. We show that in

Suz12^{GT} ESCs, PRC2 targets losing H3K27me3 with respect to wild-type cells were more likely to gain DNA methylation at CpG sites compared to regions that maintained H3K27me3 levels, suggesting that PRC2 activity is directly antagonistic to DNA methylation in *cis*.

What targets a gene for permanent repression as opposed to activation or maintenance of the poised state? PRC2 has recently been shown to recruit TET1, a dioxygenase that converts 5-methyl-cytosine into 5-hydroxymethyl-cytosine, which may safeguard developmental genes against inappropriate DNA methylation (Neri et al., 2013a). *Tet1* knockout animals display epigenetic abnormalities, but its loss does not impact embryonic or postnatal survival (Dawlaty et al., 2011), suggesting that other mechanisms contribute to regulating DNA methylation levels at PRC2 target genes. Additionally, a recent study showed that PRC2 recruits DNMT3L, a catalytically inactive DNA methyltransferase that sterically competes with active DNA methyltransferases to prevent DNA methylation at PRC2 target sites (Neri et al., 2013b). It is possible that loss of PRC2 activity in *Suz12*^{GT} cells prevents localization of TET1 or DNMT3L to promoters, leading to inappropriate DNA methylation. Each of these mechanisms could be critical for surveying the DNA methylation status at developmental genes to prevent an increase in DNA methylation that could ultimately lead to hypermethylation and to aberrant gene expression patterns (Smith and Meissner, 2013). Thus, PRC2 may play roles in preventing inappropriate DNA methylation through interaction with these and other modifiers. Our data show that while loss of PRC2 leads to an increase in promoter DNA methylation at target genes, the modest increase is not sufficient to affect changes in gene expression. Thus, we propose that loss of PRC2 activity can lead to destabilization of gene expression states during lineage commitment and that the increase in DNA methylation can promote further epigenetic instability.

In addition to its roles in development, misregulation or mutation of PcG proteins has been strongly correlated with the progression and severity of cancer. In many different types of cancer, PcG proteins,

Figure 8. PRC2 plays roles in gene regulation both in pluripotency and during lineage commitment. (A) In ESCs, PRC2 localizes largely to developmental regulator genes, and maintains them in their repressed, and yet poised, state. **(B)** Proper H3K27me3 levels are necessary to activate developmental gene programs during differentiation. **(C)** A gain in H3K27me3 during differentiation represses alternate-lineage genes, allowing for efficient lineage restriction. **(D)** PRC2 antagonizes DNA methylation in *cis*, and may play a role in preventing the premature permanent repression of developmental genes.


A
PRC2
H3K27me3
poised
developmental
regulator genes
B
H3K27me3
H3K27me3
H3K27me3
H3K27me3
H3K27me3
H3K27me3
H3K27me3
gene program
activation
on
lineage genes
C
PRC2
H3K27me3
lineage
restriction
PRC2
H3K27me3
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PRC2
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such as EZH2, are expressed at higher than normal levels, which is thought to lead to aberrant silencing of tumor suppressor genes (Collett et al., 2006; Piunti and Pasini, 2011; Raaphorst et al., 2000; Varambally et al., 2002). Indeed, forced overexpression of *Ezh2* leads to cancer phenotypes (Li et al., 2009), and inhibition of EZH2 is a promising cancer therapy (McCabe et al., 2012; Puppe et al., 2009). Conversely, decreased expression of PcG proteins has also been observed in tumor samples, such as the downregulation of *Bmi1* in melanoma (Bachmann et al., 2008), suggesting that loss of Polycomb complexes leads to activation of oncogenes. Emerging evidence also indicates that perturbation of PcG proteins in cancer may have consequences on DNA methylation patterns. For example, PRC2 target genes in ESCs are more likely to show cancer-specific promoter DNA hypermethylation, suggesting that H2K27me3 marks genes that become targets for more permanent silencing (Mohammad et al., 2009; Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007). Consistent with our results during lineage commitment, these studies suggest that loss of PRC2 activity can lead to epigenetic instability and loss of cell identity during tumorigenesis. Thus, additional studies to investigate the diverse mechanisms that PcG proteins employ to regulate cell fate transitions and cell identity are critical to further our understanding of both normal and pathologic development, and to facilitate the design of relevant therapies.

Conclusions

Loss of H3K27me3 at gene promoters in ESCs leads to gene derepression in ESCs (Figure 8A), and an inability to properly activate developmental gene programs upon loss of pluripotency (Figure 8B). We find that an inability to gain H3K27me3 over differentiation leads to failure to properly repress alternate lineage programs, leading to defects in lineage restriction (Figure 8C). We also show that PRC2/H3K27me3 is antagonistic to DNA methylation in *cis*. While loss of PRC2 is not sufficient to recruit the levels of DNA methylation necessary to repress target genes (Figure 8D), we propose that the low level seeding of inappropriate DNA methylation may lead to further epigenetic instability of cell fate, which may also explain the molecular underpinnings of PRC2 disruption in cancer. Our work provides novel insights into the role of PRC2 in mammalian development, and its effect on gene expression during lineage commitment.

Materials and Methods

ESC culture

ESCs were cultured on irradiated MEFs under standard ESC conditions. This includes E14 and *Suz12*^{GT} (ola/129 background), obtained from the Helin lab (Pasini et al., 2007); *Suz12*$^{\Delta}$ (C57/BL6 background), obtained from the Koseki lab (Lee et al., 2006a); and *Eed*^{null} (BALB/cR1 background) (Chamberlain et al., 2008), obtained from the Magnuson lab. ESCs were collected by trypsinization, incubation on cell a culture plate for 20 minutes to remove MEFs, and collection of the ESCs in suspension.

Spinal Motor Neuron differentiation

SMN differentiation was performed as described (Wichterle et al., 2002). Cells were collected at day 5, before the terminal differentiation stage, and trypsinized to single-cell suspension for use in other assays.

RNA-seq

RNA was isolated using Trizol according to manufacturer's instructions, including optional step in protocol. RNA quality was determined by Agilent Bioanalyzer. RNA-seq libraries were prepared as in (Wamstad et al., 2012). A final round of size selection by Agencourt AMPure XP beads was performed to remove small fragments such as primers. Sequencing was run on either an Illumina GA-2 or Hi-Seq. For analysis, Bowtie v. 0.12.7, Tophat v1.3.2 and Cufflinks v 1.2.1 and Cuffdiff were utilized to determine the expression levels of genes (Trapnell et al., 2012), using a NCBIN37, ENSEMBL-based annotation and flags -p 4, -r 170, --segment-length 20 --segment-mismatches 1 --solexa1.3-quals --no-novel-juncs. Cufflinks was guided using the same annotation as Tophat with flags -b -u -p 6. For regression analysis (Figures 4C-D), gene expression or read coverage fold-changes were $\log_2$-transformed, and dependent variables were regressed using a generalized linear modelling framework (glm, with an identity link function) in the R statistical environment (v. 3.0.2). Segmented

regression was performed on the resulting object using the "segmented" R package (v. 0.3-3.0), with a starting psi parameter (i.e. inflexion point) set at -0.5 for all analyses.

ChIP-seq

Chromatin immunoprecipitation (ChIP) was performed based on the protocol as described in Lee et al., 2006 (Lee et al., 2006b), with modifications and adaptations. Briefly, a Diagenode bioruptor was used for sonication of formaldehyde-crosslinked cells on high for 30s on/ 30s off for 45 cycles in sonication buffer (20mM Tris-HCl; 150mM NaCl; 2mM EDTA; 0.1mM SDS; 1% Triton X-100; protease inhibitor (Thermo-scientific)). In ChIPs to be sequenced, the Diagenode IP-star was also used for automation of the ChIP protocol, according to the manufacturer's specifications. Antibodies used for ChIP are listed in Supplementary Table ST1. After purification of DNA, samples were used for quantification via qPCR and/or used to prepare libraries for Illumina sequencing. Library preparation is performed essentially as described in Schmidt et al., 2009; the amplification and size selection steps are reversed in order, and size selection was performed using Agencourt Ampure XP beads (Schmidt et al., 2009). Sequencing was run on an Illumina Hi-Seq (barcoded). Peak calling was performed as previously described (Wamstad et al., 2012).

X-gal staining

Cells grown on cell culture plates were fixed for 4 minutes in fixing solution (4% formaldehyde, 0.5% glutaraldehyde, 0.1M NaH_2PO_4, 0.1M Na_2HPO_4), rinsed twice with PBS, and stained at 37°C until sufficiently colored, in staining solution (1mg/mL X-gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2mM $MgCl_2$, 1xPBS).

Immunoprecipitations

Protein G Dynabeads (Life Technologies) were added to the appropriate antibody and incubated in PBS + BSA for 4 hours at 4°C. Concomitantly, cells were incubated in lysis buffer (50 mM hepes pH 7.2,

250 mM NaCl, 10% (vol/vol) glycerol, 2 mM EDTA, 0.1% (vol/vol) Nonidet P-40, protease inhibitor (Thermo-scientific)) (Philipp et al., 2010) for 20 minutes on ice. In the middle of this lysis, the cells were briefly homogenized using a Tissue Tearor™ homogenizer. This lysate was then spun down 5 minutes at 16,000g at 4°C to remove debris, and the supernatant used as input. 3% of the input was boiled 10 minutes in Laemmli buffer and set aside at -20°C. The bead mixture was then added to the input, and this rotated at 4°C for 4 hours. Beads were washed 3x with lysis buffer, resuspended in Laemmli buffer, and boiled for 10 minutes before removal of beads and analysis of supernatant by Western blot.

shRNA-mediated knockdown of transcripts in ESCs

Oligonucleotides were designed such that when annealed, they would form dsDNA that would be transcribed into an RNA hairpin. Annealed hairpin dsDNA was ligated into the pLKO.1 vector. This construct was then co-transfected with packaging vectors into 293 cells, and the virus produced was filtered and used to infect ESCs. These infected cells were puromycin-selected before testing the knockdown level by qRT-PCR.

Quantitative reverse transcriptase PCR

For expression analysis, RNA was extracted using Trizol according to manufacturer's instructions, and cDNA was made using MMLV reverse transcriptase according to manufacturer's instructions, with random hexamer primers. Quantitative reverse transcriptase PCR was performed on either cDNA or ChIP template using a Roche LightCycler 480 machine, using KAPA SYBR FAST Master Mix (2X) optimized for this machine. Primers are listed in Table ST2. Reactions were prepared in triplicate and temperature cycled according to the product specifications. Analysis of data was performed by comparing each reaction of the experimental triplicate to each reaction of the control triplicate, using a 2^{-dCp} model (Schmittgen and Livak, 2008). The average and standard deviation of this set of results was then calculated.

Immunohistochemistry

Aggregated motor neurons at day 5 of the Spinal Motor Neuron differentiation were collected and fixed for 20 minutes in 10% formalin, washed with PBS, and then dehydrated in sequentially higher concentrations of ethanol for 20 minutes each (70%, 80%, 95%, 95%, 100%, 100%, 100%) and washed three times in Xylene. They were then embedded in paraffin overnight at 60°C and sectioned to 0.4uM. Parafin was removed with xylene, and the samples were rehydrated. Immunohistochemistry was performed with anti-OLIG2 antibody (Table ST2) at 1:500.

RRBS

Reduced representation bisulfite sequencing was performed as published (Gu et al., 2011) and sequenced on an Illumina Hi-Seq. The sequencing data were analyzed initially as published. Briefly, reads were mapped against an *in-silico* modified mouse genome (UCSC mm9, with inferred MspI restriction and genome-wide conversion of C to T and G to A) using maq (v 0.7.1-9) with the parameters -D -s 0 -M c -e 100 C. Resulting bam files were sorted and indexed with samtools (v.0.1.16 , r963:234) and per-position read pileups were obtained with mpileup using unmodified mm9 as a reference. Following that, for each sample, per-base read coverage and fraction of C or G-containing reads (depending on the read mapping strand) were extracted and CpG sites were summed and summarized using custom perl scripts. Genome-wide methylation levels were assessed by tallying the fraction of methylation-representative reads over read coverage in each sample for sites with 10x or higher coverage. For pairwise sample comparisons, sites meeting a 10x-read coverage in both samples were binned according to their methylation levels in both samples and displayed in matrix form. To assess relative over- or underrepresentation of a given bin, expected counts per bin were estimated by averaging pairwise replicate methylation matrices in all cell types (background model). Deviations from the expected distribution are therefore represented as the observed:expected ratios (fold enrichment). All DNA methylation values were floored at 0.01% to allow calculations for CpGs with no

methylation. Association of a CpG with a gene was determined by its proximity. Briefly, a CpG located within 4kb of a gene body was associated with that gene. Proximity to another gene-associated CpG was also used as an alternate criterion. Otherwise, it was assigned to the nearest PRC2 target gene within 200kb.

