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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SUPPLEMENTAL DATA

Supplemental figures and legends

Figure S1

A

miRNA expression:

| Chromosome | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | X |
|------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|
| Eed-/-/Wild-type |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |
| Jarid2-/-/Wild-type |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |

B

RT-qPCR

C

D

E

miRNAs at G6D-Rian-Mirg locus
Figure S1. PRC2 is required to maintain expression of maternal miRNAs and IncRNAs at the *Gtl2-Rian-Mirg* locus (related to Figure 1)

(A) Small RNA-seq demonstrates log-fold change of miRNA expression in *Eed-*/- and *Jarid2-*/- mESCs as compared to wild-type. Significantly reduced expression of a cluster of miRNAs is observed at the *Gtl2-Rian-Mirg* locus of chromosome 12 in *Eed-*/- and *Jarid2-*/- mESCs.

(B) RT-qPCR analysis of miRNA expression per chromosome shows significant reduction of the miRNAs expression from chromosome 12 in *Ezh2-*/-, *Eed-*/- and *Jarid2-*/- mESCs. Data are represented as mean +/- SEM (n=2); p-values were calculated using a 2way ANOVA; ***p <0.0001.

(C) Northern-blot confirms dramatically reduced expression of maternal miRNAs from the *Gtl2-Rian-Mirg* locus in *Ezh2-*/- mESCs. miR-130a is shown as a control.

(D-E) RT-qPCR (D) and Northern-blot (E) confirm significantly reduced expression of maternal miRNAs from the *Gtl2-Rian-Mirg* locus in *Eed-*/- and *Jarid2-*/- mESCs. miR-130a is shown as a control. For RT-qPCR, miRNA expression data are represented as mean +/- SEM (n=3); p-values were calculated using a 2 way ANOVA; ***p <0.0001, *p <0.01.

(F) No significant changes in mRNA expression of Dicer, Dgcr8, and Ago2 are observed in absence of Ezh2, Eed and Jarid2 of PRC2. Transcript levels were normalized using Gapdh. mRNA expression data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; ns (non-significant). Protein levels of Drosha and Ago2 were unaffected in wild-type and *Ezh2-*/-. Actin is shown as a loading control for Western blot.
(G) RNA-seq reveals significant reduction of Gtl2, Rian and Mirg lncRNAs expression in absence of Ezh2, Eed and Jarid2.

(H) RT-qPCR shows dramatically reduced expression of maternal miRNAs from the Gtl2-Rian-Mirg locus in an independent Ezh2/- clone. miR-130a shown as a control. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2 way ANOVA; ***p <0.0001, ns (non-significant).

(I) RT-qPCR shows dramatic reduction of maternal Gtl2, Rian and Mirg lncRNA expression in an independent Ezh2/- clone 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 2 way ANOVA; ***p <0.0001, **p <0.001, *p <0.01 and ns (non-significant).

(J) RT-qPCR shows significant reduction of maternal Gtl2, Rian and Mirg lncRNAs expression in Eed/- and Jarid2/- 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 2 way ANOVA; ***p <0.0001, **p <0.001, *p <0.01 and ns (non-significant).

(K) mRNA expression (microarray) analysis of all imprinted genes show differential expression of imprinted genes in the absence of components of PRC2. Most significant reduction was observed for Gtl2 and Rian, as well as for H19 expression, compared to other imprinted genes in the absence of PRC2 components. Reduced expression of Gtl2 and Rian was consistent in all PRC2 mutants, Ezh2, Eed and Jarid2; whereas H19 was down-regulated only in the absence of Ezh2 and Jarid2.

(L) RT-qPCR confirms reduced expression of H19 in the absence of Ezh2 and Jarid2,
same as microarray data. Three individual primer pairs of H19 yielded similar results. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2 way ANOVA; **p <0.0001.
Figure S2. Methylation of the *Gtl2-Rian-Mirg* locus in the absence of PRC2 (related to Figure 2)

(A) Several individual Ezh2 rescue clones express different levels of exogenous Ezh2; A5 and B6 express near endogenous levels of Ezh2. Actin was used as a loading control.

(B) Ezh2 rescue clone (B6) restores global H3K27me3 similar to wild-type.

(C) RT-qPCR shows that reintroduction of Ezh2 in *Ezh2/-* mESCs has no significant effect on Dlk1, Dio3 expression. Expression of Nanog and Ezh1 was used as additional controls. Ezh2 expression shows different mRNA levels in different Ezh2 rescue clones. Data are represented as mean +/- SEM (n=3); p-values were calculated using one-way ANOVA; ***p <0.0001, **P <0.01, *p <0.01, ns (non-significant).

(D) Ezh2 rescue clones, A5 and B6 that express near endogenous level of Ezh2 (Figures S2A and S2C), fail to restore the expression of maternal miRNAs from the *Gtl2-Rian-Mirg* locus (for example miR-433). miRNA expression is shown as mean +/- SEM (n=3); p-values were calculated using a one-way ANOVA; ***p <0.0001, *p <0.01, ns (non-significant). miR-135b was used as an internal control.

(E) Denaturing PAGE gel electrophoresis shows miRNA expression in Ezh2 rescue clones.

