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| As Published | http://dx.doi.org/10.1016/j.celrep.2015.07.053 |
| Publisher | Elsevier |
| Version | Final published version |
| Accessed | Sat Feb 09 23:07:33 EST 2019 |
| Citable Link | http://hdl.handle.net/1721.1/100783 |
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PRC2 Is Required to Maintain Expression of the Maternal *Gtl2-Rian-Mirg* Locus by Preventing De Novo DNA Methylation in Mouse Embryonic Stem Cells

**Graphical Abstract**

**Highlights**
- PRC2 is required to maintain expression of the maternal *Gtl2-Rian-Mirg* locus
- PRC2 transcriptionally regulates the *Gtl2-Rian-Mirg* locus through DNAme at IG-DMR
- IG-DMR serves as an enhancer of the maternal *Gtl2-Rian-Mirg* locus
- PRC2 prevents de novo DNAme at IG-DMR for maternal *Gtl2-Rian-Mirg* locus expression

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**In Brief**
Polycomb Repressive Complex 2 (PRC2) function and DNA methylation (DNAme) are both typically correlated with gene repression. Das et al. find that PRC2 prevents recruitment of Dnmt3s and DNAme at the IG-DMR element, thus allowing proper expression of the nearby maternal *Gtl2-Rian-Mirg* locus.

**Accession Numbers**
GSE58414
PRC2 Is Required to Maintain Expression of the Maternal Gtl2-Rian-Mirg Locus by Preventing De Novo DNA Methylation in Mouse Embryonic Stem Cells

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SUMMARY

Polycomb Repressive Complex 2 (PRC2) function and DNA methylation (DNAme) are typically correlated with gene repression. Here, we show that PRC2 is required to maintain expression of maternal microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) from the Gtl2-Rian-Mirg locus, which is essential for full pluripotency of iPSCs. In the absence of PRC2, the entire locus becomes transcriptionally repressed due to gain of DNAme at the intergenic differentially methylated regions (IG-DMRs). Furthermore, we demonstrate that the IG-DMR serves as an enhancer of the maternal Gtl2-Rian-Mirg locus. Further analysis reveals that PRC2 interacts physically with Dnmt3 methyltransferases and reduces recruitment to and subsequent DNAme at the IG-DMR, thereby allowing for proper expression of the maternal Gtl2-Rian-Mirg locus. Our observations are consistent with a mechanism through which PRC2 counteracts the action of Dnmt3 methyltransferases at an imprinted locus required for full pluripotency.

INTRODUCTION

Somatic cells are readily converted to an embryonic stem cell (ESC)-like state (induced pluripotent stem cells [iPSCs]) through enforced expression of a defined set of transcription factors (TFs), including Oct4, Sox2, Klf4, and c-Myc (OSKM) (Takahashi and Yamanaka, 2006). However, it remains unclear whether iPSCs are molecularly and functionally equivalent to blastocyst-derived ESCs. Overall mRNA and microRNA (miRNA) expression patterns are nearly indistinguishable between genetically
matched mouse ESCs (mESCs) and iPSCs, with the exception of a few maternally expressed long non-coding RNAs (IncRNAs Gtl2, Rian, and Mirg) and miRNAs originating from the imprinted Dlk1-Dio3 gene cluster that is silenced in the majority of iPSC clones (Stadtfeld et al., 2010). The iPSC clones with a silenced Dlk1-Dio3 gene cluster (called Gtl2OFF clones) poorly contribute to chimeras and fail to yield viable iPSC-derived mice (all-iPSC mice). In contrast, iPSC clones with proper expression of the Dlk1-Dio3 gene cluster (called Gtl2ON clones) contribute to a high grade of chimeras and generate viable all-iPSC mice (Stadtfeld et al., 2010). Moreover, ascorbic acid (vitamin C) prevents the loss of imprinting at the Dlk1-Dio3 gene cluster and facilitates generation of all-iPSC mice from differentiated B cells (Stadtfeld et al., 2012). Thus, expression of maternal IncRNAs and miRNAs from the Dlk1-Dio3 imprinted gene cluster is essential for the establishment of full pluripotency. Here we find that Polycomb Repressive Complex 2 (PRC2) is required to maintain expression of the Dlk1-Dio3 imprinted gene cluster, and that PRC2 counteracts de novo DNA methylation (DNAm) at this locus.

PRC2, which is comprised of the core components Ezh2/Ezh1, Eed, Suz12, histone chaperones Rbbp4/6, and associated other factors (e.g., Pcls and Jarid2), catalyzes H3K27me2/3, a chromatin mark correlated with transcriptional repression at silent and bivalent genes (Margueron and Reinberg, 2011). In ESCs, many PRC2 targets are bivalent and marked by both H3K4me3 and H3K27me3 at lineage-specific genes that are poised but activated upon differentiation (Boyer et al., 2006). As such, PRC2 is critical for both ESC maintenance and differentiation. Although bivalent domains initially were believed to be ESC specific, they have been identified in differentiated somatic cells at lower frequency (Bernstein et al., 2006; Mikkelsen et al., 2007). While most functions of PRC2 correlate with repression, a subset of its target genes in mESCs (Brookes et al., 2012; Ferrari et al., 2014).