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References

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jørgensen, H.F., John, R.M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M., et al. (2006). Chromatin signatures of pluripotent cell lines. Nat Cell Biol *8*, 532–538.

Bachmann, I.M., Puntervoll, H.E., Otte, A.P., and Akslen, L.A. (2008). Loss of BMI-1 expression is associated with clinical progress of malignant melanoma. Mod. Pathol. *21*, 583–590.

Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., et al. (2006). A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. Cell *125*, 315–326.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nat Cell Biol *441*, 349–353.

Bracken, A.P., Dietrich, N., Pasini, D., Hansen, K.H., and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev *20*, 1123–1136.

Cao, R., and Zhang, Y. (2004). SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell *15*, 57–67.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043.

Chamberlain, S.J., Yee, D., and Magnuson, T. (2008). Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells *26*, 1496–1505.

Collett, K., Eide, G.E., Arnes, J., Stefansson, I.M., Eide, J., Braaten, A., Aas, T., Otte, A.P., and Akslen, L.A. (2006). Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin. Cancer Res. *12*, 1168–1174.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185–196.

Dawlaty, M.M., Ganz, K., Powell, B.E., Hu, Y.-C., Markoulaki, S., Cheng, A.W., Gao, Q., Kim, J., Choi, S.-W., Page, D.C., et al. (2011). Tet1 is dispensable for maintaining pluripotency and its loss is compatible with embryonic and postnatal development. Cell Stem Cell *9*, 166–175.

Di Croce, L., and Helin, K. (2013). Transcriptional regulation by Polycomb group proteins. Nat Struct Mol Biol *20*, 1147–1155.

Faust, C., Schumacher, A., Holdener, B., and Magnuson, T. (1995). The eed mutation disrupts anterior mesoderm production in mice. Development *121*, 273–285.

Gu, H., Smith, Z.D., Bock, C., Boyle, P., Gnirke, A., and Meissner, A. (2011). Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nat Protoc *6*, 468–481.

Hagarman, J.A., Motley, M.P., Kristjansdottir, K., and Soloway, P.D. (2013). Coordinate regulation of DNA methylation and H3K27me3 in mouse embryonic stem cells. PLoS ONE *8*, e53880.

Hawkins, R.D., Hon, G.C., Lee, L.K., Ngo, Q., Lister, R., Pelizzola, M., Edsall, L.E., Kuan, S., Luu, Y., Klugman, S., et al. (2010). Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell Stem Cell *6*, 479–491.

Kirmizis, A., Bartley, S.M., Kuzmichev, A., Margueron, R., Reinberg, D., Green, R., and Farnham, P.J. (2004). Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev *18*, 1592–1605.

Ku, M., Koche, R.P., Rheinbay, E., Mendenhall, E.M., Endoh, M., Mikkelsen, T.S., Presser, A., Nusbaum, C., Xie, X., Chi, A.S., et al. (2008). Genomewide Analysis of PRC1 and PRC2 Occupancy Identifies Two Classes of Bivalent Domains. PLoS Genet *4*, e1000242.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev *16*, 2893–2905.

Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K.-I., et al. (2006a). Control of developmental regulators by Polycomb in human embryonic stem cells. Cell *125*, 301–313.

Lee, T.I., Johnstone, S.E., and Young, R.A. (2006b). Chromatin immunoprecipitation and microarray-based analysis of protein location. Nat Protoc *1*, 729–748.

Li, X., Gonzalez, M.E., Toy, K., Filzen, T., Merajver, S.D., and Kleer, C.G. (2009). Targeted overexpression of EZH2 in the mammary gland disrupts ductal morphogenesis and causes epithelial hyperplasia. Am J Pathol *175*, 1246–1254.

Lister, R., and Ecker, J.R. (2009). Finding the fifth base: genome-wide sequencing of cytosine methylation. Genome Res *19*, 959–966.

Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, C.L., Dynlacht, B.D., and Reinberg, D. (2008). Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Mol Cell *32*, 503–518.

Mazzoni, E.O., Mahony, S., Peljto, M., Patel, T., Thornton, S.R., McCuine, S., Reeder, C., Boyer, L.A., Young, R.A., Gifford, D.K., et al. (2013). Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals. Nat. Neurosci. *16*, 1191–1198.

McCabe, M.T., Ott, H.M., Ganji, G., Korenchuk, S., Thompson, C., Van Aller, G.S., Liu, Y., Graves, A.P., Pietra, Della, A., Diaz, E., et al. (2012). EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature *492*, 108–112.

Meissner, A., Gnirke, A., Bell, G.W., Ramsahoye, B., Lander, E.S., and Jaenisch, R. (2005). Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Research *33*, 5868–5877.

Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim,

T.-K., Koche, R.P., et al. (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553–560.

Mohammad, H.P., Cai, Y., Mcgarvey, K.M., Easwaran, H., van Neste, L., Ohm, J.E., O'Hagan, H.M., and Baylin, S.B. (2009). Polycomb CBX7 promotes initiation of heritable repression of genes frequently silenced with cancer-specific DNA hypermethylation. Cancer Res *69*, 6322–6330.

Mohn, F., Weber, M., Rebhan, M., Roloff, T.C., Richter, J., Stadler, M.B., Bibel, M., and Schübeler, D. (2008). Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell *30*, 755–766.

Montgomery, N.D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S.J., Otte, A.P., and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol *15*, 942–947.

Neri, F., Incarnato, D., Krepelova, A., Rapelli, S., Pagnani, A., Zecchina, R., Parlato, C., and Oliviero, S. (2013a). Genome-wide analysis identifies a functional association of Tet1 and Polycomb PRC2 in mouse embryonic stem cells. Genome Biol *14*, R91.

Neri, F., Krepelova, A., Incarnato, D., Maldotti, M., Parlato, C., Galvagni, F., Matarese, F., Stunnenberg, H.G., and Oliviero, S. (2013b). Dnmt3L Antagonizes DNA Methylation at Bivalent Promoters and Favors DNA Methylation at Gene Bodies in ESCs. Cell *155*, 121–134.

O'Carroll, D., Erhardt, S., Pagani, M., Barton, S.C., Surani, M.A., and Jenuwein, T. (2001). The polycomb-group gene Ezh2 is required for early mouse development. Mol Cell Biol *21*, 4330–4336.

Ohm, J.E., Mcgarvey, K.M., Yu, X., Cheng, L., Schuebel, K.E., Cope, L., Mohammad, H.P., Chen, W., Daniel, V.C., Yu, W., et al. (2007). A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet *39*, 237–242.

Pasini, D., Bracken, A.P., Hansen, J.B., Capillo, M., and Helin, K. (2007). The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol *27*, 3769–3779.

Pasini, D., Bracken, A.P., Jensen, M.R., Lazzerini Denchi, E., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J *23*, 4061–4071.

Pengelly, A.R., Copur, Ö., Jäckle, H., Herzig, A., and Müller, J. (2013). A histone mutant reproduces the phenotype caused by loss of histone-modifying factor Polycomb. Science *339*, 698–699.

Philipp, S., Puchert, M., Adam-Klages, S., Tchikov, V., Winoto-Morbach, S., Mathieu, S., Deerberg, A., Kolker, L., Marchesini, N., Kabelitz, D., et al. (2010). The Polycomb group protein EED couples TNF receptor 1 to neutral sphingomyelinase. Proc Natl Acad Sci USA *107*, 1112–1117.

Piunti, A., and Pasini, D. (2011). Epigenetic factors in cancer development: Polycomb group proteins. Future Oncology *7*, 57–75.

Puppe, J., Drost, R., Liu, X., Joosse, S.A., Evers, B., Cornelissen-Steijger, P., Nederlof, P., Yu, Q., Jonkers, J., van Lohuizen, M., et al. (2009). BRCA1-deficient mammary tumor cells are dependent on EZH2 expression and sensitive to Polycomb Repressive Complex 2-inhibitor 3-deazaneplanocin A. Breast Cancer Res. *11*, R63.

Raaphorst, F.M., van Kemenade, F.J., Blokzijl, T., Fieret, E., Hamer, K.M., Satijn, D.P., Otte, A.P., and Meijer, C.J. (2000). Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. Am J Pathol *157*, 709–715.

Schlesinger, Y., Straussman, R., Keshet, I., Farkash, S., Hecht, M., Zimmerman, J., Eden, E., Yakhini, Z., Ben-Shushan, E., Reubinoff, B.E., et al. (2007). Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet *39*, 232–236.

Schmidt, D., Wilson, M.D., Spyrou, C., Brown, G.D., Hadfield, J., and Odom, D.T. (2009). ChIP-seq: using high-throughput sequencing to discover protein-DNA interactions. Methods *48*, 240–248.

Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc *3*, 1101–1108.

Shen, X., Liu, Y., Hsu, Y.-J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G.-C., and Orkin, S.H. (2008). EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell *32*, 491–502.

Simon, J.A., and Kingston, R.E. (2009). Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol *10*, 697–708.

Smith, Z.D., and Meissner, A. (2013). DNA methylation: roles in mammalian development. Nat Rev Genet *14*, 204–220.

Surface, L.E., Thornton, S.R., and Boyer, L.A. (2010). Polycomb group proteins set the stage for early lineage commitment. Cell Stem Cell *7*, 288–298.

Tolhuis, B., de Wit, E., Muijrers, I., Teunissen, H., Talhout, W., van Steensel, B., and van Lohuizen, M. (2006). Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in Drosophila melanogaster. Nat Genet *38*, 694–699.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc *7*, 562–578.

Varambally, S., Dhanasekaran, S.M., Zhou, M., Barrette, T.R., Kumar-Sinha, C., Sanda, M.G., Ghosh, D., Pienta, K.J., Sewalt, R.G.A.B., Otte, A.P., et al. (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature *419*, 624–629.

Varley, K.E., Gertz, J., Bowling, K.M., Parker, S.L., Reddy, T.E., Pauli-Behn, F., Cross, M.K., Williams, B.A., Stamatoyannopoulos, J.A., Crawford, G.E., et al. (2013). Dynamic DNA methylation across diverse human cell lines and tissues. Genome Res *23*, 555–567.

Wamstad, J.A., Alexander, J.M., Truty, R.M., Shrikumar, A., Li, F., Eilertson, K.E., Ding, H., Wylie, J.N., Pico, A.R., Capra, J.A., et al. (2012). Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage. Cell *151*, 206–220.

Wichterle, H., and Peljto, M. (2008). Differentiation of mouse embryonic stem cells to spinal motor neurons. Curr Protoc Stem Cell Biol *Chapter 1*, Unit1H.1.1–1H.1.9.

Wichterle, H., Lieberam, I., Porter, J.A., and Jessell, T.M. (2002). Directed differentiation of embryonic stem cells into motor neurons. Cell *110*, 385–397.

Widschwendter, M., Fiegl, H., Egle, D., Mueller-Holzner, E., Spizzo, G., Marth, C., Weisenberger, D.J., Campan, M., Young, J., Jacobs, I., et al. (2007). Epigenetic stem cell signature in cancer. Nat Genet *39*, 157–158.

Yuan, W., Wu, T., Fu, H., Dai, C., Wu, H., Liu, N., Li, X., Xu, M., Zhang, Z., Niu, T., et al. (2012). Dense Chromatin Activates Polycomb Repressive Complex 2 to Regulate H3 Lysine 27 Methylation. Science *337*, 971–975.

Chapter 3

Discussion

The overarching goal of this thesis was to elucidate the function of Polycomb Repressive Complex 2 (PRC2) and its interactions with downstream effectors, with a focus on its role in lineage commitment. Towards this goal, Chapter 1 detailed the current state of the field regarding Polycomb group (PcG) proteins, their relevance to mammalian development, and current models for their targeting to genomic sites and function in ESCs and differentiated cells. In Chapter 2, we investigated the role of PRC2 function in transcriptional regulation and relationship to DNA methylation using an *in vitro* differentiation scheme and a reverse genetics approach. Appendices A and B extended these analyses to investigate the effects of H3K27me3 levels on PRC1, and the role JARID2 plays in normal PRC2 function, respectively. In this chapter, we will review these major findings and conclusions, put them in context of the current body of knowledge on this topic, and discuss the next logical set of questions. Finally, we will discuss the relevance of this topic and these findings to the human condition.