(F) Treatment with the Dnmt inhibitor, 5-azacitidine (5-aza) at high concentration (1µM) only partially restores Gtl2 expression in *Ezh2/-* mESCs and Ezh2 rescue clones (A5 & B6).

(G) High concentration of ascorbic acid (vitamin C) treatment fails to restore Gtl2 expression in *Ezh2/-* mESCs.
(H) ChIP-qPCR shows increased H3K9me3 occupancy at the IG-DMR locus in Ezh2−/− mESCs compared to wild-type.
Figure S3. IG-DMR/Enh1 serves as an enhancer for the Gtl2-Rian-Mirg locus (related to Figure 3)

(A) Genomic tracks display occupancy of factors and histone marks at the Dlk1-Dio3 gene cluster. Dlk1 promoter, Dio3 promoter, Gtl2 promoter, IG-DMR/Enh1, Enh2, Non-Enh1 and Non-Enh2 regions are highlighted.

(B) DNAme (%) was analyzed at IG-DMR/Enh1 and Enh2 in Ezh2/- mESCs as compared to wild-type. The Enh2 region is unmethylated. DNAme levels at the Nanog and Oct4 proximal promoters were used as controls. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2 way ANOVA; ***p <0.0001, ns (non-significant).

(C) 3C demonstrates that both Enh1 and Enh2 loop into proximity with Gtl2 promoter in Ezh2 independent manner. No interaction was observed between Enh2 and the Dio3 promoter.

(D) Biallelic deletion of the Dlk1 promoter reveals no effect on the Gtl2-Rian-Mirg locus.
Figure S4

A

B

C

D

E

F

Superox 6 gel-filtration fractions

Wild-type mESCs:

Wild-type mESCs:

Wild-type mESCs:
Figure S4. PRC2 physically interacts with Dnmt3a/3l (related to Figure 4)

(A) Expression of Dnmt3a and Dnmt3l is up-regulated in Eed-/ and Jarid2-/ mESCs compared to wild-type. Transcript levels were normalized to Gapdh. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; ***p <0.0001, **p <0.001, *p <0.01, ns (non-significant).

(B) mRNA expression of Ezh2 is unchanged in absence of Dnmts. Individual Dnmt knockout (KO) mESCs show significant down-regulation of the corresponding Dnmts. Transcript levels were normalized to Gapdh. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; **p <0.001, ns (non-significant).

(C) Flag-Biotin tagged-Ezh2 was immunoprecipitated from mESCs nuclear extract using streptavidin beads; specific interaction between Ezh2 and Dnmt3a/Dnmt3l was observed.

(D) Western blot analysis of Superose 6 gel-filtration fractions. Whole-cell lysates from mESCs were fractionated. Dnmt3a and Dnmt3l both were eluted in the same fractions as PRC2 components- Ezh2, Jarid2 and Suz12.

(E) Endogenous Jarid2 immunoprecipitated from mESCs nuclear extract shows specific interaction with components of PRC2 (Ezh2 and Suz12) and Dnmt3a.

(F) RNA immunoprecipitation (RIP) demonstrates interaction of Gtl2 IncRNA with Ezh2, Eed and Suz12 of PRC2 complex. U1 RNA was used as control. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; ***p <0.0001, **p <0.001, *p <0.01, ns (non-significant).
Figure S5. PRC2 antagonizes de novo DNAme at the IG-DMR through distinct mechanism (related to Figure 5)

(A-E) Genomic tracks display occupancy of histone marks, RNA Pol II, PRC2 (Ezh2, Suz12, Jarid2 and H3K27me3) and DNAme levels at selected down-regulated (Gtl2, H19, Sgce and Peg10), up-regulated (Qpct) and unchanged (Th) imprinted gene loci upon depletion of Ezh2/PRC2. Occupancy of H3K4me3 and H3K27ac is reduced at both the imprinting control regions- IG-DMR (for Gtl2-Rian-Mir locus) and ICR (for H19) in the absence of Ezh2 (A, B), and relates to the reduced expression of Gtl2, Rian and H19. PRC2/H3K27me3 occupies strongly at the ICR of H19, and ICR gains DNAme in absence of Ezh2/PRC2, whereas the IG-DMR weakly occupied by Ezh2/PRC2 and with no detectable H3K27me3 deposition, and gains DNAme (A, B). Two other down-regulated imprinted genes, Sgce and Peg10, are occupied with PRC2/H3K27me3, but fail to gain DNAme in absence of Ezh2/PRC2 (C). Th, an imprinted gene whose expression is unchanged in the absence of Ezh2/PRC2, does not reveal changes in factor binding, histone marks and DNAme (D). The up-regulated imprinted gene, Qpct, shows loss of H3K27me3 without gain of DNAme upon loss of Ezh2/PRC2 (E), indicating that PRC2 most probably regulates these imprinted gene loci in a different fashion, directly or indirectly.

(F) Analysis of 24 CpGs at the ICR of H19 shows gain of DNAme (%) in Ezh2/- mESCs compared to wild-type. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; ***p <0.0001.