The mechanism by which PRC2 is recruited to its target genes is incompletely understood. In Drosophila, Polycomb response elements (PREs) are responsible for PRC2 recruitment (Simon and Kingston, 2008). However, in mammals this is not the case. Instead, PRC2 is recruited at highly enriched CpG islands (Ku et al., 2008). Recent findings also posit that IncRNAs are important for PRC2 recruitment and its function. In mammals, X chromosome inactivation (XCI) initiates expression of the ~17-kb IncRNA Xist, which binds to PRC2 and catalyzes H3K27me3 in cis to control chromosome-wide silencing (Zhao et al., 2008). Also, repression of the Hox-D locus appears to be regulated in trans by Hotair that is generated from the Hox-C locus and binds to PRC2 (Rinn et al., 2007). In addition, a class of short RNAs (50–200 nt) plays an important role in association with PRC2 to regulate its target genes (Kanhere et al., 2010). Genome-wide analysis using RNA immunoprecipitation (RIP) sequencing demonstrates >9,000 IncRNAs (>200 nt in size) are associated with PRC2 (Zhao et al., 2010). The PRC2-interacting transcriptome consists of numerous transcripts, such as Xist, H19, IGF2, Air, IGF2r, Kcnq1, and Gtl2, that originate from genomic imprinted loci (Zhao et al., 2010). Genomic imprinting is an epigenetic phenomenon in which genes are expressed either from the paternally or maternally inherited allele (Edwards and Ferguson-Smith, 2007). The majority of imprinted genes are clustered in the genome and usually contain protein-coding genes as well as at least one non-coding RNA (ncRNA) (Edwards and Ferguson-Smith, 2007). Each cluster is under the control of a cis-regulatory element, termed the imprinting control region (ICR). ICRs generally acquire DNAm during oogenesis or spermatogenesis in germ cells and that leads to imprinting of one of the parental alleles (da Rocha et al., 2008). The detailed functions of PRC2 IncRNAs in mediating the regulation of genomic imprinting are largely unknown. For example, PRC2-Gtl2 IncRNA represses Dlk1 expression in cis (Zhao et al., 2010); similarly, Kcnq1ot1 IncRNA interacts with PRC2 and silences genes in the Kcnq1 domain in cis (Pandey et al., 2008).

Contrary to the conventional role of PRC2 in maintenance of repression, we demonstrate here that PRC2 is required to maintain expression of maternal miRNAs and IncRNAs from the Gtl2-Rian-Mirg locus within the Dlk1-Dio3 imprinted gene cluster in mESCs. In the absence of Ezh2/PRC2, the entire Gtl2-Rian-Mirg locus becomes transcriptionally silent due to gain of de novo DNAm at the IG-DMR, a critical cis-regulatory element that controls expression of the maternal Gtl2-Rian-Mirg locus. In the presence of PRC2, the maternal IG-DMR is lowly methylated and acts as an enhancer of the maternal Gtl2-Rian-Mirg locus. Further analysis shows that PRC2 prevents Dnmt3 methytransferase recruitment and subsequent de novo DNAm at the IG-DMR, thereby allowing proper expression of the maternal Gtl2-Rian-Mirg locus. These findings reveal an unanticipated function of PRC2 as well as the complex interplay between PRC2 function and DNAm. Our observations suggest a mechanism through which PRC2 antagonizes de novo DNAm at an imprinted locus.

RESULTS

PRC2 Is Required to Maintain Expression of Maternal miRNAs and IncRNAs at the Gtl2-Rian-Mirg Locus

To further investigate the role of PRC2 in gene regulation in mESCs, we conducted both RNA and size-selected small RNA expression profiling using high-throughput sequencing of Ezh2−/− and wild-type mESCs. We observed a striking reduction in expression of a cluster of miRNAs in Ezh2−/− mESCs at the Gtl2-Rian-Mirg locus within the Dlk1-Dio3 imprinted gene cluster on chromosome 12qf1 (Figures 1A and 1B). The Gtl2-Rian-Mirg locus harbors IncRNA genes (Gtl2, Rian, and Mirg), miRNAs, and small nucleolar RNAs (snoRNAs) that are expressed from the maternally inherited chromosome, whereas protein-coding genes Dlk1 and Dio3 are expressed from the paternally inherited chromosome (Figure 1B; da Rocha et al., 2008). Furthermore, global qRT-PCR analysis of total miRNA expression per chromosome revealed a significant reduction in miRNA expression from chromosome 12 in Ezh2−/− mESCs (Figure S1B), as the majority of the miRNAs reside at the maternal Gtl2-Rian-Mirg locus of chromosome 12. We also observed a reduced expression of maternal miRNAs derived from the Gtl2-Rian-Mirg locus, as well as from chromosome 12, in Eed−/− and Jarid2−/− mESCs (Figures S1A and S1B).
Figure 1. PRC2 Is Required to Maintain Expression of Maternal miRNAs and lncRNAs at the Gtl2-Rian-Mirg Locus

(A) Small RNA-seq demonstrates log-fold changes of miRNA expression in Ezh2−/− mESCs compared to wild-type. Significantly reduced expression of a cluster of miRNAs is observed at the Gtl2-Rian-Mirg locus of chromosome 12 in Ezh2−/− mESCs compared to wild-type.

(B) Schematic representation of the Dlk1-Dio3 imprinted gene cluster. The lncRNA genes (Gtl2, Rian, and Mirg), miRNAs, and snoRNAs are expressed from maternally inherited chromosome, whereas protein-coding genes, Dlk1, Dio3, and Rtl1, are expressed from paternally inherited chromosome. Empty boxes (legend continued on next page)
miR-410, miR-431, and miR-433) in Ezh2-/-, as well as in Eed-/- and Jarid2-/-, mESCs as compared to wild-type (Figures 1C and S1C–S1E). Another independent Ezh2-/- clone showed similar levels of reduction of all these maternal miRNAs (Figure S1H). To exclude possible effects on miRNA biogenesis in the absence of PRC2, we examined expression of Dicer, Drosha, and Ago2. Expression of these critical factors for miRNA biogenesis was unchanged in the absence of PRC2 (Figure S1F). Next we examined expression of IncRNAs Gtl2 (also known as Meg3), Rian, and Mirg, as well as protein-coding genes Dlk1 and Dio3. RNA sequencing (RNA-seq) and qRTPCR revealed a marked reduction in expression of the maternal Gtl2, Rian, and Mirg IncRNAs in two independent Ezh2-/- mESC clones, similar to the observed deficit in miRNA expression in these cells. However, expression of the paternal Dlk1 and Dio3 alleles was unaffected (Figures 1D, S1G, and S1I). Similarly, expression of Gtl2, Rian, and Mirg IncRNAs also was reduced in Eed-/- and Jarid2-/- mESCs (Figures S1G and S1J). The deficit in expression of miRNAs and IncRNAs was greater in the absence of Ezh2 as compared to Eed or Jarid2 loss. Marked reduction in expression of maternal miRNAs and IncRNAs from the Gtl2-Rian-Mirg locus in the absence of several PRC2 components implies that transcription of the entire locus was affected in the absence of intact PRC2.