Conclusions and Future Directions

We previously determined that a pre-existing "*Suz12 null*" ESC line (Pasini et al., 2007) maintained partial H3K27me3 levels, unlike other PRC2 core component knockout ESCs (Mazzoni et al., 2013). Despite this, this mutation is embryonic lethal during gastrulation, similar to other PRC2 core component knockouts (Pasini et al., 2004). Our work suggested that this allele of *Suz12*, which we named *Suz12GT*, functioned as a hypomorph *in vitro* in that it retained partial catalytic function of PRC2 and allowed low-efficiency *in vitro* differentiation – unlike total loss of H3K27me3 – but did not restrict the lineage pathways as vigorously as did the wild-type H3K27me3 profile. We recognized that this *Suz12GT* ESC line was a novel and invaluable tool to study the role of PRC2 during differentiation, as other PRC2 loss of function ESC lines characterized thus far are not capable of directed differentiation *in vitro*.

While we found, consistent with the literature (Hawkins et al., 2010), that wild-type ESCs gain significant enrichment of H3K27me3 over differentiation, *Suz12GT* ESCs did not show the same levels of this modification. These data support a model in which SUZ12GT-PRC2 can maintain a stable level of H3K27me3 in ESCs but cannot mediate the steep increase of H3K27me3 seen during differentiation. This phenotype allowed us to address a major open question in the field: what is the role of PRC2 in regulating gene expression during differentiation? We found that genes that normally gain H3K27me3 over differentiation are repressed in a wild-type system, but that these genes failed to be repressed when the levels of H3K27me3 were compromised in Suz12GT cells. Genes that should be activated upon differentiation, on the other hand, can still be activated at the correct time, albeit less efficiently, suggesting that gene program activation is unstable in the absence of PRC2. New methodologies that can now measure the transcript levels of single cells (Wu et al., 2013a) should help to better understand how altered PRC2 activity can affect cell fate. Together, these data indicate that regulation of H3K27me3 levels is necessary for proper activation of gene programs in response to developmental cues and for repression of alternate pathways to maintain lineage fidelity. Future *in vivo* studies of inactivation of PRC2 at discrete points in time during mammalian development will even further describe the role of this complex in differentiation, lineage commitment, and development.

Emerging evidence supports a mutual antagonism between PRC2 and DNA methylation in ESCs. However, the relationship between these two critical epigenetic regulatory mechanisms had been difficult to study during lineage commitment because PRC2 null ESCs do not undergo proper directed differentiation. The observation that *Suz12GT* ESCs can differentiate, albeit less efficiently, and harbor regions of variable H3K27me3 levels in the genome compared to wild-type cells, makes these cells a useful model to study this relationship. As such, we examined the changes in DNA methylation in wild-type and in several PRC2 mutant ESC lines at nucleotide-resolution using RRBS. We showed that in *Suz12GT* ESCs, PRC2 target regions losing H3K27me3 with respect to wild type cells gained DNA

methylation, suggesting that H3K27me3 is antagonistic to DNA methylation in *cis*. Supporting our observation, a recent study showed that PRC2 can recruit DNMT3L, a catalytically inactive DNA methyltransferase family member, to its target sites; DNMT3L then sterically competes with active DNA methyltransferases to prevent DNA methylation at PRC2 target sites (Neri et al., 2013).

While some PRC2-mediated changes are observed in DNA methylation in differentiated cells, overall DNA methylation in CpG islands (which are thought to recruit PRC2) does not exhibit a strong directional change over differentiation, whereas CpGs that are not contained within CpG islands show striking changes in their methylation state: unmethylated CpGs becoming fully methylated, or vice versa. This is consistent with the observation several years ago that promoter regions remained mainly stable over differentiation, while CpGs in non-promoter regions showed the majority of the change observed genome-wide (Meissner et al., 2008). Interestingly, this trend also holds true upon partial depletion of H3K27me3, indicating that the gross changes in DNA methylation state over differentiation are largely PRC2-independent, while the positioning of DNA methylation with respect to PRC2 target sites is partially regulated by PRC2.

While we do observe small but significant changes at a subset of CpG sites, most CpGs in the genome do not change, either between wild-type and PRC2 mutant cells, or over differentiation. The stability of the DNA methylome is largely maintained upon loss or reduction of PRC2 repression. Thus, as with any other complex system, there are multiple safeguards protecting the epigenome from loss of any particular mechanism. A growing body of evidence suggests that DNA methylation correlates more strongly with epigenetic features of the genome, such as various histone marks, than it does with the DNA sequence itself (Meissner et al., 2008). As such, studies of combinatorial histone modifications may be able to shed further light on the regulation of DNA methylation in pluripotency and lineage commitment, and the role of PRC2 therein. Additionally, it has been suggested that gene body DNA

methylation may have an activating effect on gene expression, as opposed to the repressive effect of promoter DNA methylation (Wu et al., 2010). Thus, comparing how DNA methylation changes at promoters relative to gene bodies in *Suz12GT* cells will be critical for understanding how these pathways coordinate to regulate complex networks of genes during development.

Prior studies suggested that H3K27me3-enriched genes in ESCs are more likely to gain DNA methylation upon differentiation (Mohn et al., 2008). We tested this hypothesis, and found that H3K27me3-enriched genes were more likely than non-enriched to gain DNA methylation over differentiation ($p<5E-4$); however, it must be noted that this is not a sweeping trend. Thus, while it is an appealing model, our data suggest that while H3K27me3 may anticipate DNA methylation over developmental time at some genes, it is neither necessary nor sufficient for the subsequent gain of DNA methylation. The relationship is much more complicated than that, and will require significant future study for elucidation.

We also found, as shown in Appendix A, that PRC1 is partially recruited to target sites in an H3K27me3-dependent manner, consistent with prior studies (Gao et al., 2012; Tavares et al., 2012; Wu et al., 2013b). Additionally, we found a surprising decrease in RING1B genomic localization over differentiation, which is consistent with a recently proposed model of H2AK119ub-mediated loss of PRC1 and derepression of target genes upon loss of pluripotency (Richly et al., 2010). However, in our hands, this loss of RING1B does not show a concomitant change in gene expression. It is worth noting the possibility that this observed loss of RING1B binding could be explained by a change in chromatin condensation and is not actually a loss but rather a figment of the method of measurement. It is possible that parallel gene repression pathways can compensate for the loss of RING1B binding at this stage. A possibility that merits future testing is that our study did not offer fine enough resolution over time to detect a PRC1-mediated change in gene expression over differentiation. It is possible that

PRC1 and H2AK119ub are lost immediately upon loss of pluripotency, and that that would be the correct time to check for change in gene expression. Additionally, conditional knockout and knockdown of *Ring1b* both before and after loss of pluripotency would test the precise roles of RING1B in gene regulation.

JARID2 and its role in mediating PRC2 recruitment and catalytic activity have been the subject of several studies (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). However, while all agree that JARID2 aids in PRC2 recruitment, its effects on PRC2 activity remain controversial. Our data in Appendix B showed that JARID2 negatively affects PRC2 activity. Thus, JARID2 manipulation should provide an excellent tool for parsing the effects of PRC2 recruitment into its non-catalytic and catalytic activities, which remains a major unresolved question in the field.

Relevance of the study of PcG proteins

Knowledge of Polycomb group repression in the context of lineage commitment and cell identity is critical for gaining further insights into regulation of cell fate. The very origins of our knowledge about PcG proteins are entrenched in homeotic transformation, a process whereby body patterning goes wrong and cells form the wrong structures, by virtue of misexpression of key genes that regulate entire developmental gene programs. Knockout of any PRC2 core component is embryonic lethal, as is *Ring1b* or any DNA methyltransferase (Li et al., 1992; Montgomery et al., 2005; O'Carroll et al., 2001; Okano et al., 1999; Pasini et al., 2004; Voncken et al., 2003). PRC- and DNA methylation-mediated repression are crucial components of the regulatory machinery that controls proper development by establishing proper chromatin states that enable cells to activate lineage programs while restricting cell identity (Smith and Meissner, 2013; Surface et al., 2010). When things go wrong with PcG-mediated repression during development, they can go drastically wrong. A human mutation of *CBX2*,

a homolog of Drosophila *M33* and component of H3K27me3-dependent PRC1 varieties, can cause complete sex reversal: a physiologically normal female with an XY genotype (Biason-Lauber et al., 2009). Indeed, studies of mammalian development are directly relevant to each and every one of us. Over 30% of conceptions end in miscarriage, more than 20% before they are even clinically recognized as a pregnancy (Wang et al., 2003; Wilcox et al., 1988). The many causes of fetal inviability have not been exhaustively elucidated, but developmental defects that result from disruption of transcriptional networks are a major factor (Regan and Rai, 2000). Thus, it is critical to precisely define how epigenetic regulators establish proper gene expression states during development, as the more we know about this intricately regulated process, the more we can do to rectify these issues.

Moreover, in addition to its roles in development, misregulation or mutation of PcG proteins is strongly associated with progression and severity of cancer. Overwhelming evidence has shown that PcG proteins are often misregulated in cancer cells. PcG proteins have been shown to be expressed at higher than normal levels, which is thought to lead to aberrant silencing of tumor suppressor genes (Piunti and Pasini, 2011). For example, PRC2 histone methyltransferase gene *Ezh2* is overexpressed in prostate cancer (Varambally et al., 2002), breast cancer (Collett et al., 2006; Kleer et al., 2003), lymphomas (Raaphorst et al., 2000; van Kemenade et al., 2001), and bladder cancer (Weikert et al., 2005), to name just a few. Indeed, ectopic overexpression of *Ezh2* leads to cancerous cell morphologies and phenotypes (Li et al., 2009), while inhibition of EZH2 is being tested as a promising cancer therapy (McCabe et al., 2012; Puppe et al., 2009). This effect is not limited to PRC2, as PRC1 component *Bmi1* is also upregulated in a variety of cancer subtypes (Hoenerhoff et al., 2009; Mohty et al., 2007; van Kemenade et al., 2001; van Leenders et al., 2007). Conversely, downregulation of PcG proteins has also been observed in tumor samples, suggesting the possibility of an inappropriate derepression of oncogenes; for example, *Bmi1* is downregulated in melanoma (Bachmann et al., 2008).

Emerging evidence also indicates that perturbation of PcG proteins in cancer may also have consequences on DNA methylation patterns. As discussed in depth in Chapter 1, PcG proteins and DNA methylation are thought to have an antagonistic effect at promoters; however, this relationship may disintegrate in cancer cells (Statham et al., 2012). For example, studies have shown that in cancer cells, PcG-associated genes can recruit DNA methyltransferases and are hypermethylated (Mohammad et al., 2009; Schlesinger et al., 2007). If the relationship between PcG-mediated silencing and DNA methylation is one of the many checks and balances that are broken in cancer, it behooves us to understand the normal state such that we can treat the problem.

In conclusion, PcG proteins occupy key positions in the transcriptional regulatory circuitry in diverse cell types and their faulty regulation can lead to developmental failure or diseases such as cancer. Thus, detailed studies to investigate the diverse mechanisms that PcG proteins employ to regulate cell fate transitions and cell identity are critical to further our understanding of both normal and pathologic development, and to facilitate the design of relevant therapies.

References

Bachmann, I.M., Puntervoll, H.E., Otte, A.P., and Akslen, L.A. (2008). Loss of BMI-1 expression is associated with clinical progress of malignant melanoma. Mod. Pathol. *21*, 583–590.

Biason-Lauber, A., Konrad, D., Meyer, M., DeBeaufort, C., and Schoenle, E.J. (2009). Ovaries and female phenotype in a girl with 46,XY karyotype and mutations in the CBX2 gene. Am. J. Hum. Genet. *84*, 658–663.

Collett, K., Eide, G.E., Arnes, J., Stefansson, I.M., Eide, J., Braaten, A., Aas, T., Otte, A.P., and Akslen, L.A. (2006). Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin. Cancer Res. *12*, 1168–1174.

Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., and Reinberg, D. (2012). PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. Mol Cell *45*, 344–356.

Hawkins, R.D., Hon, G.C., Lee, L.K., Ngo, Q., Lister, R., Pelizzola, M., Edsall, L.E., Kuan, S., Luu, Y., Klugman, S., et al. (2010). Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. Cell Stem Cell *6*, 479–491.

Hoenerhoff, M.J., Chu, I., Barkan, D., Liu, Z.-Y., Datta, S., Dimri, G.P., and Green, J.E. (2009). BMI1 cooperates with H-RAS to induce an aggressive breast cancer phenotype with brain metastases. Oncogene *28*, 3022–3032.

Kleer, C.G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S.A., Ghosh, D., Sewalt, R.G.A.B., Otte, A.P., Hayes, D.F., et al. (2003). EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci USA *100*, 11606–11611.

Landeira, D., Sauer, S., Poot, R., Dvorkina, M., Mazzarella, L., Jørgensen, H.F., Pereira, C.F., Leleu, M., Piccolo, F.M., Spivakov, M., et al. (2010). Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. Nat Cell Biol *12*, 618–624.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell *69*, 915–926.