(G) Regions of statistical enrichment and depletion for Ezh2 binding were determined by comparing Ezh2 ChIP-Seq to input reads with SICER using a window size of 1kb. For
each condition, the histogram compares the distributions of the log-fold change of the fraction of methylated dimers (in Ezh2/- compared to wild type) across all regions. Ezh2-enriched regions show enhanced methylation and these two distributions are different, as determined by a KS test, with a p-value less than 2.2e-16.
Figure S6

A

B

C

D

E

F
Figure S6. PRC2 protects IG-DMR from de novo DNAme to allow proper expression of the maternal Gtl2-Rian-Mirg locus (related to Figure 6)

(A) Time course experiment of knockdown of Ezh2, where Ezh2 protein level was significantly reduced. Gapdh used as an internal control.

(B) Knockdown of Ezh2 shows reduced expression of Gtl2 and increased expression of Dnmt3a. Transcript levels were normalized to Gapdh. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; ***p <0.0001, **p <0.01 and ns (non-significant).

(C) Analysis of 29 CpGs at the IG-DMR shows no significant changes of DNAme (%) levels upon knockdown of Ezh2 at different time points. Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; ns (non-significant).

(D) Deletion of Dnmt3a (Dnmt3a-/-) shows mild increase of Gtl2 expression (RT-qPCR), but no significant change of DNAme level at the IG-DMR (E). Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; *p <0.01, ns (non-significant).

(F) Depletion of Ezh2 in Dnmt3a-/- mESCs show reduced Gtl2 expression (RT-qPCR). Data are represented as mean +/- SEM (n=3); p-values were calculated using a 2-way ANOVA; *p <0.01.
Figure S7

A

[Bar chart showing relative expression of different conditions]
Figure S7. Overexpression of Ezh2 increases efficiency of “Gtl2\textsuperscript{ON} clones” during somatic cell reprogramming (related to Figure 7)

Overexpression of Ezh2 increases Gtl2 expression and efficiency of “Gtl2\textsuperscript{ON} clones” (1 iPSCs clone out of 10 iPSCs clones with OSKM+empty; and 5 iPSCs clones out of 8 iPSCs clones with OSKM+Ezh2, i.e increase efficiency of Gtl2\textsuperscript{ON} iPSCs clones up to ~50-60%) during somatic cell reprogramming.
Supplemental tables

Table S1.

High-throughput small RNA-Seq and RNA-seq data summary (related to Figure 1)

| Sample names                          | Total reads sequenced | Total aligned to mm9 |
|---------------------------------------|-----------------------|----------------------|
| Wild-type_smRNASeq_R1                | 128345588             | 117065061            |
| Wild-type_smRNASeq_R2                | 148608928             | 129251255            |
| Ezh2/−/−_smRNASeq_R1                 | 130155426             | 115823634            |
| Ezh2/−/−_smRNASeq_R2                 | 142044658             | 128072042            |
| Eed/−/−_smRNASeq_R1                  | 124417057             | 114288285            |
| Eed/−/−_smRNASeq_R2                  | 164454580             | 152960461            |
| Jarid2/−/−_smRNASeq_R1               | 68950485              | 62259756             |

| Sample names                          | Total Sequenced Reads |
|---------------------------------------|-----------------------|
| Wild-type_RNASeq_R1                   | 84740020              |
| Wild-type_RNASeq_R2                   | 151053542             |
| Ezh2/−/−_RNASeq_R1                    | 93154862              |
| Ezh2/−/−_RNASeq_R2                    | 137561873             |
| Eed/−/−_RNASeq_R1                     | 37489298              |
| Eed/−/−_RNASeq_R2                     | 110000921             |
| Jarid2/−/−_RNASeq_R1                  | 56476890              |
| Jarid2/−/−_RNASeq_R2                  | 140278885             |

Table S2. High-throughput ChIP-Seq data summary (related to Figure 3)

| Sample names                          | Total Sequenced Reads |
|---------------------------------------|-----------------------|
| H3K4me3 wild-type                     | 144281126             |
| H3K4me3 Ezh2/−                        | 108742136             |
| H3K27me3 wild-type                    | 15793532              |
| H3K27me3 Ezh2/−                       | 108098497             |
| H3K27ac wild-type                     | 128603319             |
| H3K27ac Ezh2/−                        | 116156052             |
| RNA Pol II wild-type                  | 113379190             |
| RNA Pol II Ezh2/−                     | 47463655              |
| Wild-type input DNA_R1                | 50043014              |
| Wild-type input DNA_R2                | 51639593              |
| Wild-type input DNA_R3                | 44768252              |
| Ezh2/−/− input DNA                    | 169073268             |
Table S3. Published high-throughput ChIP-Seq data summary (related to Figure 3)