To establish this, we performed chromatin immunoprecipitation sequencing (ChIP-seq) analysis of RNA Polycrom II (Pol II), which revealed a significant reduction of RNA Pol II occupancy at the entire Gtl2-Rian-Mirg locus (~220 kb) in Ezh2-/- mESCs compared to wild-type (Figure 1E). Thus, the entire maternal Gtl2-Rian-Mirg locus is repressed in the absence of Ezh2/PRC2. Interestingly, RNA Pol II co-occupied with H3K36me3 and H3K79me2 elongation marks at the Gtl2-Rian-Mirg locus (Zhou et al., 2011; Figure 1E). This continuous stretch of co-occupancy of RNA Pol II, H3K36me3, and H3K79me2 and sense-strand specificity of maternal miRNAs and IncRNAs indicate that the maternal Gtl2-Rian-Mirg locus may act as a single transcriptional unit, and most likely maternal miRNAs and IncRNAs are processed from this single transcript. Moreover, a global view of mRNA expression analysis of all imprinted genes showed differential expression of selected imprinted genes in the absence of PRC2 components (Figure S1K). The most pronounced reduction in expression was observed at the Gtl2-Rian-Mirg locus in the absence of Ezh2, Eed, and Jarid2 of the PRC2 components; H19 expression was significantly reduced, but only in the absence of Ezh2 or Jarid2 (Figures S1K and S1L).

**Methylation of the Gtl2-Rian-Mirg Locus in the Absence of PRC2**

To explore mechanisms by which PRC2 loss might lead to repression of the Gtl2-Rian-Mirg locus, we first attempted to rescue Ezh2 expression in Ezh2-/- mESCs. Individual Ezh2 rescue clones expressing different levels of exogenous Ezh2 were examined (Figures S2A and S2C). Ezh2 rescue clones with low-level Ezh2 expression failed to rescue expression of maternal IncRNAs and miRNAs (Figures 2A and 2B). Even Ezh2 rescue clones (clones A5 and B6) that expressed at a near-endogenous level of Ezh2 and restored global H3K27me3 failed to rescue maternal Gtl2, Rian, and Mirg IncRNAs, as well as miRNA expression from the Gtl2-Rian-Mirg locus (Figures 2A, 2B, and S2B–S2E).

To study the basis for highly inefficient rescue of maternal IncRNAs and miRNAs upon re-expression of Ezh2, we assessed DNAme level at the IG-DMR, an important regulatory element located ~12 kb upstream of the Gtl2 promoter involved in regional imprinting at the Gtl2-Rian-Mirg locus. The IG-DMR of the paternally inherited chromosome was heavily methylated. In contrast, the IG-DMR on the maternally inherited chromosome remained unmethylated (Figure 1B; Lin et al., 2003; da Rocha et al., 2008). As expected, the IG-DMR was 45% DNA methylated in wild-type mESCs. However, the methylation level increased to 92% in Ezh2-/- mESCs. Ezh2 rescue clones A5 and B6, which expressed near-endogenous levels of Ezh2, retained 92% and 88% DNAme at the IG-DMR, respectively (Figure 2C). Furthermore, treatment with high concentrations of the Dnmt inhibitor 5-azacitidine (5-aza) failed to restore Gtl2 expression in Ezh2-/- mESCs and Ezh2 rescue clones (Figure S2F). Similarly, high concentrations of ascorbic acid (vitamin C) failed to restore Gtl2 expression in Ezh2-/- (Figure S2G). Thus, DNAme at the IG-DMR is both dense and stable in the absence of Ezh2. Moreover, we observed a small increase in H3K9me3 occupancy at the IG-DMR locus in Ezh2-/- mESCs as compared to wild-type (Figure S2H), suggesting that co-operation between DNAme and H3K9me3 may lead to stable and long-term silencing of the maternal Gtl2-Rian-Mirg locus (Epstein-Litman et al., 2008; Dong et al., 2008; Smith and Meissner, 2013) in the absence of Ezh2. These data imply that DNAme is stable at the IG-DMR in the absence of Ezh2 and causes repression of IncRNAs and miRNAs. Once DNAme is established, re-expression of Ezh2 is unable to erase DNAme from the IG-DMR. Taken together, these results indicate that PRC2 is required to maintain expression of the maternal Gtl2-Rian-Mirg locus, most likely through preventing DNAme at the IG-DMR.

represents genes that are repressed. Imprinting is regulated by IG-DMR, which is methylated in paternally inherited chromosome, but unmethylated in maternally inherited chromosome. Therefore, by default, all IncRNAs, miRNAs, and snoRNAs from paternally inherited chromosome are repressed due to hypermethylation at IG-DMR, and only maternal ones are expressed.

(C) The qRTPCR confirms dramatically reduced expression of maternal miRNAs from the Gtl2-Rian-Mirg locus in Ezh2-/- mESCs; miR-130a is shown as a control. miRNA expression is represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001, *p < 0.01.

(D) The qRTPCR shows a dramatic reduction of maternal Gtl2, Rian, and Mirg IncRNA expression in Ezh2-/- mESCs as compared to wild-type. Dlk1 and Dio3 mRNA expression is unaltered in Ezh2-/- mESCs. Transcript levels were normalized to Gapdh. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001; ns, non-significant.