Li, G., Margueron, R., Ku, M., Chambon, P., Bernstein, B.E., and Reinberg, D. (2010). Jarid2 and PRC2, partners in regulating gene expression. Genes Dev *24*, 368–380.

Li, X., Gonzalez, M.E., Toy, K., Filzen, T., Merajver, S.D., and Kleer, C.G. (2009). Targeted overexpression of EZH2 in the mammary gland disrupts ductal morphogenesis and causes epithelial hyperplasia. Am J Pathol *175*, 1246–1254.

Mazzoni, E.O., Mahony, S., Peljto, M., Patel, T., Thornton, S.R., McCuine, S., Reeder, C., Boyer, L.A., Young, R.A., Gifford, D.K., et al. (2013). Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals. Nat. Neurosci. *16*, 1191–1198.

McCabe, M.T., Ott, H.M., Ganji, G., Korenchuk, S., Thompson, C., Van Aller, G.S., Liu, Y., Graves, A.P., Pietra, Della, A., Diaz, E., et al. (2012). EZH2 inhibition as a therapeutic strategy for lymphoma with

EZH2-activating mutations. Nature *492*, 108–112.

Meissner, A., Mikkelsen, T.S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B.E., Nusbaum, C., Jaffe, D.B., et al. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature *454*, 766–770.

Mohammad, H.P., Cai, Y., Mcgarvey, K.M., Easwaran, H., van Neste, L., Ohm, J.E., O'Hagan, H.M., and Baylin, S.B. (2009). Polycomb CBX7 promotes initiation of heritable repression of genes frequently silenced with cancer-specific DNA hypermethylation. Cancer Res *69*, 6322–6330.

Mohn, F., Weber, M., Rebhan, M., Roloff, T.C., Richter, J., Stadler, M.B., Bibel, M., and Schübeler, D. (2008). Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell *30*, 755–766.

Mohty, M., Yong, A.S.M., Szydlo, R.M., Apperley, J.F., and Melo, J.V. (2007). The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. Blood *110*, 380–383.

Montgomery, N.D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S.J., Otte, A.P., and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol *15*, 942–947.

Neri, F., Krepelova, A., Incarnato, D., Maldotti, M., Parlato, C., Galvagni, F., Matarese, F., Stunnenberg, H.G., and Oliviero, S. (2013). Dnmt3L Antagonizes DNA Methylation at Bivalent Promoters and Favors DNA Methylation at Gene Bodies in ESCs. Cell *155*, 121–134.

O'Carroll, D., Erhardt, S., Pagani, M., Barton, S.C., Surani, M.A., and Jenuwein, T. (2001). The polycomb-group gene Ezh2 is required for early mouse development. Mol Cell Biol *21*, 4330–4336.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247–257.

Pasini, D., Bracken, A.P., Hansen, J.B., Capillo, M., and Helin, K. (2007). The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol *27*, 3769–3779.

Pasini, D., Bracken, A.P., Jensen, M.R., Lazzerini Denchi, E., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J *23*, 4061–4071.

Pasini, D., Cloos, P.A.C., Walfridsson, J., Olsson, L., Bukowski, J.-P., Johansen, J.V., Bak, M., Tommerup, N., Rappsilber, J., and Helin, K. (2010). JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. Nature *464*, 306–310.

Peng, J.C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A., and Wysocka, J. (2009). Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell *139*, 1290–1302.

Piunti, A., and Pasini, D. (2011). Epigenetic factors in cancer development: Polycomb group proteins. Future Oncology *7*, 57–75.

Puppe, J., Drost, R., Liu, X., Joosse, S.A., Evers, B., Cornelissen-Steijger, P., Nederlof, P., Yu, Q., Jonkers, J., van Lohuizen, M., et al. (2009). BRCA1-deficient mammary tumor cells are dependent on EZH2

expression and sensitive to Polycomb Repressive Complex 2-inhibitor 3-deazaneplanocin A. Breast Cancer Res. *11*, R63.

Raaphorst, F.M., van Kemenade, F.J., Blokzijl, T., Fieret, E., Hamer, K.M., Satijn, D.P., Otte, A.P., and Meijer, C.J. (2000). Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. Am J Pathol *157*, 709–715.

Regan, L., and Rai, R. (2000). Epidemiology and the medical causes of miscarriage. Baillieres Best Pract Res Clin Obstet Gynaecol *14*, 839–854.

Richly, H., Rocha-Viegas, L., Ribeiro, J.D., Demajo, S., Gundem, G., Lopez-Bigas, N., Nakagawa, T., Rospert, S., Ito, T., and Di Croce, L. (2010). Transcriptional activation of polycomb-repressed genes by ZRF1. Nature *468*, 1124–1128.

Schlesinger, Y., Straussman, R., Keshet, I., Farkash, S., Hecht, M., Zimmerman, J., Eden, E., Yakhini, Z., Ben-Shushan, E., Reubinoff, B.E., et al. (2007). Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet *39*, 232–236.

Shen, X., Kim, W., Fujiwara, Y., Simon, M.D., Liu, Y., Mysliwiec, M.R., Yuan, G.-C., Lee, Y., and Orkin, S.H. (2009). Jumonji Modulates Polycomb Activity and Self-Renewal versus Differentiation of Stem Cells. Cell *139*, 1303–1314.

Smith, Z.D., and Meissner, A. (2013). DNA methylation: roles in mammalian development. Nat Rev Genet *14*, 204–220.

Statham, A.L., Robinson, M.D., Song, J.Z., Coolen, M.W., Stirzaker, C., and Clark, S.J. (2012). Bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq) directly informs methylation status of histone-modified DNA. Genome Res *22*, 1120–1127.

Surface, L.E., Thornton, S.R., and Boyer, L.A. (2010). Polycomb group proteins set the stage for early lineage commitment. Cell Stem Cell *7*, 288–298.

Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., et al. (2012). RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. Cell *148*, 664–678.

van Kemenade, F.J., Raaphorst, F.M., Blokzijl, T., Fieret, E., Hamer, K.M., Satijn, D.P., Otte, A.P., and Meijer, C.J. (2001). Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. Blood *97*, 3896–3901.

van Leenders, G.J.L.H., Dukers, D., Hessels, D., van den Kieboom, S.W.M., Hulsbergen, C.A., Witjes, J.A., Otte, A.P., Meijer, C.J., and Raaphorst, F.M. (2007). Polycomb-group oncogenes EZH2, BMI1, and RING1 are overexpressed in prostate cancer with adverse pathologic and clinical features. Eur. Urol. *52*, 455–463.

Varambally, S., Dhanasekaran, S.M., Zhou, M., Barrette, T.R., Kumar-Sinha, C., Sanda, M.G., Ghosh, D., Pienta, K.J., Sewalt, R.G.A.B., Otte, A.P., et al. (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature *419*, 624–629.

Voncken, J.W., Roelen, B.A.J., Roefs, M., de Vries, S., Verhoeven, E., Marino, S., Deschamps, J., and van

Lohuizen, M. (2003). Rnf2 (Ring1b) deficiency causes gastrulation arrest and cell cycle inhibition. Proc Natl Acad Sci USA *100*, 2468–2473.

Wang, X., Chen, C., Wang, L., Chen, D., Guang, W., and French, J. (2003). Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study. Fertil. Steril. *79*, 577–584.

Weikert, S., Christoph, F., Köllermann, J., Müller, M., Schrader, M., Miller, K., and Krause, H. (2005). Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. Int. J. Mol. Med. *16*, 349–353.

Wilcox, A.J., Weinberg, C.R., O'Connor, J.F., Baird, D.D., Schlatterer, J.P., Canfield, R.E., Armstrong, E.G., and Nisula, B.C. (1988). Incidence of early loss of pregnancy. N. Engl. J. Med. *319*, 189–194.

Wu, A.R., Neff, N.F., Kalisky, T., Dalerba, P., Treutlein, B., Rothenberg, M.E., Mburu, F.M., Mantalas, G.L., Sim, S., Clarke, M.F., et al. (2013a). Quantitative assessment of single-cell RNA-sequencing methods. Nat Meth.

Wu, H., Coskun, V., Tao, J., Xie, W., Ge, W., Yoshikawa, K., Li, E., Zhang, Y., and Sun, Y.E. (2010). Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science *329*, 444–448.

Wu, X., Johansen, J.V., and Helin, K. (2013b). Fbxl10/Kdm2b recruits polycomb repressive complex 1 to CpG islands and regulates H2A ubiquitylation. Mol Cell *49*, 1134–1146.

Appendix A

H3K27me3-dependent PRC1 recruitment contributes no additive effect to H3K27me3-mediated gene repression

Author contributions for Appendix A:

Experiments designed by Seraphim Thornton and Laurie Boyer, and performed by Seraphim Thornton.

Summary

Although many studies have been done on Polycomb Repressive Complex 1 recruitment, the picture is a complex and multifaceted process, and thus not yet completely understood. However, at least part of PRC1 recruitment seems to occur through the canonical pathway where H3K27me3 is recognized and bound by a PRC1 subunit, such as a CBX-family protein. Thus, we wanted to determine what role PRC1 might play in PRC2-mediated gene repression, both during pluripotency and during differentiation. Here, we show a dose-dependent loss of RING1B localization with loss of H3K27me3. However, this H3K27me3-dependent PRC1 recruitment does not seem to play an additive role in gene repression in ESCs. Additionally, we observe a decrease in RING1B localization over differentiation, which may be partially dependent upon gain of H3K27me3; however, loss of RING1B does not appear to derepress target genes on the timescale studied. Thus, our data would suggest a model in which H3K27me3-dependent PRC1 exerts its gene repressive effect through the same pathway as does PRC2.

Introduction

Many of the gene promoters to which PRC2 localizes in ESCs also are enriched for Polycomb Repressive Complex 1 (PRC1) (Ku et al., 2008). PRC1 has been shown to exist in multiple varieties, containing multiple different combinations of subunits, and these varieties of PRC1 may be recruited through different mechanisms and play different roles in regulating gene expression. PRC1 is known to catalyze the histone mark H2AK119ub through action of its E3 ubiquitin ligase RING1B, and to a lesser extent, RING1A (Buchwald et al., 2006; Cao et al., 2005); however, it is still a matter of debate which varieties of PRC1 actually functionally contribute to H2AK119ub in the genome. Loss of RING1B, and thus the majority of H2AK119ub, leads to derepression of target genes – important developmental regulators – in ESCs (van der Stoop et al., 2008). However, while canonically thought of as a repressive mark in ESCs, this ubiquityl mark also shows recent evidence of being involved in activation of genes upon exit from pluripotency (Richly and Di Croce, 2011; Richly et al., 2010). Additionally, PRC1 is implicated in chromatin compaction, which has been shown to be independent of its catalytic ubiquitylation activity, although compaction may be insufficient to properly repress genes in the absence of H2AK119ub (Endoh et al., 2012; Eskeland et al., 2010; Francis et al., 2004). Indicative of these crucial roles in gene program regulation during mammalian development, loss of *Ring1b* is embryonic lethal in mice, and leads to an inability to properly differentiate *in vitro* (van der Stoop et al., 2008; Voncken et al., 2003).

It is becoming increasingly clear that the recruitment of PRC1 to its target genomic sites is a complex process, with multiple mechanisms acting on multiple varieties of PRC1. The canonical hypothesis is that PRC1 is recruited to chromatin by its CBX subunits, whose chromodomain recognizes and binds to H3K27me3 (Kaustov et al., 2011). In keeping with this, PRC1 has been shown to depend on H3K27me3 for proper genomic localization in some contexts (Agger et al., 2007; Boyer et al., 2006;

Morey et al., 2012; Wang et al., 2004). However, as loss of PRC2 doesn't completely ablate H2AK119ub, and PRC1 can be found at promoters without PRC2 – and vice versa – there are clearly additional mechanisms that function in directing its localization to genomic sites (Ku et al., 2008; Leeb et al., 2010; Schoeftner et al., 2006; Sing et al., 2009; Tavares et al., 2012). Recent studies showed that varieties of PRC1 containing RYBP and FBXL10 are able to localize to target sites independently of H3K27me3, and that these versions of the complex catalyze the majority of H2AK119ub (Gao et al., 2012; Wu et al., 2013). Additionally, RYBP and CBX-family proteins associate with distinct RING1B-containing PRC1 complexes; the latter family correlates with overlap of PRC1 and H3K27me3, in support of its biochemical role in binding said histone mark (Gao et al., 2012; Tavares et al., 2012). RYBP, on the other hand, stimulates catalysis of H2AK119ub (Gao et al., 2012). Collectively, the data suggest that this mark is predominantly if not exclusively catalyzed by PRC1 that is recruited independently of H3K27me3.