| ChIP Samples | GEO Accession | Publications |
|--------------|---------------|--------------|
| Lsd1 R1      | GSM687281     | Whyte 2012(Whyte et al., 2012) |
| Lsd1 R2      | GSM687282     | Whyte 2012   |
| Med12 R1     | GSM560344     | Kagey 2010 (Kagey et al., 2010) |
| Med12 R2     | GSM560345     | Kagey 2010   |
| Med1 R1      | GSM560347     | Kagey 2010   |
| Med1 R2      | GSM560348     | Kagey 2010   |
| Smc1 R1      | GSM560341     | Kagey 2010   |
| Smc1 R2      | GSM560342     | Kagey 2010   |
| Smc3 R1      | GSM560343     | Kagey 2010   |
| Smc3 R2      | GSM560344     | Kagey 2010   |
| H3K79me2 R1  | GSM307150     | Marson 2008 (Marson et al., 2008) |
| H3K79me2 R2  | GSM307151     | Marson 2008   |
| H3K36me3 R1  | GSM307152     | Marson 2008   |
| H3K36me3 R2  | GSM307153     | Marson 2008   |
| Nanog R1     | GSM307140     | Marson 2008   |
| Nanog R2     | GSM307141     | Marson 2008   |
| Pou5f1/Oct4  | GSM307137     | Marson 2008   |
| Sox2 R1      | GSM307138     | Marson 2008   |
| Sox2 R2      | GSM307139     | Marson 2008   |
| Input DNA    | GSM307155     | Marson 2008   |
| Input DNA    | GSM307154     | Marson 2008   |
| Klf          | GSM288354     | Chen 2008(Chen et al., 2008) |
| Esrrb        | GSM288355     | Chen 2008     |
| GFP (control)| GSM288358     | Chen 2008     |
| Suz12        | GSM1199188    | Kaneko 2013(Kaneko et al., 2013) |
| Suz12        | GSM1199189    | Kaneko 2013   |
| Ezh2 2 R1    | GSM1199182    | Kaneko 2013   |
| Ezh2 2 R2    | GSM1199183    | Kaneko 2013   |
| Input DNA    | GSM1199187    | Kaneko 2013   |
| H3K4me1      | GSM723016     | Shen 2012(Shen et al., 2012) |
| Input DNA    | GSM723020     | Shen 2012     |
| Jarid2       | GSM465889     | Peng 2009(Peng et al., 2009) |
| Input DNA    | GSM480164     | Peng 2009     |
| Ezh2 1       | GSM480161     | Peng 2009     |

Summary of downloaded datasets, and associated GEO Accessions and publications.
Table S4. ChIP-Seq peak identification and alignment methods (related to Figure 3)

| Sample               | Other Bowtie Parameters | SICER Window/Gap Size (bp) | ChIP-Seq Control |
|----------------------|-------------------------|----------------------------|------------------|
| H3K4me3              | -1 50 -n 2              | 200/200**                  | Our Input        |
| H3K27me3             | -1 50 -n 2              | 200/600**                  | Our Input        |
| H3K27ac              | -1 50 -n 2              | 200/600                    | Our Input        |
| RNA Pol II           | -1 50 -n 2              | 200/600                    | Our Input        |
| Lsd1                 | -1 26 -n 1              | 200/400                    | Our Input        |
| Med12                | -1 26 -n 1              | 200/400                    | Our Input        |
| Med1                 | -1 26 -n 1              | 200/400                    | Our Input        |
| Smc1                 | -1 26 -n 1              | 200/400                    | Our Input        |
| Smc3                 | -1 26 -n 1              | 200/400                    | Our Input        |
| H3K79me2             | -1 26 -n 1              | 200/600                    | Marson Input     |
| H3K36me3             | -1 26 -n 1              | 200/600                    | Marson Input     |
| Nanog                | -1 26 -n 1              | 200/400                    | Marson Input     |
| Pou5f1/Oct4          | -1 26 -n 1              | 200/400                    | Marson Input     |
| Sox2                 | -1 26 -n 1              | 200/400                    | Marson Input     |
| Klf                  | -1 26 -n 1              | 200/400                    | GFP ChIP         |
| Esrrb                | -1 26 -n 1              | 200/400                    | GFP ChIP         |
| Ezh2 (Kaneko)        | -1 46 -n 2              | 200/400                    | Kaneko Input     |
| Suz12                | -1 46 -n 2              | 200/400                    | Kaneko Input     |
| H3K4me1              | -1 26 -n 1              | 200/200                    | Shen Input       |
| Jarid2               | -1 45 -n 2              | 200/400                    | Peng Input       |
| Ezh2 (Peng)          | -1 26 -n 1              | 200/400                    | Peng Input       |