(E) ChIP-seq analysis of RNA Pol II illustrates log-fold changes of RNA Pol II occupancy in Ezh2-/- mESCs compared to wild-type. RNA Pol II occupancy is significantly reduced at the entire Gtl2-Rian-Mirg locus (~220 kb) in Ezh2-/- mESCs compared to wild-type. RNA Pol II co-occupancy with H3K36me3 and H3K79me2 (elongation marks) suggests that the maternal Gtl2-Rian-Mirg locus acts as a single transcriptional unit. See also Figure S1.
IG-DMR/Enhancer1 Serves as an Enhancer for the Gtl2-Rian-Mirg Locus

DNAme at the IG-DMR has been established as essential for proper imprinting control (Lin et al., 2003; da Rocha et al., 2008). However, the role of histone modifications at the IG-DMR in imprinting is less well understood. We examined the binding landscape of ESC-specific pluripotency factors, cohesion, mediators, histone marks, and PRC2 components at the entire Dlk1-Dio3 gene cluster (Figures 3A and S3A). The IG-DMR was co-occupied by ESC-specific TFs (e.g., Oct4, Nanog, Sox2, Klf4, and Esrrb), mediator (Med1/12), cohesin (Smc1/3), Lsd1, H3K27ac, and H3K4me1 (Figures 3A–3C). Taken together, these characteristics are consistent with this region serving as an enhancer (Kagey et al., 2010; Whyte et al., 2012). We designated this region Enhancer1 (Enh1). A similar region (Enhancer2 [Enh2]), located farther downstream (~450 kb) of Enh1, showed
similar binding patterns (Figure 3D). Both Enh1 and Enh2 exhibited strong enhancer activity in reporter assays (Figure 3E). Interestingly, we observed that the H3K27ac mark was significantly reduced at Enh1 and Enh2 in Ezh2−/− mESCs. This finding correlates with reduced expression of maternal IncRNAs and miRNAs from the Gtl2-Rian-Mirg locus in the absence of Ezh2, suggesting that the H3K27ac active histone mark is an indicator of transcription activity of this imprinted locus (Figures 3C and 3D; Xie et al., 2012). Of note, we observed reduced marking with H3K27ac and H3K4me3, as well as the Gtl2 promoter in the absence of Ezh2/PRC2. Strikingly, we found weak occupancy of Ezh2, Jarid2, and no binding of Suz12 of PRC2 components at IG-DMR/Enh1 and Enh2, and we failed to observe detectable H3K27me3 deposition (Figures 3C and 3D). We cannot exclude the possibility that the weak binding of Ezh2/PRC2 we saw derives from the paternal allele.

The similarities between Enh1 and Enh2 led us to consider how together they might regulate the maternal Gtl2-Rian-Mirg locus. However, unlike IG-DMR/Enh1, Enh2 is not hypermethylated in the absence of Ezh2 (Figure 3B). We investigated whether Enh1 and Enh2 loop into proximity with the Gtl2 promoter to regulate the Gtl2-Rian-Mirg locus. Chromosomal conformation capture (3C) revealed that both Enh1 and Enh2 interact with the Gtl2 promoter in the presence and absence of Ezh2 (Figure S3C), suggesting that Ezh2 does not interfere with looping between Gtl2 promoter and Enh1/Enh2. To determine a requirement for IG-DMR/Enh1 and Enh2 in regulation of the Gtl2-Rian-Mirg locus, we deleted Enh1 (7 kb) and Enh2 (7 kb) using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system (Cong et al., 2013). Biallelic deletion of Enh2 (Enh2−/−) failed to affect expression of the Gtl2-Rian-Mirg locus. In contrast, biallelic deletion of IG-DMR/Enh1 (IG-DMR/Enh1−/−) abrogated expression of maternal Gtl2, Rian, and Mirg (Figure 3F), demonstrating that IG-DMR/Enh1 is an essential regulatory element for the maternal Gtl2-Rian-Mirg locus (Lin et al., 2003). We identified strong co-occupancy of PRC2 and H3K27me3 at the Dlk1 promoter (Figures 3A and 3B). Therefore, we hypothesized that PRC2 might distally regulate the Gtl2-Rian-Mirg locus. To test this possibility, we deleted the Dlk1 promoter region (3 kb) in CRISPR/Cas9. Biallelic deletion of the Dlk1 promoter showed no effect on the locus (Figure S3D), indicating that PRC2 does not distally regulate the Gtl2-Rian-Mirg locus. Collectively, these results demonstrate that the IG-DMR/Enh1 is an important cis-regulatory element that serves as an enhancer for the maternal Gtl2-Rian-Mirg locus.

**PRC2 Physically Interacts with Dnmt3a/3l in a Gtl2 IncRNA-Independent Manner, and the Interaction between Gtl2 IncRNA-Ezh2 Inhibits Binding of Ezh2/PRC2 at the IG-DMR**

Our results demonstrate that, in the absence of PRC2, the entire maternal Gtl2-Rian-Mirg locus is transcriptionally repressed in association with DNA hypermethylation at the IG-DMR (Figures 1 and 2). These data hint at a strong connection between DNAme and PRC2 in regulation of this locus. To explore this relationship further, we examined expression of DNA methyltransferases (Dnmts) in Ezh2−/− and wild-type mESCs. We found that expression of the de novo Dnmts, particularly Dnmt3a and Dnmt3l, were upregulated in Ezh2−/− mESCs (Figures 4A and 4B). Expression of Dnmt1, which is responsible for DNAme maintenance, was not significantly altered in Ezh2−/− mESCs (Figures 4A and 4B). Additionally, we observed upregulation of Dnmt3a and Dnmt3l in Eed−/− and Jarid2−/− mESCs (Figure S4A). Ezh2 expression was unaffected in the absence of any Dnmts (Figure S4B). Co-immunoprecipitation revealed that Ezh2, as well as Jarid2, interacts with Dnmt3a/Dnmt3l proteins (Figures 4C, S4C, and S4E). Moreover, Dnmt3a and Dnmt3l were both eluted in the same fractions as PRC2 components (Ezh2, Jarid2, and Suz12) (Figure S4D), consistent with interaction between PRC2 and Dnmt3a/3l.

We asked whether interaction between Ezh2 and Dnmt3a/3l is dependent on Gtl2 IncRNA. To test this, we used biallelic IG-DMR−/− mESCs, in which expression of maternal Gtl2 IncRNA is abrogated (Figures 3F and 4D). Interaction between Ezh2 and Dnmt3a/Dnmt3l was observed in the absence of Gtl2 IncRNA (Figure 4D). Nonetheless, Gtl2 IncRNA bound to PRC2 components (Ezh2, Eed, and Suz12), but not detectably to Dnmt3a (Figures 4E and S4F). Thus, the interaction between PRC2 and Dnmt3a/3l is Gtl2 IncRNA independent. Of note, interactions between Gtl2 IncRNA and PRC2 components (Ezh2, Eed, and Suz12) (Figure S4F), as well as interactions between PRC2 components (Figure S4E), suggest that assembly or interactions of PRC2 complex components are not prevented in the presence of Gtl2 IncRNA.