While this thesis focuses mainly on the role of PRC2, understanding the downstream effectors – or "readers" – of H3K27me3 is critical to understanding its function in regulating lineage decisions during development. PRC1 may in some cases act as an important downstream effector of PRC2, and as such, we investigated its possible ties to H3K27me3 and its function during differentiation.

Genomic RING1B localization is partially dependent upon H3K27me3

Recent studies paint a picture of PRC1 recruitment with many paths, some of which are H3K27me3-dependent. To test the hypothesis that some, but not all, RING1B is dependent upon H3K27me3 for its recruitment, we performed ChIP-seq for RING1B in wild-type, *Suz12GT*, *Suz12Δ*, and *Eed*^{null} ESCs and day 5 differentiated motor neurons. As described in more detail in Chapter 2 of this thesis, *Suz12GT* ESCs maintain an intermediate level of H3K27me3, but with a similar genomic localization pattern to wild-type ESCs; *Suz12Δ*, and *Eed*^{null} ESCs, on the other hand, lose nearly all detectable H3K27me3. In

support of the model, RING1B localization was only slightly lost in Suz12GT ESCs, whereas its levels were more significantly decreased – and yet, still not ablated – in Suz12Δ and Eed-/- ESCs (Figure 1). These data are in agreement with the decrease in PRC1 seen in other studies upon depletion of PRC2 (Tavares et al., 2012; Wu et al., 2013). Furthermore, by leveraging the power of the *Suz12GT* ESC line, we were able to ask whether the magnitude of loss of H3K27me3 at a given promoter correlated with that of loss of RING1B localization. We binned genes according to their gain or loss of H3K27me3 in *Suz12GT* ESCs with respect to the wild type, and compared the distribution of concomitant change in RING1B (Figure 2). We found that overall, more severe loss of H3K27me3 correlated with a more severe loss of RING1B. Thus, in ESCs, average RING1B levels were affected in a roughly dose-dependent manner by H3K27me3 levels, indicating that H3K27me3 does play a role in localization, albeit not one necessary for all RING1B localization.

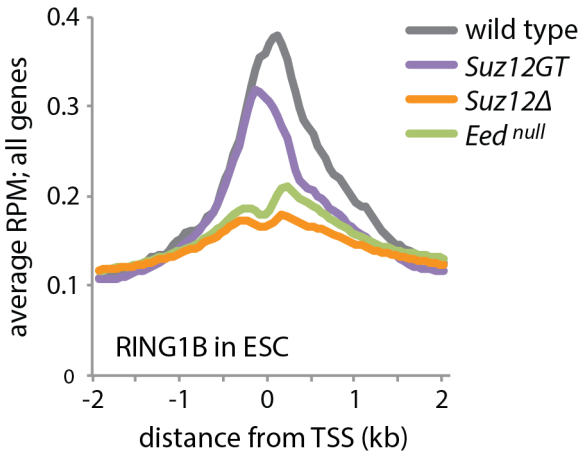

Figure 1. ChIP-Seq for RING1B was performed on wild-type, *Suz12GT*, *Suz12Δ*, and *Eed*^{null} ESCs. These data are shown in a metagene analysis: the average signal over all genes at +/-2kb from the TSS.

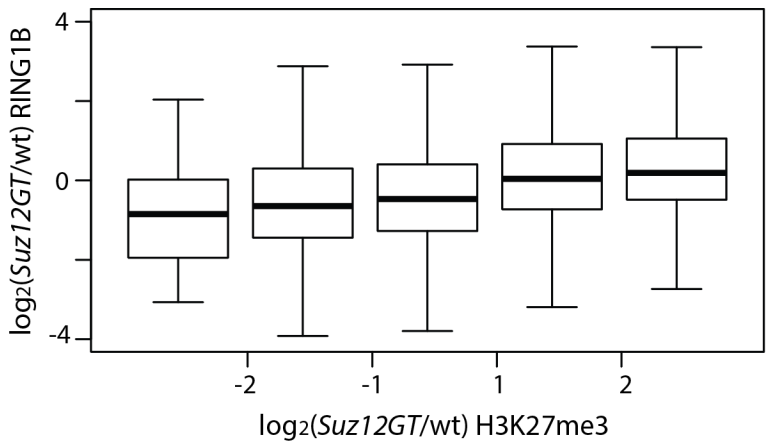

Figure 2. ChIP-Seq for RING1B and H3K27me3 was performed on wild-type and *Suz12GT* ESCs. All genes were binned by their change in H3K27me3 in *Suz12GT* vs. wild-type ESCs, and the distribution of change in RING1B in *Suz12GT* vs. wild-type ESCs is plotted on the y-axis.

To determine if PRC1 is a downstream effector of PRC2 in gene repression, we next asked whether loss of PRC1 at a given gene had an additive effect in the derepression caused by loss of PRC2. Using our ChIP-seq data for H3K27me3 and RING1B, as well as RNA-Seq data, in wild-type and *Suz12GT* ESCs, we separated PRC2 target genes into three categories: those that lose both H3K27me3 and RING1B in Suz12GT ESCs with respect to the wild type, those that lose only H3K27me3 and not RING1B, and those that maintain wild-type levels of both. As shown in Chapter 2 and in the literature (Boyer et al., 2006), genes losing H3K27me3 are significantly upregulated with respect to genes not losing H3K27me3 (Figure 3). Intriguingly, however, we observed no significant difference in upregulation between H3K27me3-losing genes that lose RING1B and those that do not (Figure 3). Thus, these data suggest that H3K27me3 participates in localization of RING1B-containing PRC1 to target genes in ESCs; however, this H3K27me3-dependent PRC1 localization fails to result in an observable effect on gene repression.

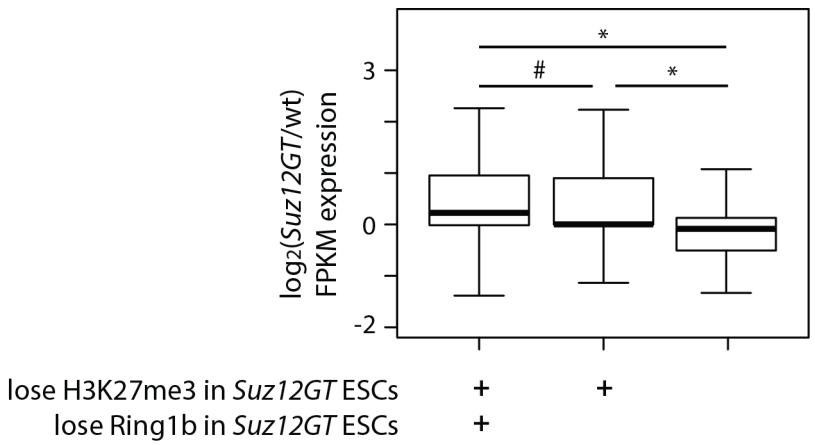

Figure 3. ChIP-Seq for RING1B and H3K27me3, and RNA-Seq, were performed on wild-type and *Suz12GT* ESCs. All genes were binned according to the categories described at bottom ("lose": $\log_2$<-1), and the distribution of change in expression in *Suz12GT* vs. wild-type ESCs is plotted on the y-axis. #: p=0.94; *: p<5E-12

Genomic RING1B levels decline upon ESC differentiation towards SMNs

Our data (Chapter 2) suggest that H3K27me3 levels are critical to maintaining the fidelity of lineage commitment. Given that we have supported a role for H3K27me3 in RING1B localization in ESCs, we asked whether PRC1 levels were tied to H3K27me3 over differentiation. Thus, we performed ChIP-seq for RING1B in ESCs and at day 5 of SMN differentiation. Surprisingly, in wild-type cells, while H3K27me3 is largely gained during differentiation, RING1B showed a shift towards loss of enrichment upon differentiation (Figure 4). The more than seven thousand genes that decrease RING1B levels twofold over SMN differentiation are primarily enriched for biological processes involving gene regulation and neuron differentiation (data not shown). This could reflect a disparity in the roles of PRC2 and PRC1 in this lineage: while PRC2 has an important role for repressing alternate lineage pathways during differentiation as shown in chapter 2, RING1B-containing PRC1 may play its repressive role more during pluripotency than SMN lineage commitment. It must be noted, however,

that we cannot exclude the possibility that our measurement of RING1B binding at different differentiation stages, and thus potentially different levels of chromatin compaction, causes an observed loss of binding that in reality pertains to the possibility that one PRC1 complex can cover more DNA length in condensed chromatin than in uncondensed chromatin.

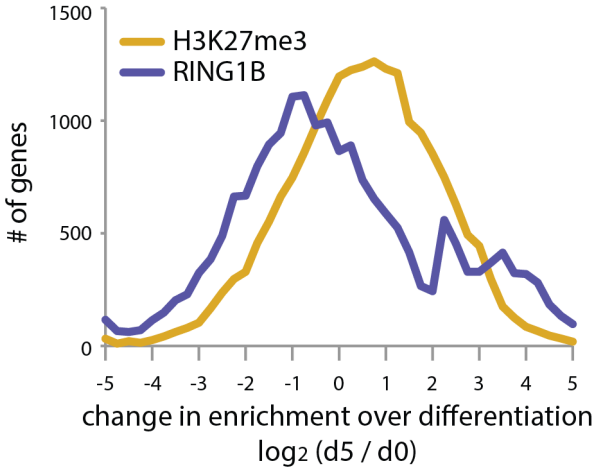

Figure 4. ChIP-Seq for RING1B and H3K27me3 was performed on wild-type ESCs and day 5 MNs. A histogram of the respective changes in enrichment over differentiation at promoter regions is shown here.

As such, we next wanted to know whether this normal loss of RING1B localization over differentiation actually had an effect on gene expression levels. Surprisingly, when we compared the change in RING1B localization to the change in expression of genes over differentiation, we found no significant association (Figure 5). Thus, it is possible that the loss of RING1B localization during differentiation is compensated for by some other mechanism, preventing the de-repression of target genes.

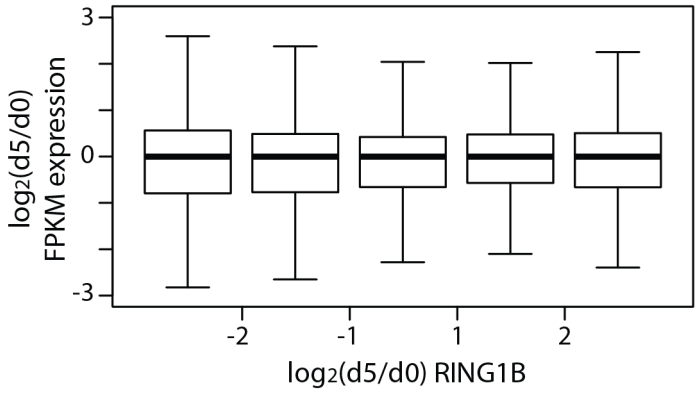

Figure 5. ChIP-Seq for RING1B and RNA-Seq were performed on wild-type ESCs and day 5 MNs. All genes were binned according to their change in RING1B levels over differentiation. The distribution of change in expression over differentiation was plotted on the y-axis. No significant difference was found between any bins.

To take a more in-depth look at whether PRC1 is recruited downstream of PRC2 during differentiation, we used our loss-of-function system. Curiously, upon directed differentiation, not as much Ring1b is lost on average in Suz12GT cells as in wild-type cells (Figure 6). This might be explained by our observation that fewer cells commit to the SMN lineage in Suz12GT cells than in the wild type. Alternatively, PRC1 loss over differentiation could be an effect of H3K27me3 gain, which is much weaker in Suz12GT cells than in the wild type. To test this second possibility, we binned genes by their ranked change in H3K27me3 levels over differentiation, and observed the distribution of RING1B change in these bins (Figure 7). Notably, the quintile of genes that gain the most H3K27me3 over differentiation also lose significantly more RING1B localization at their promoters (Figure 7, right). This result would support an antagonism between PRC2 and PRC1 during differentiation, despite their cooperative and mutually supportive roles during pluripotency.