ChIP-Seq methods: The following provides information on the parameters used to align reads and SICER peak identification parameters. For bowtie, the –l length parameter was chosen to be consistent with the shortest read length for the pool of reads associated with that dataset. The number of mismatches was chosen to be 1 for reads of length of about 25, and 2 for reads of length of about 50.
| Name of the RT primers | Sequences               |
|-----------------------|-------------------------|
| PPD_Gt12_RT_F         | TTGCACATTTCCTGTGGGAC    |
| PPD_Gt12_RT_R         | AAGCACCATGAGCCACTAGG    |
| PPD_Rian_RT_F         | CGGTGTGTTGTGTTGTGTTG    |
| PPD_Rian_RT_R         | GCCAAGGTCTCTACCAAGCAG   |
| PPD_Mirg_RT_F         | GGCAGAGTCTAGGATGAGA     |
| PPD_Mirg_RT_R         | CGCCAGCTTCTGAAATCTCC    |
| PPD_Dlk1_RT_F         | GACCTGGAGAAAGGCAAGTA    |
| PPD_Dlk1_RT_R         | AGGGAGAAACCATTGTACAGC   |
| PPD_Dio3_RT_F         | CCCATGACACAGATGAGCAC    |
| PPD_Dio3_RT_R         | CTTGAGAGCAAGCCAAACAC    |
| PPD_Ezh1_RT_F         | CAATAACTATGATGGAAGAAGCCACAC |
| PPD_Ezh1_RT_R         | CTTCTCTCACAGATCTGGTT    |
| PPD_Ezh2_RT_F         | GGGAGAGCAAAATGATAAAGAAGAG |
| PPD_Ezh2_RT_R         | ATTCTCAGGAGTTCAATATTTTG |
| PPD_Nanog_RT_F        | AGGGTCTGTGCTAGGATGCT    |
| PPD_Nanog_RT_R        | CAAACACTGATTTTCTGCAAC   |
| PPD_Pou5f1_RT_F       | CTGAGGAGCCAGCAGGACAGCAG |
| PPD_Pou5f1_RT_R       | CGTAGGGAGGGCTCCGGGCABC |
| PPD_Dnmt1_RT_F        | GGAAGCTACCTGTGCAAATG    |
| PPD_Dnmt1_RT_R        | ATTTGAGCTCTGGAGTCTTCT   |
| PPD_Dnmt3a_RT_F       | GAGATGCGCAAATCAGATGTTG  |
| PPD_Dnmt3a_RT_R       | GAGGACTTCGTAAGGCTGTTT   |
| PPD_Dnmt3b_RT_F       | CAGGGATACCTGTTGAGTTT    |
| PPD_Dnmt3b_RT_R       | ATCCGGCTGCTGCAACTGAGAT  |
| PPD_Dnmt3L_RT_F       | GATAAAGCTTCTGGAGTCTTCTT |
| PPD_Dnmt3L_RT_R       | TGCCACACACTCAGAAACAGTAG |
| PPD_Dicer_RT_F        | AAAGACCTGCGCCACTGAGCAG  |
| PPD_Dicer_RT_R        | CTGACGGGCTGACACTGTTT    |
| PPD_Drosha_RT_F       | CGGAGTCCGAGAAGCACAGCAG  |
| PPD_Drosha_RT_R       | GGCTCAGAGCAACTTGGTAAG   |
| PPD_Ago2_RT_F         | CACCCGGAGAACAATCAACAAC |
| PPD_Ago2_RT_R         | ACTCTCGAGGGCTTCTCTC     |
| PPD_U1_RT_F           | ATACCTACCTGAGGAGGAGGAG  |
| PPD_U1_RT_R           | CAGGGAGAAAGGCGGAAAGCA   |
| PPD_Gapdh_RT_F        | AAATTCAACGGCACATGAGCA   |
| PPD_Gapdh_RT_R        | CCACCACATTGATGTGAGA     |
### Table S6. miRNA RT primer sequences (related to Figure 1 & 2)

| Name of the RT primers | RT primer sequences |
|------------------------|---------------------|
| PPD_rt_miR127_rv       | CTCAACTGGTGTCGTCGTCGGAATTACGTTGAG-AGCCAAGGC |
| PPD_rt_miR127_fw       | ACACTTCAGCTGGG-TGGGATCCGTCGTCGAGC |
| PPD_rt_miR134_rv       | ACACTTCAGCTGGG-ATGACTGTTGACCA |
| PPD_rt_miR134_fw       | ACACTTCAGCTGGG-ATGACTGTTGACCA |
| PPD_rt_miR323-3p_rv    | CTCAACTGCTGCTGAGTCGGAAATTCAGTTGAG-AGAGGTTCG |
| PPD_rt_miR323-3p_fw    | ACACTTCAGCTGGG-CACATTACACGGGCTGA |
| PPD_rt_miR410_rv       | CTCAACTGCTGCTGAGTCGGAAATTCAGTTGAG-ACAGGCCA |
| PPD_rt_miR410_fw       | ACACTTCAGCTGGG-ATATATAACACAGAGG |
| PPD_rt_miR431_rv       | CTCAACTGCTGCTGAGTCGGAAATTCAGTTGAG-TGCACTGAC |
| PPD_rt_miR431_fw       | ACACTTCAGCTGGG-TGCTTGCAGGCGGTGC |
| PPD_rt_miR433_rv       | ACACTTCAGCTGCTGAGTCGGAAATTCAGTTGAG-ACACCGAG |
| PPD_rt_miR433_fw       | ACACTTCAGCTGGG-ATCATGATGGGTCTCC |
| PPD_rt_miR130a_rv      | CTCAACTGCTGCTGAGTCGGAAATTCAGTTGAG-ATGCCCTT |
| PPD_rt_miR130a_fw      | ACACTTCAGCTGGG-CAGTGCAATGTAAA |
| PPD rt_universal       | CTCAAGTGTCGTCGTCGTCGCAAA |

### Table S7. miRNA northern probes (related to Figure 1)

| miRNAs | miRNA sequences | Northern probes |
|--------|----------------|-----------------|
| miR-127 | UCGGAUCUCGUGCUGAGCUUGGCC | AGCCAAGCTCAGACGGGATCCGAGA |
| miR-134 | UGUGAUCUGGUUGACACAGAGGG | CCCCTCTGATCAACCAGTCACA |
| miR-323-3p | CACAGUUAACGAGGGACCCU | AGGCCAGCCTATTGATTGAGG |
| miR-410 | AAUUAUAAACAGAUGGGCGCU | ACAGGGCACTCAGTTTATATT |
| miR-431 | UGCUUUCAGGCGGCUAGUGCA | TGCACTGACGGCCTGGGAAGCA |
| miR-433 | AUCAGAUGUGGCUCCUGCGGU | ACACCGAGGAGCCCATACGTAG |
| miR-130a | CAGUGCAUAAUUGUUAAAGGGCAU | ATGCCCTTTTAACATGCTGACAG |
| U6 snRNA | GUGCUCGCUCUGGCGACGACA | TGCTGTGGCAGGAGCAC |