PRC2 transcriptome analysis identified a genome-wide pool of >9,000 PRC2-interacting RNAs, including Gtl2 IncRNA, in mESCs (Zhao et al., 2010). The majority of these PRC2-interacting RNAs recruit PRC2 itself at their targets for gene repression (Margueron and Reinberg, 2011). However, recent studies demonstrated that Ezh2/PRC2 is located at a large fraction of active promoters, where it binds to the nascent RNAs that somehow reduce deposition of H3K27me3 (Davidovich et al., 2013; Kaneko et al., 2013). Interestingly, these active promoters reveal low-level occupancy by Ezh2 (Kaneko et al., 2013). Further studies showed that deletion of PRC2-interacting RNA/s rescued PRC2-mediated deposition of H3K27me3 (Kaneko et al., 2014), implying that PRC2 activity is inhibited by interaction with nascent transcripts. We hypothesized that similar binding of nascent Gtl2 IncRNA to Ezh2 (Figure 4E) inhibits the interaction of Ezh2/PRC2 at the IG-DMR and subsequent deposition of H3K27me3. To support this, we showed that Gtl2 promoter deletion disrupts the formation of Gtl2 IncRNA and is associated with increased binding of Ezh2 at the IG-DMR locus (Figures 4F and 4G). We did not observe, however, a significant increase in H3K27me3 at the IG-DMR (Figure 4H).

**PRC2 Antagonizes De Novo DNAme at the IG-DMR through a Distinct Mechanism**

Next we determined the occupancy of Dnmt3a, Dnmt3b, Dnmt3l, and Dnmt1 at the IG-DMR locus in the absence of PRC2. Occupancy of Dnmt3a, Dnmt3b, and Dnmt3l was markedly increased at the IG-DMR locus in the absence of Ezh2 or Jarid2 (Figures 5A and 5B). We noted that recruitment of Dnmt3a/3b/3l was higher at the IG-DMR in the absence of Ezh2 as compared to the absence of Jarid2, which may indicate
that components of PRC2 have different capacities to modulate de novo Dnmt3s occupancy/recruitment at the IG-DMR. We pursued this observation further by examining DNA me levels at the IG-DMR in Ezh2−/−, Eed−/−, and Jarid2−/− mESCs. Indeed, different extents of DNA hypermethylation were observed at the IG-DMR in the absence of the distinct PRC2 components (Figure 5C). Importantly, DNA hypermethylation levels at the IG-DMR correlated with reduced expression levels of maternal lncRNAs and miRNAs at the Gtl2-Rian-Mirg locus in the absence of Ezh2, Eed, and Jarid2 (Figures 1 and S1). In summary, these data suggest that PRC2 prevents recruitment of Dnmt3s for de novo Dnmt3 occupancy/recruitment at the IG-DMR. We propose that components of PRC2 have different capacities to modulate Gtl2 expression, but no significant change in DNAme at the IG-DMR (Figures S6D and S6E). In addition, depletion of Ezh2 in Dmnt3a−/− mESCs reduced Gtl2 expression (Figure S6F), indicating a positive function of Ezh2/PRC2 at the maternal Gtl2-Rian-Mirg locus.

To exclude the trivial possibility that increased binding of Dnmt3 methyltransferases and DNAme at the IG-DMR is due to increased levels of de novo Dnmt3 methyltransferases in the absence of Ezh2, we performed global DNAme analysis from Ezh2−/− and wild-type mESCs using reduced-representation bisulfite sequencing (RRBS). We observed a gain of DNAme globally in the absence of Ezh2 (Figure 5D). Particularly, DNAme was gained at Ezh2-binding sites, in the absence of Ezh2 (Figure S5G). These data indicate the Ezh2 antagonizes Dnmt3 methyltransferase activity and DNAme in mESCs.

To investigate whether this mechanism is restricted to the maternal Gtl2-Rian-Mirg imprinted locus, we examined histone marks, PRC2 occupancy, and DNAme at several differentially regulated imprinted loci, including H19, whose expression also significantly was reduced in the absence of PRC2 (Figure S1K). Occupancy of H3K27ac and H3K4me3 was significantly reduced at both the ICRs, IG-DMR (for Gtl2-Rian-Mirg locus) and ICR (for H19) in the absence of Ezh2, correlating with reduced expression of Gtl2, Rian, and H19. Interestingly, the ICR of H19 was strongly occupied by Ezh2/PRC2 with corresponding H3K27me3 deposition and acquired DNAme in the absence of Ezh2/PRC2, whereas the IG-DMR was weakly occupied by Ezh2/PRC2 without H3K27me3 yet gained DNAme in the absence of Ezh2/PRC2 (Figures S5A–S5F). These findings are consistent with antagonism between PRC2 and DNAme at both loci, but they hint at differences in mechanistic detail.

**PRC2 Protects IG-DMR from De Novo DNAme to Allow Proper Expression of the Maternal Gtl2-Rian-Mirg Locus**

We demonstrated that Gtl2 lncRNA inhibits strong Ezh2/PRC2 occupancy and subsequent H3K27me3 deposition at the IG-DMR locus (Figures 3 and 4F–4H). Therefore, we proposed that Ezh2 occupancy is weak at the IG-DMR, and it may be present in the vicinity of the locus in association with Gtl2 lncRNA. To address the mechanistic details of how Ezh2 prevents Dnmt3s occupancy/recruitment and DNAme at the IG-DMR locus, first we performed a time-course experiment after knockdown of Ezh2. Knockdown of Ezh2 showed reduced expression of Gtl2 lncRNA and increased expression of Dmnt3a (Figures S6A and S6B), similar to, but quantitatively less extreme than, the pattern observed upon complete deletion of Ezh2 (Figures 1D, 4B, S6A, and S6B). However, knockdown of Ezh2 did not increase the DNAme level at the IG-DMR, as we observed in Ezh2−/− mESCs (Figures S6C and 2C). On the other hand, deletion of Dmnt3a (Dmnt3a−/−) showed a modest increase in Gtl2 expression, but no significant change in DNAme at the IG-DMR (Figures S6D and S6E). In addition, depletion of Ezh2 in Dmnt3a−/− mESCs reduced Gtl2 expression (Figure S6F), indicating a positive function of Ezh2/PRC2 at the maternal Gtl2-Rian-Mirg locus.