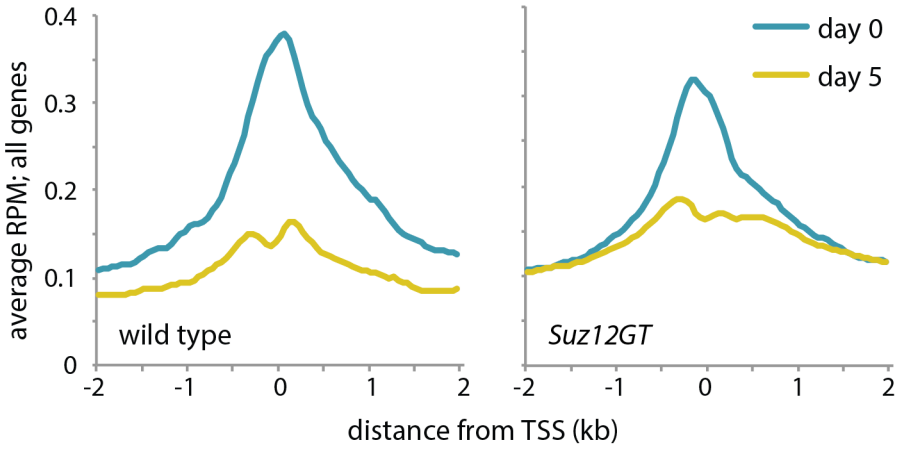

Figure 6. ChIP-Seq for RING1B was performed on wild-type and *Suz12GT* ESCs and day 5 MNs. These data are shown in a metagene analysis: the average signal over all genes at +/-2kb from the TSS.

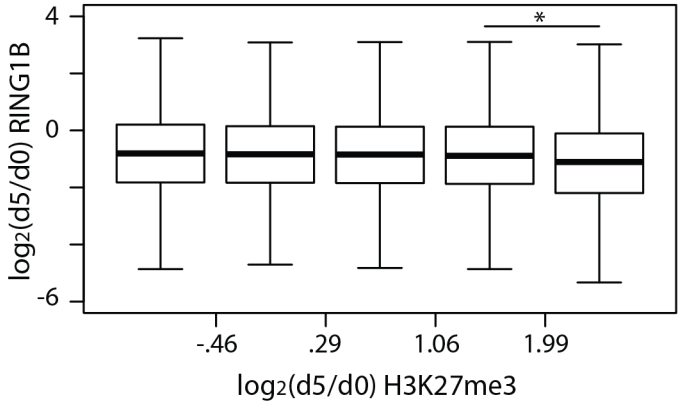

Figure 7. ChIP-Seq for RING1B and H3K27me3 were performed on wild-type ESCs and day 5 MNs. All genes were ranked according to their change in H3K27me3 levels over differentiation, and binned into quintiles. The distribution of change in RING1B levels over differentiation was plotted on the y-axis. *: p<5E-8

Concluding Remarks

Here, we show supporting data for the prevailing model that some, but not all, PRC1 is recruited by H3K27me3-mediated mechanisms in ESCs. In Suz12GT ESCs, which do not lose all H3K27me3 and thus see fewer secondary effects, RING1B localization is still partially lost. *Suz12Δ* and *Eed*^{null} ESCs lose RING1B localization to a greater extent, although still not completely. This is consistent with what would be expected if H3K27me3-dependent RING1B localization were part, but not all, of the story. Additionally, it has been shown that some PRC1 varieties are recruited in an H3K27me3-dependent manner, while others are not (Gao et al., 2012; Tavares et al., 2012); our data are also consistent with that model. Furthermore, several recent studies observed that the PRC1 varieties that were recruited to the genome independent of H3K27me3 actually catalyzed the majority of H2AK119ub (Gao et al., 2012; Tavares et al., 2012; Wu et al., 2013). As the non-catalytic functions of RING1B may be insufficient to repress target genes (Endoh et al., 2012), this supports our observation that H3K27me3-dependent loss of RING1B does not add to gene repression in ESCs. Overall, thus, our data support the intriguing model that in ESCs, RING1B-containing PRC1 complexes fall into two distinct categories: those that interact with H3K27me3, and those that catalyze H2AK119ub, leading to gene repression.

The recent suggestion that some H3K27me3 maintenance could, in turn, be RING1B-dependent(Endoh et al., 2012; Morey et al., 2012), although not RYBP-dependent (Gao et al., 2012), is intriguing in that it would allow the for a mutually-reinforcing Polycomb feedback loop. While many previous studies (Endoh et al., 2008; Gao et al., 2012; Stock et al., 2007) have shown that depletion of PRC1 leads to derepression of developmentally important target genes, the possibility remains that this is mediated primarily through H3K27me3-independent PRC1. This would seem to suggest separate roles for the H3K27me3-dependent and -independent categories of PRC1: CBX-containing PRC1 is recruited by H3K27me3 and functions to promote maintenance of said H3K27me3 and thus its

downstream gene repression pathway, while RYBP/FBXL10-containing PRC1 is recruited independently of H3K27me3 and catalyzes H2AK119ub, which in turn mediates its own gene repression pathway. If H3K27me3-dependent RING1B causes its effects on gene repression through modifying H3K27me3 levels, it would not show an additive effect on gene repression, which would be consistent with our data shown in Figure 3.

The possibility has also been raised that PRC1 acts to maintain the repressive state of target genes during pluripotency, but upon receiving the appropriate signals to exit pluripotency and differentiate, is in fact a targeted mechanism for selective gene program activation. PRC1 is lost and, through the same mechanism, histone de-ubiquitylases are recruited to remove H2AK119ub (Richly et al., 2011; 2010). Our data support this model very nicely, with RING1B showing a loss over differentiation at many gene promoters. *Suz12GT* ESCs also display this loss of RING1B localization over differentiation, although to a lesser extent; this could indicate a partial dependence on H3K27me3 of this directed loss of PRC1, either at the ESC stage in recruiting the necessary variety of PRC1 in the first place, or over differentiation as H3K27me3 is gained in the wild type. However, while our data do support a directed loss of RING1B localization over differentiation, they do not support a concomitant derepression of target genes. It remains possible that a comparison between day 0 and day 5 does not provide adequate resolution to detect the effects on gene expression of RING1B, especially if this loss of RING1B takes place immediately upon loss of pluripotency of ESCs and not during the lineage commitment pathway.

Thus, further study of the role of PRC1 during loss of pluripotency and lineage commitment is definitely merited, and should further illuminate the role of PcG proteins in regulating gene expression programs during mammalian development.

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Materials and Methods

As described in Chapter 2.

References

Agger, K., Cloos, P.A.C., Christensen, J., Pasini, D., Rose, S., Rappsilber, J., Issaeva, I., Canaani, E., Salcini, A.E., and Helin, K. (2007). UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature *449*, 731–734.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nat Cell Biol *441*, 349–353.

Buchwald, G., van der Stoop, P., Weichenrieder, O., Perrakis, A., van Lohuizen, M., and Sixma, T.K. (2006). Structure and E3-ligase activity of the Ring-Ring complex of polycomb proteins Bmi1 and Ring1b. Embo J *25*, 2465–2474.

Cao, R., Tsukada, Y.-I., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol Cell *20*, 845–854.

Endoh, M., Endo, T.A., Endoh, T., Fujimura, Y.-I., Ohara, O., Toyoda, T., Otte, A.P., Okano, M., Brockdorff, N., Vidal, M., et al. (2008). Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity. Development *135*, 1513–1524.

Endoh, M., Endo, T.A., Endoh, T., Isono, K.-I., Sharif, J., Ohara, O., Toyoda, T., Ito, T., Eskeland, R., Bickmore, W.A., et al. (2012). Histone H2A Mono-Ubiquitination Is a Crucial Step to Mediate PRC1-Dependent Repression of Developmental Genes to Maintain ES Cell Identity. PLoS Genet *8*, e1002774.

Eskeland, R., Leeb, M., Grimes, G.R., Kress, C., Boyle, S., Sproul, D., Gilbert, N., Fan, Y., Skoultchi, A.I., Wutz, A., et al. (2010). Ring1B compacts chromatin structure and represses gene expression independent of histone ubiquitination. Mol Cell *38*, 452–464.

Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science *306*, 1574–1577.

Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., and Reinberg, D. (2012). PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. Mol Cell *45*, 344–356.

Kaustov, L., Ouyang, H., Amaya, M., Lemak, A., Nady, N., Duan, S., Wasney, G.A., Li, Z., Vedadi, M., Schapira, M., et al. (2011). Recognition and specificity determinants of the human cbx chromodomains. J Biol Chem *286*, 521–529.

Ku, M., Koche, R.P., Rheinbay, E., Mendenhall, E.M., Endoh, M., Mikkelsen, T.S., Presser, A., Nusbaum, C., Xie, X., Chi, A.S., et al. (2008). Genomewide Analysis of PRC1 and PRC2 Occupancy Identifies Two Classes of Bivalent Domains. PLoS Genet *4*, e1000242.

Leeb, M., Pasini, D., Novatchkova, M., Jaritz, M., Helin, K., and Wutz, A. (2010). Polycomb complexes act redundantly to repress genomic repeats and genes. Genes Dev *24*, 265–276.

Morey, L., Pascual, G., Cozzuto, L., Roma, G., Wutz, A., Benitah, S.A., and Di Croce, L. (2012). Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. Cell Stem Cell *10*, 47–62.

Richly, H., Aloia, L., and Di Croce, L. (2011). Roles of the Polycomb group proteins in stem cells and cancer. Cell Death Dis *2*, e204.

Richly, H., and Di Croce, L. (2011). The flip side of the coin: role of ZRF1 and histone H2A ubiquitination in transcriptional activation. Cell Cycle *10*, 745–750.

Richly, H., Rocha-Viegas, L., Ribeiro, J.D., Demajo, S., Gundem, G., Lopez-Bigas, N., Nakagawa, T., Rospert, S., Ito, T., and Di Croce, L. (2010). Transcriptional activation of polycomb-repressed genes by ZRF1. Nature *468*, 1124–1128.

Schoeftner, S., Sengupta, A.K., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., Jenuwein, T., and Wutz, A. (2006). Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. Embo J *25*, 3110–3122.

Sing, A., Pannell, D., Karaiskakis, A., Sturgeon, K., Djabali, M., Ellis, J., Lipshitz, H.D., and Cordes, S.P. (2009). A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. Cell *138*, 885–897.

Stock, J.K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A.G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat Cell Biol *9*, 1428–1435.

Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., et al. (2012). RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. Cell *148*, 664–678.

van der Stoop, P., Boutsma, E.A., Hulsman, D., Noback, S., Heimerikx, M., Kerkhoven, R.M., Voncken, J.W., Wessels, L.F.A., and van Lohuizen, M. (2008). Ubiquitin E3 ligase Ring1b/Rnf2 of polycomb repressive complex 1 contributes to stable maintenance of mouse embryonic stem cells. PLoS ONE *3*, e2235.

Voncken, J.W., Roelen, B.A.J., Roefs, M., de Vries, S., Verhoeven, E., Marino, S., Deschamps, J., and van Lohuizen, M. (2003). Rnf2 (Ring1b) deficiency causes gastrulation arrest and cell cycle inhibition. Proc Natl Acad Sci USA *100*, 2468–2473.

Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004). Hierarchical recruitment of polycomb group silencing complexes. Mol Cell *14*, 637–646.

Wu, X., Johansen, J.V., and Helin, K. (2013). Fbxl10/Kdm2b recruits polycomb repressive complex 1 to CpG islands and regulates H2A ubiquitylation. Mol Cell *49*, 1134–1146.

Appendix B

JARID2 has a negative modulatory effect on PRC2 activity in ESCs

Author contributions for Appendix B:

All experiments designed by Seraphim Thornton and Laurie Boyer, and performed by Seraphim Thornton.

Summary

JARID2, a protein that can complex with PRC2 under the correct circumstances, has much-debated effects on the activity of PRC2. Although the literature largely agrees that JARID2 aids in PRC2 recruitment to mutual target sites, there is evidence supporting both a positive and a negative effect on the catalysis of H3K27me3 by PRC2 subunit EZH2. Here, we use the *Suz12GT* cell line, with its already-reduced levels of H3K27me3, to show an enhancement of H3K27me3 upon JARID2 depletion. Thus, our data support a model in which JARID2 attenuates PRC2's catalytic ability.

Introduction

Although the core components of PRC2 are expressed ubiquitously, there are several different forms of the complex that include other associated proteins or alternative isoforms of the core components. JARID2, for example, has recently been found to associate with PRC2 in ESCs (Landeira and Fisher, 2011). JARID2 is part of the Jumonji family of histone lysine demethylases, but is thought to be catalytically inactive (Klose et al., 2006). Its loss leads to embryonic lethality, and mutations in *Jarid2* are associated with several human disorders (Landeira and Fisher, 2011). Although studies agree that JARID2 assists in recruitment of PRC2 to its target sites (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010a; Peng et al., 2009; Shen et al., 2009), there remains a lack of consensus in the field regarding its function, as it has been shown to both activate and repress PRC2's catalytic activity (Landeira et al., 2010; Li et al., 2011; Montgomery et al., 2005; Pasini et al., 2007; 2010b; Peng et al., 2009; Shen et al., 2009; 2008). Thus, this optional PRC2 subunit may be important for regulating the effects of PRC2-mediated gene silencing, through H3K27me3.