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Small RNA sequencing (RNA-Seq) analyses

Small RNA sequencing was performed with the SOLiD platform. Reads were trimmed and aligned in colorspace. Trimming of the 3’ adapter was performed with the software cutadapt using the command:

cutadapt --trim-primer -z -c -e 0.3 -a 33020103031312312 -m 10 -o reads.fastq reads.csfasta reads.qual

Reads were then aligned to mouse genome version mm9 using bowtie. Alignments were also performed in colorspace, using the command:

bowtie -C -S -a -k 10 -m 10 --best -l 20 -q mm9 c reads.fastq > reads.sam

Size selected small RNAs (18-40 nt) were sequenced from wild-type (CJ7), Ezh2/-, Eed/- and Jarid2/- mESCs. A summary of the libraries sequenced is given in Table S1.

RNA sequencing (RNA-Seq) analyses

RNA was sequenced with the Illumina pipeline, aligned to mm9 and mm10 genome using TopHat v2.0.9. Differential expression was determined using Cuffdiff v2.2.1 and the GRCm38 (mm10) Ensembl genome annotation downloaded from the UCSC Genome Bioinformatics website “Tables” tool. Alignments were made with --no-novel-juncs and --no-coverage-search. Please see Table S2 for data summary.
Co-Immunoprecipitation (Co-IP) and Western blot

For each IP, cells were harvested from a 15 cm dish, and washed twice with ice cold PBS. Cell pellet was allowed to swell in twice the volume of hypotonic solution (10 mM HEPES- pH 7.3, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 1 mM PMSF and protease inhibitors), and passed through a 26½-gauge needle 5 times, followed by centrifugation at 14,000 rpm for 20-30 seconds. The cloudy supernatant cytoplasmic fraction was removed, and then the cell pellet was resuspended in the same volume of high salt buffer (20 mM HEPES-pH 7.3, 1.5 mM MgCl₂, 420 mM KCl, 0.2 mM EDTA, 30% Glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors) and rotated for 1-2 hour at 4°C. Then, neutralizing buffer “without salt” (20 mM HEPES-pH 7.3, 0.2 mM EDTA, 20% Glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors) was added to the nuclear extract (NE) to bring the salt concentration to around 150 mM. NE was centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was collected. The volume of supernatant was increased to 1ml with IP buffer (combining high salt buffer and neutralizing buffer to make a final concentration of 150 mM, with 1 mM DTT, 1 mM PMSF and protease inhibitors). The supernatant was pre-cleared with either Protein-A or G agarose beads (Roche). The 5%-10% of cleared supernatant was collected as an input, and rest of the supernatant was incubated with antibody overnight at 4°C. The next day either Protein-A or G agarose beads were added to it (depending upon the antibody), and incubated for 2-3 hours at 4°C to IP endogenous protein against the specific antibody used. Subsequently, IP-ed protein-beads were washed 3 times with IP buffer, each for 5 minutes at 4°C. IP-ed proteins and their interacting partners were eluted from beads in
XT buffer (Bio-Rad) by heating at 95°C for 10 minutes, and resolved on a 4-12% gradient Bis-Tris gel (Bio-Rad) and analyzed by western blot using specific antibodies.

**Antibodies**

Ezh2 (D2C9 XP: 5246S, Cell signaling) (AC22: 3147, Cell signaling) (ChIPAb+ Ezh2, clone AC22 (17-662), Millipore); Suz12 (P-15: sc-46264, Santa Cruz); Jarid2 (NB100-2214, Novus Biologicals); H3K4me3 (ab1012, Abcam); H3K9me3 (ab8898, Abcam); H3K27me3 (ab6002, Abcam); H3K27ac (ab4729, Abcam); RNA Pol II 8WG16 (MMS-126R, Covance); Actin (Clone C4: MAB1501R, Millipore); Dnmt1 (39204, Active motif); Dnmt3a (IMG-268A, Imgenex) (ab13888, abcam) (ab2850, abcam); Dnmt3b (IMG-184A, Imgenex) (ab122932, abcam); Dnmt3l (IMG-6809A, Imgenex) (12309, Cell signaling).