Furthermore, we overexpressed Ezh2 and Dmnt3a in wild-type mESCs. Overexpression of neither Ezh2 nor Dmnt3a altered Gtl2 expression and DNAme at the IG-DMR (Figures 6A–6F). In addition, overexpression of Ezh2 in Dmnt3a−/− mESCs showed no significant change in Gtl2 expression and DNAme at the IG-DMR (Figures 6G–6I), implying that Ezh2 does not function as an activator at the IG-DMR locus. Taken together, these data support that Ezh2 functions to protect the IG-DMR locus from Dnmt3s/DNAme and, thereby, serves to maintain expression of the maternal Gtl2-Rian-Mirg locus.

**DISCUSSION**

The precise mechanisms regulating imprinting at the Dlk1-Dio3 domain have remained largely unknown (da Rocha et al., 2008). Here we demonstrate that PRC2 is required for proper expression of the maternal Gtl2-Rian-Mirg locus, a cluster essential for successful IPSC reprogramming (Figure S7A; Stadtfeld et al., 2010). Absence of PRC2 results in markedly elevated DNAme at the IG-DMR, leading to transcriptional repression of the entire maternal Gtl2-Rian-Mirg locus (Figures 1 and 2). The maternal IG-DMR is lowly methylated/hypomethylated and acts as an enhancer of the maternal Gtl2-Rian-Mirg locus due to co-occupancy of ESC-specific TFs, mediators, cohesin, Lsd1, H3K27ac, and H3K4me1 (Figure 3). This finding is consistent with antagonism between PRC2 and DNAme at both loci, but they hint at differences in mechanistic detail.

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**Figure 3. IG-DMR/Enh1 Serves as an Enhancer for the Gtl2-Rian-Mirg Locus**

(A–D) Co-occupancy of ESC-specific TFs (e.g., Oct4, Nanog, Sox2, Klf4, and Esrrb), mediator (Med12/12), cohesin (Smc1/3), Lsd1, H3K27ac, and H3K4me1 at IG-DMR/Enh1 and Enh2 fulfills criteria for putative enhancer regions of the Gtl2-Rian-Mirg locus (A). The magnified shaded regions show Dlk1 promoter (B), IG-DMR/Enh1, Gtl2 promoter (C), and Enh2 (D) regions, which are occupied with several factors and histones marks in Ezh2−/− and wild-type mESCs. Multiple individual ChIP-seq genomic tracks of PRC2 components show weak occupancy of Ezh2 and Jarid2 and no binding of Suz12 of PRC2 components at the IG-DMR/Enh1 and Enh2, and we failed to observe detectable H3K27me3 deposition.

(E) Luciferase reporter assays of Enh1 and Enh2 demonstrate strong enhancer activity as Nanog enhancer. Non-Enh1 and Non-Enh2 (lacks binding of any of the factors and histone marks, see Figure S3A) both were used as controls. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001, **p < 0.001, *p < 0.01.

(F) Biallelic deletion of Enh2 (Enh2−/−) (~7 kb) reveals no effect on the Gtl2-Rian-Mirg locus, whereas, biallelic deletion of IG-DMR/Enh1 (IG-DMR/Enh1−/−) (~7 kb) abrogates expression of maternal Gtl2, Rian, and Mirg. Non-Enh2−/− (~7 kb) was used as a control. mRNA expression of Dlk1, Dio3, Gtl2, Rian, and Mirg were examined from undifferentiated wild-type, IG-DMR−/−, Enh2−/−, and Non-Enh2−/− mESCs. mRNA expressions are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001, **p < 0.001, *p < 0.01; ns, non-significant. See also Figure S3.
A. **Ezh2-/- vs Wild-type FPKMs for differentially expressed genes**

B. **Fold change (mRNA level)**

C. **Wild-type mESCs:**

D. **IG-DMR-/- mESCs:**

RT-PCR

E. **EZH2 enrichment**

F. **RT-PCR:**

G. **Ezh2 chip**

H. **H3K27me3 chip**

(legend on next page)
consistent with the observation that lowly methylated regions (LMRs) serve as distal regulatory regions and act as enhancers (Stadler et al., 2011).

Since Gtl2 lncRNA binds to Ezh2, the occupancy of Ezh2 is weak and H3K27me3 deposition does not take place at the IG-DMR of the maternal Gtl2-Rian-Mirg locus (Figures 5A, 3C, and 4E–4H). Nonetheless, we propose that the presence of Ezh2/PRC2 protects the IG-DMR locus from recruitment of Dnmt3s and subsequent DNAme. Several lines of evidence support this model. First, Ezh2 and Dnmt3a/3l physically interact (Figure 4C). Second, Dnmt3s binding to the IG-DMR is increased (Figures 5A and 5B) and DNA is strongly methylated in the absence of Ezh2 (and PRC2) (Figures 2C and 5C). Third, DNAme is globally increased at Ezh2-binding sites in the absence of Ezh2 (Figure 5D). Finally, neither overexpression of Ezh2 or Dnmt3a in wild-type ESCs nor overexpression of Ezh2 in Dnmt3a−/−/− ESCs alters DNAme at the IG-DMR and Gtl2 lncRNA expression (Figure 6). In effect, Ezh2/PRC2 then protects the IG-DMR locus from Dnmt3s and its activity (i.e., DNAme).