JARID2 maintains some association with Suz12GT-PRC2

In Chapter 2, we showed that EZH2 and EED can form a complex in Suz12GT ESCs. Hypomorphic systems are often useful tools in which to observe weak phenotypes; as such, If JARID2 associates with PRC2 in *Suz12GT* cells, this may prove useful in investigating the effect of JARID2 on PRC2. Thus, to determine whether JARID2 still localizes to PRC2 target sites, we performed ChIP-qPCR for JARID2 on *Suz12GT* ESCs. We found a much-decreased presence of the protein at its target genes in *Suz12GT* ESCs (Figure 1). However, when we depleted *Suz12GT* ESCs of JARID2 using an shRNA-mediated knockdown system (Figure 2), we found that the ChIP-qPCR signal for JARID2 was even further ablated, indicating that some JARID2 remained at target genes in *Suz12GT* ESCs (Figure 1). This lends support for the model that JARID2 binds to the N-terminal end of SUZ12, since the *Suz12GT* allele only includes exons 1-7 of *Suz12* (Peng et al., 2009).

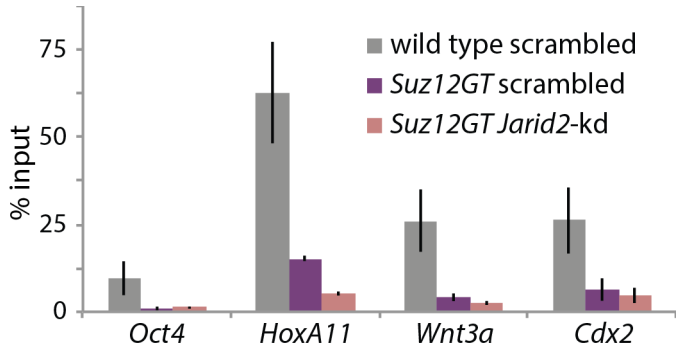

Figure 1. ChIP for JARID2 was performed on *Suz12GT* ESCs expressing scrambled shRNA and shRNA targeted to *Jarid2*, and wild-type ESCs expressing scrambled shRNA. JARID2 localization was tested by qPCR at the promoter regions of four genes: *Oct4*, *HoxA11*, *Wnt3a*, and *Cdx2*; the first is a negative control, while the last three are canonical JARID2 target genes. Error bars show the standard deviation of three qPCR technical replicates.

Figure 2. *Suz12GT* ESCs expressing scrambled shRNA and shRNA targeted to *Jarid2*, respectively, were created. RNA was collected and qRT-PCR was performed to validate the level of *Jarid2* knockdown. *Jarid2* expression in each cell line is calculated relative to expression of housekeeping gene *GAPDH*, and normalized to the level of *Jarid2* expression in *Suz12GT* scrambled. Error bars show the standard deviation of three qPCR technical replicates.

JARID2 may act to depress PRC2 function

After learning that JARID2 still associated with PRC2 in *Suz12GT* ESCs, we wanted to use this system to determine the effect of JARID2 loss on the catalytic activity of PRC2. As such, we performed ChIP-qPCR for H3K27me3 on *Jarid2*-knockdown *Suz12GT* ESCs, as well as a control line for shRNA expression. Interestingly, JARID2 depletion caused an elevation in H3K27me3 levels, in agreement with Shen et al. (Figure 3) (Shen et al., 2009). The effect of *Jarid2* depletion on wild-type PRC2 has already been tested, to mixed results; here, we add new data to the debate over the function of JARID2. SUZ12GT-PRC2 acts like a hypomorph in that it deposits a lower level of H3K27me3 in the genome. In this background, JARID2 is still able to exert a positive effect on the catalytic abilities of PRC2. Thus, of the several models proposed by the literature, these additional data would support the model that JARID2 assists in PRC2 recruitment – a part of the model that is not contested – but also represses the catalytic activity of PRC2, thus depressing the level of H3K27me3.

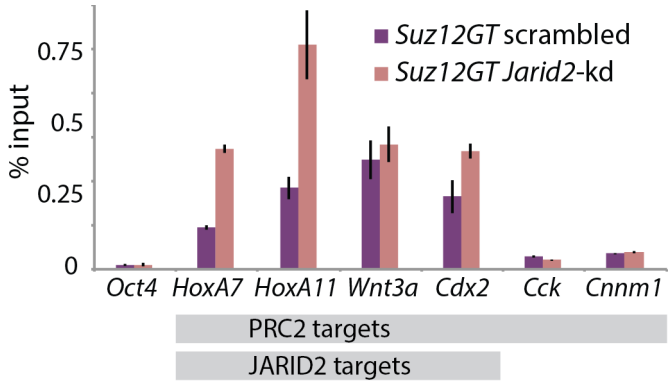

Figure 3. ChIP for H3K27me3 was performed on *Suz12GT* ESCs expressing scrambled shRNA and shRNA targeted to *Jarid2*. H3K27me3 levels were tested by qPCR at the promoter regions of seven genes. Canonical PRC2 and JARID2 target genes are indicated below the graph. Error bars show the standard deviation of three qPCR technical replicates.

Concluding Remarks

If the sole purpose of PRC2 were to catalyze H3K27me3 at the correct time and the correct place in the genome, what would be the purpose of a protein that allows the cell to keep H3K27me3 levels in check, while still keeping PRC2 in its position? Recent studies are bringing to light the possibility that PRC2 has functions beyond H3K27me3, even if loss of this histone mark seems to recapitulate the loss of Polycomb phenotype in *Drosophila* (Pengelly et al., 2013). Notably, for example, PRC2 has been shown to recruit DNMT3L to its target sites, which in turn blocks access of DNMT3a and DNMT3b to these PRC2 target promoters (Neri et al., 2013). However, if the catalysis of H3K27me3 by PRC2 cannot be modulated, the cell has only two options: recruit PRC2, set up strong H3K27me3-mediated gene silencing, and prevent DNA methylation-mediated gene silencing; or, remove PRC2, allow H3K27me3 and its associated repression pathway to lapse, and allow repressive DNA methylation. The introduction of JARID2, with its own subset of recruitment sites, allows a third possibility: bind PRC2 to its target, but keep its level of catalytic activity in check, thus reducing H3K27me3 levels, while still competitively inhibiting the binding of active DNA methyltransferases to the locus. This provides an elegant mechanism for fine-tuning gene expression in response to cellular signals during loss of pluripotency, lineage commitment, and differentiation.

Materials and Methods

As described in Chapter 2.

References

Klose, R.J., Kallin, E.M., and Zhang, Y. (2006). JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet *7*, 715–727.

Landeira, D., and Fisher, A.G. (2011). Inactive yet indispensable: the tale of Jarid2. Trends Cell Biol *21*, 74–80.

Landeira, D., Sauer, S., Poot, R., Dvorkina, M., Mazzarella, L., Jørgensen, H.F., Pereira, C.F., Leleu, M., Piccolo, F.M., Spivakov, M., et al. (2010). Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. Nat Cell Biol *12*, 618–624.

Li, G., Margueron, R., Ku, M., Chambon, P., Bernstein, B.E., and Reinberg, D. (2010). Jarid2 and PRC2, partners in regulating gene expression. Genes Dev *24*, 368–380.

Li, X., Isono, K.-I., Yamada, D., Endo, T.A., Endoh, M., Shinga, J., Mizutani-Koseki, Y., Otte, A.P., Casanova, M., Kitamura, H., et al. (2011). Mammalian polycomb-like Pcl2/Mtf2 is a novel regulatory component of PRC2 that can differentially modulate polycomb activity both at the Hox gene cluster and at Cdkn2a genes. Mol Cell Biol *31*, 351–364.

Montgomery, N.D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S.J., Otte, A.P., and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol *15*, 942–947.

Neri, F., Krepelova, A., Incarnato, D., Maldotti, M., Parlato, C., Galvagni, F., Matarese, F., Stunnenberg, H.G., and Oliviero, S. (2013). Dnmt3L Antagonizes DNA Methylation at Bivalent Promoters and Favors DNA Methylation at Gene Bodies in ESCs. Cell *155*, 121–134.

Pasini, D., Bracken, A.P., Hansen, J.B., Capillo, M., and Helin, K. (2007). The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol *27*, 3769–3779.

Pasini, D., Cloos, P.A.C., Walfridsson, J., Olsson, L., Bukowski, J.-P., Johansen, J.V., Bak, M., Tommerup, N., Rappsilber, J., and Helin, K. (2010a). JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. Nature *464*, 306–310.

Pasini, D., Malatesta, M., Jung, H.R., Walfridsson, J., Willer, A., Olsson, L., Skotte, J., Wutz, A., Porse, B., Jensen, O.N., et al. (2010b). Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. Nucleic Acids Research *38*, 4958–4969.

Peng, J.C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A., and Wysocka, J. (2009). Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell *139*, 1290–1302.

Pengelly, A.R., Copur, Ö., Jäckle, H., Herzig, A., and Müller, J. (2013). A histone mutant reproduces the phenotype caused by loss of histone-modifying factor Polycomb. Science *339*, 698–699.

Shen, X., Kim, W., Fujiwara, Y., Simon, M.D., Liu, Y., Mysliwiec, M.R., Yuan, G.-C., Lee, Y., and Orkin, S.H. (2009). Jumonji Modulates Polycomb Activity and Self-Renewal versus Differentiation of Stem Cells. Cell *139*, 1303–1314.

Shen, X., Liu, Y., Hsu, Y.-J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G.-C., and Orkin, S.H. (2008). EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell *32*, 491–502.

Appendix C

Supplemental Information to Chapter 2

Author contributions for Appendix C:

See Chapter 2

ST1: Table of antibodies

Protein	**Use**	**Antibody**
SUZ12	Western Blot	Bethyl A302-407A
H3K27me3	ChIP / Western Blot	Millipore 07-449
EED	IP	Abcam ab4469
EZH2	Western Blot	Millipore 07-689
β-actin	Western Blot	Abcam ab8226
OLIG2	IHC	Millipore AB9610
RING1B	ChIP	Koseki lab (Atsuta et al., 2001)
JARID2	ChIP	Novus Biologicals NB100-2214

ST2: Table of primers and oligonucleotides

Use	Gene	Forward primer	Reverse primer
Expression qRT-PCR	*Suz12* 5′	TACCCTGGAAGTCCTGCTTG	AACTGCCAGGGATGGAAAAT
Expression qRT-PCR	*Suz12* 3′	CTTGTTTGCAGGCCAAAAAGAAC	ATTTCTTGTGGCCGAAGAGGTAA
Expression qRT-PCR	*Gapdh*	CAT CTT CTT GTG CAG TGC CAG	GGC AAC AAT CTC CAC TTT GCC
ChIP qRT-PCR	*Oct4*	ACACCAGTGATGCGTGAAAA	CCAGTCACACCCAACCTCTT
ChIP qRT-PCR	*HoxA7*	TTG CCT GAG AGC CGC CAG GA	GCT GGC TGG CAG CAA CTC CA
ChIP qRT-PCR	*HoxA11*	AGTAGCAGTGGGCCAGATTG	GATTTCTCCAGCCTCCCTTC
ChIP qRT-PCR	*Wnt3a*	CTGTCCAGCCTCTCCAAGAC	CGCTGTCGCCTAGCTTCTAC
ChIP qRT-PCR	*Cdx2*	ATTGAGACCGTGGGCTACC	TACCCGGACTACGGTGGTTA
ChIP qRT-PCR	*Cck*	AGCTTCTGCAGGGACTACCG	ACTTCTGTGTGCGGGACTTT
ChIP qRT-PCR	*Cnnm1*	TGCTTGACTTCGCCACTGTA	AAAGGCCAAGTCTTTGACGA
ChIP qRT-PCR	*Lhx9*	TTAGCGGCTCCTTGACTTGG	TGGAGGGCTCGGATTCACTA
ChIP qRT-PCR	*Inhbb*	GCT CTG GAG ACT GAA GCG AA	ACA GCT CTC TCT CCT CCC G
Expression qRT-PCR	*Oct4*	GCT CAC CCT GGG CGT TCT C	GGC CGC AGC TTA CAC ATG TTC
Expression qRT-PCR	*Pax6*	GAC CGG AAG CTG GGG CAC AC	TGG CAG CCA TCT TGC GTG GG
Expression qRT-PCR	*Olig2*	CTC CGC AGC GAG CAC CTC AAA	TCA GCT CTG GCG GGC AGT CG
Expression qRT-PCR	*Isl1*	TGC AAA TGG CAG CCG AAC CCA	AGG TCC GCA AGG TGT GCA GC
Expression qRT-PCR	*Hlxb9 (Hb9)*	AGA TGC CGG ACT TCA GCT CCC AG	TCT CGG TGA GCA TGA GCG AGG T
shRNA cloning	scr	CCGGCCTAAGGTTAAGTCGCCCTCCTCGAGGAGGGCGACTTAACCTTAGGTTTTTG	AATTCAAAAACCTAAGGTTAAGTCGCCCTCCTCGAGGAGGGCGACTTAACCTTAGG
shRNA cloning	*5′ Suz12*	CCGGAAAGTTGATGATATGTTATCACTCGAGTGATAACATATCATCAACTTTTTTTTG	AATTCAAAAAAAAGTTGATGATATGTTATCACTCGAGTGATAACATATCATCAACTTT
shRNA cloning	*LacZ*	CCG GAA ACT GTG GAG CGC CGA AAT CCT CGA GGA TTT CGG CGC TCC ACA GTT TTT TTT G	AAT TCA AAA AAA ACT GTG GAG CGC CGA AAT CCT CGA GGA TTT CGG CGC TCC ACA GTT T
shRNA cloning	*Ezh2*	CCG GAA ATC TGA GAA GGG ACC GGT TCT CGA GAA CCG GTC CCT TCT CAG ATT TTT TTT G	AAT TCA AAA AAA ATC TGA GAA GGG ACC GGT TCT CGA GAA CCG GTC CCT TCT CAG ATT T
shRNA cloning	*Ezh1*	CCGGTTCTACACAAGTGTATAACTACCTCGAGGTAGTTATACACTTGTGTAGAATTTTTG (Shen et al., 2008)	AATTCAAAAATTCTACACAAGTGTATAACTACCTCGAGGTAGTTATACACTTGTGTAGAA (Shen et al., 2008)