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed as described previously with some modifications (Das et al., 2014). Input genomic DNA was used for the reference sample. Briefly, cells were crosslinked directly on 15 cm dishes with 37% formaldehyde solution (Calbiochem) to a final concentration of 1% for 8 min at room temperature with gentle shaking. The reaction was quenched by adding 2.5M glycine to a final concentration of 0.125 M. Cells were washed twice with PBS, trypsinized and washed twice with PBS. Cell pellet was resuspended in SDS-ChIP buffer (20 mM Tris-HCl pH 8.0, 150mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and protease inhibitor), and chromatin was sonicated to around 200-500 bp. Sonicated chromatin were pre-cleared with either Protein-A or Protein-G agarose (Roche)
beads. Cleared samples were incubated with 5-10 µg antibody overnight at 4°C. After overnight incubation, protein A or G agarose beads were added to the ChIP reactions and incubated for 2-3 hours at 4°C to IP chromatin. Subsequently, beads were washed twice with 1 ml of low salt wash buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with 1 ml of high salt wash buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once with 1 ml of LiCl wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% NP-40, 250 mM LiCl), and twice with 1 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0). The chromatin was eluted and reverse-crosslinked at 65°C overnight in SDS elution buffer (300 µl) (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). The next day, an equal volume of TE was added (300 µl). ChIP DNA was treated with 1 µl of RNaseA (10 mg/ml) for 1 hour, and with 3 µl of proteinase K (20 mg/ml) for 3 hours at 37°C, and purified using phenol-chloroform extraction, followed by QIAquick PCR purification spin columns (Qiagen). Finally, ChIP-DNA was eluted from the column with 40 µl of water. For several factors we used multiple individual ChIPs. At the end, all eluted ChIP-DNA samples were pooled and precipitated to enrich the ChIP-DNA material to make libraries for sequencing. Input ChIP samples were reserved after the pre-clear step and continued from reverse cross-linking step until the end, same as other ChIP samples. All ChIP primers are listed here.
ChIP primer sequences:

| Primer names     | Sequences                     |
|------------------|-------------------------------|
| PPD_IGDMR_a_F    | AC CCCAAGACAGAGAATTT         |
| PPD_IGDMR_a_R    | GCAGTGACACTTCCTCAT           |
| PPD_IGDMR_b_F    | CCTTCTACCTTAAAAGATGCTACGA    |
| PPD_IGDMR_b_R    | CGTAACTATGTTGACTTGTGCATC     |
| PPD_IGDMR_c_F    | CCTCAGCACACGATACTTTGA        |
| PPD_IGDMR_c_R    | GCTGAAACGGACGTGTA            |
| PPD_IGDMR_d_F    | GTCAGTTTCAAGTGGCTA           |
| PPD_IGDMR_d_R    | TACTGCTCTGTGCCGTGAAG         |
| PPD_IGDMR_e_F    | CAAGTCAACAGCCCTACTCTGTAGT    |
| PPD_IGDMR_e_R    | CATATACGGTGAATGCGATGTA       |
| PPD_IGDMR_f_F    | GCTATGCTTTTTCTTTCTTTTCTTT   |
| PPD_IGDMR_f_R    | ATTCCCAATCTGAGAACGTCCATT     |
| PPD_IGDMR_g_F    | AATGGGATCACGCGAGTAAG         |
| PPD_IGDMR_g_R    | GCTCCATGCTATTGGAGGC          |
| PPD_IGDMR_h_F    | GCTTGGAAATTCGTGAAGGA         |
| PPD_IGDMR_h_R    | TCAGCTCACAATCGTCATT          |
| Nanog_0.2up_F    | AATGAGGTAAAGGCTCTTTTTTGG    |
| Nanog_0.2up_R    | ACCATGGACATTGTAAATGCAAAA     |
| Klf4_2_F         | GTGCGAGTGGTGGACTTGTA         |
| Klf4_2_R         | AAGGAAGGCGCTCCAGATTT        |

**ChIP library generation and sequencing**

Purified ChIP DNA was measured in Qubit (Invitrogen). 2-10 ng of purified ChIP DNA was used to prepare sequencing libraries, using NEB next generation ChIP sequencing Kit (NEB) and Illumina ChIP seq kit (Illumina) according to the manufacture’s instructions. All libraries were checked through a Bio-analyzer for quality control purposes. ChIP sequencing was performed using Illumina Hiseq 2000. Raw data were processed through Illumina software pipeline.
**ChIP sequencing (ChIP-Seq) analyses**

All ChIP-seq samples were aligned with Bowtie v0.12.9 to the mm9 genome index with the following parameters: -S -m 1 -a -best -q. Custom options are found in the Table S5. Significant peaks were identified with SICER v1.1, and the following parameters: FDR 0.05, redundancy threshold: 1, fragment size: 150, species: mm9, effective genome size: .79 for samples with aligned read length of 46 or 50, .70 for those with aligned read length of 26 (Koehler et al., 2011). Window sizes for SICER were 200, and gap sizes were chose as per recommendations in associated publication (Zang et al., 2009) to be 200 for H3K4me3, 600 for H3K27me3, and similarly for other chromatin marks. For transcription factors and sharply peaked binding proteins we used a gap length of 400. All ChIP-Seq data used for this study are summarized in Table S3 and S4.