Generally the presence of Ezh2/PRC2 correlates with gene repression (Margueron and Reinberg, 2011). However, two recent reports demonstrated that PRC2 localizes not only at the promoter regions of repressed genes but also at the promoters of the active genes. Remarkably, PRC2 weakly occupies active promoter regions with reduced level of H3K27me3 and binds to the 5’ terminus of nascent transcripts, which originate from active genes (Davidovich et al., 2013; Kaneko et al., 2013). These results suggest that PRC2 senses the transcription activity of genes through nascent RNA binding that tempers Ezh2/PRC2 activity (Kaneko et al., 2013). This scenario may allow continuous expression of active genes by cell-type-specific TFs, activators, despite the presence of PRC2. A similar phenomenon may drive continuous expression of the maternal Gtl2-Rian-Mirg locus in association with ESC-specific TFs, mediators, cohesion, H3K27ac, and H3K4me1 at the IG-DMR, despite the presence of Ezh2/PRC2 in association with Gtl2 lncRNA (Figure 7).

Our findings focus attention on the relationship of polycomb function and DNAme. Both pathways are involved in the establishment and maintenance of epigenetic gene silencing. Some evidence points to a cooperative relationship between DNAme and PRC2, where PRC2 facilitates binding (or recruitment) of Dnmts at PRC2 target promoters to promote DNAme (Viré et al., 2008). This scenario has been proposed in colon cancer, where Ezh2/PRC2 has been reported to recruit Dnmts for de novo DNAme to silence genes that are critical for normal colonic epithelium development (Schlesinger et al., 2007). Additionally, reduced levels of H3K27me3 and DNA hypomethylation concurrently activate gene expression in pediatric gliomas (Bender et al., 2013), implying that PRC2-mediated de novo DNAme contributes to carcinogenesis. In contrast, other evidence supports antagonism between DNAme and polycomb function. For example, genome-wide studies in mESCs revealed gain of H3K27me3 and DNAme upon loss of Dnmts and PRC2, respectively (Brinkman et al., 2012; Hagarman et al., 2013). Furthermore, developmentally related genes containing CpG islands that are silenced by PRC2 in normal cells acquire DNAme with loss of PRC2 marks in prostate cancer (Gal-Yam et al., 2008). Also, loss of Dnmt3a leads to an increased level of H3K27me3 in neural stem cells (Wu et al., 2010). Of particular note, a recent study implicated PRC2 in direct regulation of Dnmt3l (Basu et al., 2014), which is consistent with our observation of increased expression of Dnmt3s upon the loss of Ezh2/PRC2 (Figures 4A and 4B). In addition, links between DNA hypomethylation and accumulation and/or spreading of H3K27me3 have been proposed in cancer (Reddington et al., 2014). Thus, the relationship between DNAme and PRC2 may be critical in both normal and cancer cells.

Our data provide additional insights into the relationship between PRC2 and Dnmts. PRC2 interacts physically with Dnmt3a/3l in a Gtl2 lncRNA-independent manner and prevents Dnmt3s recruitment and subsequent DNAme at the IG-DMR of the maternal Gtl2-Rian-Mirg locus (Figures 4, 5, and 6). Dnmt3a/3l forms a tetramer for de novo DNAme (Jia et al., 2007). Dnmt3l shares homology with Dnmt3a and Dnmt3b, but lacks enzymatic activity, although Dnmt3l cooperates with Dnmt3a and Dnmt3b to establish maternal imprinting (Hata et al., 2002). Furthermore, Dnmt3l has been shown to enhance

Figure 4. PRC2 Physically Interacts with Dnmt3a/3l in a Gtl2 lncRNA-Independent Manner and the Interaction between Gtl2 lncRNA-Ezh2 Inhibits Binding of Ezh2/PRC2 at the IG-DMR

(A) Scatterplot representing differentially expressed genes from Ezh2−/− mESCs compared to wild-type. Red dots represent significantly up- and downregulated genes in Ezh2−/− mESCs with a q value < 0.01. Genes of interest are labeled in the scatterplot.

(B) The mRNA expression shows significant upregulation of Dnmt3a, Dnmt3b, and Dnmt3l, but not Dnmt1, in Ezh2−/− mESCs as compared to wild-type. Transcript levels were normalized to Gapdh. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001, *p < 0.01; ns, non-significant.

(C) Anti-Ezh2 antibody was used to immunoprecipitate endogenous Ezh2 from mESC nuclear extracts, showing a specific interaction between Ezh2 and Dnmt3a/3l.

(D) The qRT-PCR shows that biallelic deletion of IG-DMR−/− causes abrogation of maternal Gtl2 and Rian IncRNAs in mESCs. Endogenous Ezh2 maintains interaction with Dnmt3a/Dnmt3l in the absence of Gtl2 lncRNA.

(E) RNA immunoprecipitation (RIP) demonstrates a strong interaction of Gtl2 lncRNA with Ezh2, but not with Dnmt3a. U1 RNA and Oct4 mRNA were used as controls. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001; ns, non-significant.

(F) The qRT-PCR shows that biallelic deletion of Gtl2 promoter (−7 kb) disrupts the formation of Gtl2 lncRNA.

(G) ChIP-qPCR shows increased Ezh2 occupancy at the IG-DMR in the absence of Gtl2 lncRNA. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; **p < 0.01, *p < 0.05; ns, non-significant.

(H) ChIP-qPCR shows no significant increase in binding of H3K27me3 at the IG-DMR in the absence of Gtl2 lncRNA. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ns, non-significant.

See also Figure S4.
the de novo DNAme activity of Dnmt3a (Chedin et al., 2002), which implicates Dnmt3l as an important cofactor for Dnmt3a. In addition, conditional mutants of Dnmt3a and Dnmt3l in germ cells display indistinguishable phenotypes; however, conditional mutants of Dnmt3b demonstrate no apparent phenotype, indicating that Dnmt3a and Dnmt3l function together for DNAme at many of the imprinted loci in germ cells (Kaneda et al., 2004).

Figure 5. PRC2 Antagonizes De Novo DNAme at the IG-DMR through Distinct Mechanism

(A and B) ChIP-qPCR shows that Dnmt3a, Dnmt3b, and Dnmt3l occupancy at IG-DMR is significantly increased in the absence of Ezh2 (A) and Jarid2 (B), but occupancy of Dnmt1 remains unchanged. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001; ns, non-significant.