ST3: Table of Chromatin Immunoprecipitations

Cell type	**Factor**	**Reference**	**sample name (if sequenced)**
E14 ESC	H3K27me3	NA	ST45_E14d0_K27
E14 d5 MN	H3K27me3	NA	ST46_E14d5_K27
Suz12GT ESC	H3K27me3	NA	ST1331_Suz12GTd0_K27
Suz12GT d5 MN	H3K27me3	NA	ST1312_Suz12GTd5_K27
Suz12Δ ESC	H3K27me3	NA	ST1324_Suz12Dd0_K27
Suz12Δ d5 MN	H3K27me3	NA	ST1328_Suz12Dd5_K27
Eed^{null} ESC	H3K27me3	NA	ST1333_Eed-d0_K27
Eed^{null} d5 MN	H3K27me3	NA	ST1334_Eed-d5_K27
E14 ESC	RING1B	NA	ST29_E14d0_Ring1b
E14 d5 MN	RING1B	NA	ST31_E14d5_Ring1b
Suz12GT ESC	RING1B	NA	ST1310_Suz12GTd0_Ring1b
Suz12GT d5 MN	RING1B	NA	ST1314_Suz12GTd5_Ring1b
Suz12Δ ESC	RING1B	NA	ST1326_Suz12Dd0_Ring1b
Suz12Δ d5 MN	RING1B	NA	ST1330_Suz12Dd5_Ring1b
Eed^{null} ESC	RING1B	NA	ST1303_Eed-d0_Ring1b
Eed^{null} d5 MN	RING1B	NA	ST1307_Eed-d5_Ring1b
Suz12GT scr ESC	H3K27me3	NA	NA
Suz12GT Ezh2-kd ESC	H3K27me3	NA	NA
Suz12GT Ezh1-kd ESC	H3K27me3	NA	NA
V6.5 ESC	H3K27me3	(Mazzoni et al., 2013)	NA
Suz12GT ESC	H3K27me3	(Mazzoni et al., 2013)	NA
Suz12GT ESC	H3K27me3	NA	ST7_Suz12GT_K27
E14 scr ESC	JARID2	NA	NA
Suz12GT scr ESC	JARID2	NA	NA
Suz12GT Jarid2-kd ESC	JARID2	NA	NA

ST4: Table of Gene Ontology categories

GO Biological Processes category	**Bonferroni**
PRC2 target genes maintaining <50% H3K27me3 in *Suz12GT* ESCs wrt wild type	
GO:0007156~homophilic cell adhesion	6.82E-15
GO:0016337~cell-cell adhesion	2.20E-12
GO:0007155~cell adhesion	4.56E-11
GO:0022610~biological adhesion	4.90E-11
GO:0031644~regulation of neurological system process	5.10E-04
GO:0044057~regulation of system process	0.001403159
GO:0001944~vasculature development	0.00211641
GO:0048514~blood vessel morphogenesis	0.003883989
GO:0009719~response to endogenous stimulus	0.004012146
GO:0001568~blood vessel development	0.004317515
PRC2 target genes maintaining >90% H3K27me3 in *Suz12GT* ESCs wrt wild type	
GO:0007389~pattern specification process	6.95E-47
GO:0003002~regionalization	1.07E-42
GO:0006355~regulation of transcription, DNA-dependent	6.66E-38
GO:0048598~embryonic morphogenesis	3.10E-37
GO:0051252~regulation of RNA metabolic process	7.18E-37
GO:0009952~anterior/posterior pattern formation	4.13E-32
GO:0045449~regulation of transcription	3.36E-28
GO:0030182~neuron differentiation	9.95E-28
GO:0048562~embryonic organ morphogenesis	1.38E-25
GO:0048568~embryonic organ development	2.37E-25
PRC2 target genes doubling H3K27me3 in wild-type cells over differentiation	
GO:0006355~regulation of transcription, DNA-dependent	6.03E-41
GO:0051252~regulation of RNA metabolic process	1.77E-40
GO:0030182~neuron differentiation	1.46E-39
GO:0007389~pattern specification process	9.23E-36
GO:0048598~embryonic morphogenesis	8.32E-32
GO:0006357~regulation of transcription from RNA polymerase II promoter	7.04E-31
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	3.85E-29
GO:0045893~positive regulation of transcription, DNA-dependent	1.74E-28
GO:0051254~positive regulation of RNA metabolic process	3.65E-28
GO:0007267~cell-cell signaling	6.00E-28
PRC2 target genes losing at least half H3K27me3 in wild type cells over differentiation	
GO:0007156~homophilic cell adhesion	5.19E-07
GO:0016337~cell-cell adhesion	3.91E-05
GO:0048667~cell morphogenesis involved in neuron differentiation	8.10E-05
GO:0030182~neuron differentiation	1.27E-04
GO:0007411~axon guidance	2.03E-04
GO:0048666~neuron development	3.49E-04
GO:0007409~axonogenesis	5.08E-04
GO:0000904~cell morphogenesis involved in differentiation	8.18E-04
GO:0048568~embryonic organ development	0.001224233
GO:0031175~neuron projection development	0.001233972

PRC2 target genes doubling H3K27me3 in *Suz12GT* cells over differentiation	
Lhx9	
Gm973	
Fezf2	
Otx2	
Crebbp	
Rorb	
Wt1	
Prdm16	
Foxd3	
Cacna2d1	
Pdgfra	
Hoxa13	
Foxp2	
Cav1	
St7	
Evx1	
Foxl1	
PRC2 target genes gaining H3K27me3 over differentiation in *Suz12GT* cells	
GO:0006355~regulation of transcription, DNA-dependent	4.24E-31
GO:0051252~regulation of RNA metabolic process	2.03E-30
GO:0045449~regulation of transcription	1.43E-25
GO:0006357~regulation of transcription from RNA polymerase II promoter	5.56E-22
GO:0007389~pattern specification process	2.28E-19
GO:0045893~positive regulation of transcription, DNA-dependent	6.11E-18
GO:0051254~positive regulation of RNA metabolic process	8.47E-18
GO:0045941~positive regulation of transcription	1.50E-17
GO:0045165~cell fate commitment	2.24E-17
GO:0010628~positive regulation of gene expression	5.33E-17
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	7.67E-17
GO:0003002~regionalization	8.20E-17
GO:0048598~embryonic morphogenesis	8.62E-17
…	
GO:0030182~neuron differentiation	3.10E-11
GO:0001501~skeletal system development	3.22E-10
GO:0048736~appendage development	1.07E-09
GO:0060173~limb development	1.07E-09
GO:0035113~embryonic appendage morphogenesis	1.46E-09
GO:0030326~embryonic limb morphogenesis	1.46E-09
GO:0030900~forebrain development	3.86E-09
GO:0035108~limb morphogenesis	4.69E-09
GO:0035107~appendage morphogenesis	4.69E-09
GO:0009952~anterior/posterior pattern formation	2.79E-08
PRC2 target genes losing at least 50% H3K27me3 over differentiation in *Suz12GT* cells	
GO:0030001~metal ion transport	2.86E-13
GO:0006812~cation transport	2.86E-12
GO:0001501~skeletal system development	1.71E-11

GO:0006811~ion transport	1.88E-11
GO:0007156~homophilic cell adhesion	3.11E-09
GO:0007389~pattern specification process	1.51E-08
GO:0003002~regionalization	1.58E-08
GO:0006813~potassium ion transport	2.11E-08
GO:0030182~neuron differentiation	2.89E-08
GO:0009952~anterior/posterior pattern formation	2.97E-08
PRC2 target genes losing >50% H3K27me3 over diff in *Suz12GT* cells, not losing in wt	
GO:0030001~metal ion transport	3.65E-17
GO:0006812~cation transport	1.16E-16
GO:0006811~ion transport	2.19E-16
GO:0006813~potassium ion transport	1.22E-10
GO:0015672~monovalent inorganic cation transport	4.02E-10
GO:0006816~calcium ion transport	2.20E-07
GO:0001501~skeletal system development	1.30E-05
GO:0007267~cell-cell signaling	6.97E-05
GO:0015674~di-, tri-valent inorganic cation transport	8.56E-05
GO:0044057~regulation of system process	1.15E-04
GO:0030182~neuron differentiation	3.36E-04
PRC2 target genes in quintile gaining most H3K27me3 over differentiation, wild type	
GO:0006355~regulation of transcription, DNA-dependent	1.16E-65
GO:0051252~regulation of RNA metabolic process	5.22E-65
GO:0045449~regulation of transcription	1.62E-47
GO:0007389~pattern specification process	1.89E-43
GO:0006357~regulation of transcription from RNA polymerase II promoter	1.04E-41
GO:0048598~embryonic morphogenesis	6.45E-39
GO:0045893~positive regulation of transcription, DNA-dependent	1.48E-36
GO:0003002~regionalization	2.04E-36
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	2.23E-36
GO:0051254~positive regulation of RNA metabolic process	2.85E-36
GO:0051173~positive regulation of nitrogen compound metabolic process	8.87E-36
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.58E-35
GO:0030182~neuron differentiation	7.67E-35
PRC2 target genes in quintile losing most H3K27me3 over differentiation, wild type	
GO:0030182~neuron differentiation	1.64E-39
GO:0007389~pattern specification process	2.09E-32
GO:0006355~regulation of transcription, DNA-dependent	1.96E-29
GO:0051252~regulation of RNA metabolic process	1.03E-28
GO:0007267~cell-cell signaling	1.48E-28
GO:0048598~embryonic morphogenesis	2.33E-26
GO:0006357~regulation of transcription from RNA polymerase II promoter	2.79E-26
GO:0003002~regionalization	5.18E-26
GO:0001501~skeletal system development	2.19E-25
GO:0048568~embryonic organ development	1.28E-24
PRC2 target genes gaining twofold DNA methylation in *Suz12GT* ESCs vs. wild type	
GO:0048598~embryonic morphogenesis	2.64E-04

GO:0030182~neuron differentiation	0.005452039
GO:0048666~neuron development	0.016523564
GO:0048729~tissue morphogenesis	0.02627389
GO:0016331~morphogenesis of embryonic epithelium	0.04121866
Genes gaining twofold DNA methylation in *Suz12GT* day 5 differentiated cells vs. wild type	
GO:0019226~transmission of nerve impulse	0.006369025
GO:0007268~synaptic transmission	0.038216629

References

Atsuta, T., Fujimura, S., Moriya, H., Vidal, M., Akasaka, T., and Koseki, H. (2001). Production of monoclonal antibodies against mammalian Ring1B proteins. Hybridoma *20*, 43–46.

Mazzoni, E.O., Mahony, S., Peljto, M., Patel, T., Thornton, S.R., McCuine, S., Reeder, C., Boyer, L.A., Young, R.A., Gifford, D.K., et al. (2013). Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals. Nat. Neurosci. *16*, 1191–1198.

Shen, X., Liu, Y., Hsu, Y.-J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G.-C., and Orkin, S.H. (2008). EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell *32*, 491–502.