(C) Analysis of 29 CpGs at the IG-DMR shows different DNAme (%) levels in the absence of PRC2 components. Data are represented as mean ± SEM (n = 3); p values were calculated using a two-way ANOVA; ***p < 0.0001.

(D) Global DNAme analysis from Ezh2−/− and wild-type mESCs, using reduced-representation bisulfite sequencing (RRBS), represented as a heatmap of genome-wide methylation patterns. The genome was divided into non-overlapping 10-kb windows and the fraction of methylated CpGs in each window was computed for wild-type and Ezh2−/− mutants. The hue represents the number of genomic windows with a given fractional methylation in Ezh2−/− versus wild-type. Trends suggest significantly increased global DNAme in Ezh2−/−. See also Figure S5.
Although our findings are consistent with a model in which Ezh2 protects the IG-DMR locus from Dnmt3s recruitment and subsequent DNAme to maintain proper expression of the maternal Gtl2-Rian-Mirg locus, further study is needed to address more specific mechanistic issues. For one, it remains to be determined how and to what extent other PRC2 components, such as Eed, Suz12, and Jarid2, are involved in protecting the IG-DMR from DNAme. Second, the mechanism by which Gtl2 lncRNA inhibits binding of Ezh2/PRC2 at the IG-DMR and contributes to decreased H3K27me3 activity merits further clarification. Moreover, precisely how Gtl2 lncRNA recruits Ezh2/PRC2 at the IG-DMR and maintains its own expression through a feedback loop is not fully understood.

In conclusion, we find that Gtl2 lncRNA inhibits binding of Ezh2/PRC2 at the maternal IG-DMR locus, while Ezh2/PRC2 maintains its presence in the vicinity of the IG-DMR locus. In this manner, Ezh2/PRC2 protects the maternal IG-DMR locus by preventing recruitment of Dnmt3s and subsequent DNAme, thereby serving to maintain proper expression of the maternal Gtl2-Rian-Mirg locus in the presence of ESC-specific TFs and activators (Figure 7). In the absence of Ezh2, Dnmt3s is then recruited to and methylates the IG-DMR, leading to transcription repression of the maternal Gtl2-Rian-Mirg locus (Figure 7). Our findings also suggest that individual PRC2 components have different capacities to modulate Dnmt3s occupancy/recruitment and subsequent de novo DNAme at the IG-DMR (Figure 5), which ultimately sets different levels of expression of maternal IncRNAs and miRNAs from the Gtl2-Rian-Mirg locus (Figure 1).

See also Figure S6.
### EXPERIMENTAL PROCEDURES

#### mESC Culture

Mouse C57 (wild-type), Ezh2−/−, Eed−/−, Jarid2−/−, and other mESC lines were maintained in the following ES medium: DMEM (Life Technologies) supplemented with 15% fetal calf serum (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma), 2 mM L-glutamine (Life Technologies), 0.1 mM nonessential amino acid (Life Technologies), 1% nucleoside mix (Sigma), 1,000 U/ml recombinant leukemia inhibitory factor (LIF, Chemicon), and 50 U/ml penicillin/streptomycin (Life Technologies). Ezh2−/−, Eed−/−, and Jarid2−/− mESCs were established previously (Shen et al., 2008, 2009; Xie et al., 2014).

#### Small RNA-Seq

Total RNA was isolated from undifferentiated mESCs using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (10 µg) from C57 (wild-type), Ezh2−/−, Eed−/−, and Jarid2−/− mESCs were size selected to 18–40 nt on a denaturing polyacrylamide gel, and small RNA libraries were prepared according to the manufacturer’s instructions (SOLID small RNA library preparation kit, Life Technologies). All libraries were sequenced using SOLiD instrument (Life Technologies).

#### RNA-Seq

Total RNA was isolated from undifferentiated mESCs using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (1 µg) was used from C57 (wild-type), Ezh2−/−, Eed−/−, and Jarid2−/− mESCs to prepare the mRNA libraries according to the manufacturer’s instructions (Directional [strand-specific] mRNA-Seq sample preparation kit, Illumina). All libraries were sequenced using HiSeq 2000 sequencing system (Illumina).

#### qRT-PCR

Total RNA (10 µg) was resolved in denaturing PAGE, transferred to a nitrocellulose membrane, and hybridized with DNA or locked nucleic acid (LNA) probes specific to each miRNA, as described previously (Das et al., 2008).

#### ChiP

ChiP was performed as described elsewhere (Das et al., 2014). Detailed procedures and a list of antibodies are available in the Supplemental Experimental Procedures.

#### ChiP-Seq and Library Generation

Purified ChiP DNA was used to prepare Illumina multiplexed sequencing libraries. New England Biolabs next-generation sequencing kit was used to prepare the libraries.

#### ChiP-Seq Data Analyses

All ChiP-seq samples were aligned with Bowtie v0.12.9 to the mm9 genome assembly, where only uniquely mappable reads were reported. Significant peaks were found by pairing each ChiP-seq sample with the appropriate input and running SICER v1.1, with a false discovery rate (FDR) of 0.05. See the Supplemental Experimental Procedures for more detail.

### DNAme Analysis

Genomic DNA was bisulfate converted and analyzed by EpigenDx using the following assays: IG-DMR (ADS-1452), Oct4 promoter (ASY-585), and Nanog promoter (ASY-590).

### ACCESSION NUMBERS

The accession number for the RNA-seq, small RNA-seq, ChiP-seq, and microarray data reported in this paper is GEO: GSE58414.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.053.

### AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We thank Pratibha Tripathi; Glenn MacLean for reagents; Fieda Abderazzaq and Renee Rubia at the Center for Computational Biology (CCCB) sequencing facility, DFCI, for Illumina HiSeq2000 Sequencing; and the late Edward Fox at the Microarray Core of DFCI for microarray and small RNA-seq. This work was supported by funding from NIH grant HLBI U01HL100001. S.H.O. is an Investigator of the HHMI. M.C.C. was supported by NIH grant F30 DK103359-01A1.

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