**Genomic deletion using the CRISPR/Cas9 nuclease system**

**CRISPR design and creation:**

Single-guide RNA (sgRNA)-specifying oligo sequences were chosen to minimize likelihood of off-target cleavage based on publicly available online tools. “CACC” was added to the 5’ end of the sgRNA-specifying oligo sequence and “AAAC” was added to the 5’ end of the reverse complement of the sgRNA-specifying oligo for cloning using the BbsI restriction enzyme. G was added immediately following CACC if the first nucleotide was A, T, or C (in these cases C was added at the 3’ end of the reverse complement oligo). The two oligos were phosphorylated and annealed using the following conditions: guide sequence oligo (10 µM), guide sequence reverse complement
oligo (10 µM), T4 ligation buffer (1X) (New England Biolabs), and T4 polynucleotide kinase (5U) (NEB) with the following temperature conditions: 37°C for 30 minutes; 95°C for 5 minutes and then ramp down to 25°C at 5°C/minute. The annealed oligos were cloned into pSpCas9(BB) (pX330; Addgene plasmid ID: 42230) using a “Golden Gate Assembly” strategy with the following conditions: 100 ng of circular pX330 vector, annealed oligos (0.2 µM), NEB 2.1 buffer (1X) (NEB), BbsI restriction enzyme (20 U) (NEB), ATP (0.2 mM) (NEB), BSA (1X) (NEB), and T4 DNA ligase (750 U) (NEB) with the cycling conditions of 20 cycles of 37°C for 5 minutes, 20°C for 5 minutes; 80°C for 20 minutes.

Single-guide RNA (sgRNA) oligos for CRISPRs:

| Primer names | Sequences |
|--------------|-----------|
| PPD_IGDMR/Enh1_5end-F | caccgCTAATAAAGAGTAGGGCGAGC |
| PPD_IGDMR/Enh1_5end-R | aaacGCTCGCCTACTTTATTAAGc |
| PPD_IGDMR/Enh1_3end-F | caccGTGTGAAACGTATGGCCACG |
| PPD_IGDMR/Enh1_3end-R | aaacGTCGGCCATACGTTCAACAC |
| PPD_Enh2_5end-F | caccAGGAGATGGAATCAGCGGGT |
| PPD_Enh2_5end-R | aaacACCCGCTGATTCATCTCTCt |
| PPD_Enh2_3end-F | caccGCTCCGTCGAAAAAGGTCGCC |
| PPD_Enh2_3end-R | aaacGCGACCTTTTGACGAGAC |
| PPD_Non-Enh2_5end-F | caccGGAGACAGACTACAGCCCCG |
| PPD_Non-Enh2_5end-R | aaacACCGGCTGATGCTCTGTC |
| PPD_Non-Enh2_3end-F | caccTGAGCGCGGTCCGTCCACT |
| PPD_Non-Enh2_3end-R | aaacAGTGAGCGGAGGCCGCTC |
| PPD_Dlk1 prom_5end-F | caccGAGTGATACATTTATGGGCC |
| PPD_Dlk1 prom_5end-R | aaacGGCCCAATAAAGTGATACCTC |
| PPD_Dlk1 prom_3end-F | caccGACGCTCGCAGAATCCATA |
| PPD_Dlk1 prom_3end-R | aaacGTATGAGTTCTCGGAGGTC |
| PPD_Gtl2 prom_5end-F | caccGAAAATAAGGATGGGTAACGG |
| PPD_Gtl2 prom_5end-R | aaacCGTATACCCCATCCTATT |
| PPD_Gtl2 prom_3end-F | caccGTCGCAAGCGGTTCCGAC |
| PPD_Gtl2 prom_3end-R | aaacGTCGGAAACCGCTTGCGAC |
**Screening for biallelic deletion clones:** Mouse ES cells (mESCs) were cultured as described above. 1-2 x 10^6 cells were electroporated with 1 µg pmaxGFP plasmid (Lonza), and 2 µg of each pX330-guide RNA plasmid using the ECM 830 Square Wave Electroporation System (Harvard Apparatus). Specifically, cells were resuspended in 100 µl of mouse electroporation buffer (Lonza), and electroporated at 250 V for 5 ms, in 2 mm cuvettes (Harvard Apparatus). 500 µl of mESCs medium was immediately added to the cells after electroporation, and mESCs were plated on irradiated mouse fibroblasts. The highest 3-5% GFP^+ cells were sorted using a FACS Aria cell sorter (BD Biosciences) 2-3 days post-electroporation. Approximately 15,000 GFP^+ mESCs were plated on 10 cm irradiated mouse fibroblasts to obtain single cell-derived clones. After 7-10 days, single clones were picked and transferred to a 96-well plate. Individual clones were then screened for CRISPR-mediated biallelic deletion. Genomic DNA (gDNA) was extracted using 50 µL QuickExtract DNA Extraction Solution per well (Epicentre) for 65°C, 6 minutes and 98°C, 2 minutes. Polymerase chain reaction (PCR) was performed using two sets of primers (deletion and non-deletion primer pair): one set to amplify a sequence overlapping the segment to be deleted (“non-deletion band”) and another set that only amplify in the presence of a deletion (“deletion band”) using the Qiagen HotStarTaq 2x master mix (Qiagen), following cycling conditions: 95°C for 15 minutes; 35 cycles of 95°C for 15 seconds, 60°C for 1 minute, 72°C for 1 minute; 72°C for 10 minutes. Mono-allelic deletion clones were defined as having PCR amplification of both the non-deletion band and deletion band. Biallelic deletion clones were defined as having PCR amplification of the deletion band and absence of the non-deletion band. Deletion and non-deletion amplicons from non-deletion, mono-allelic, and biallelic deletion clones
were subjected to Sanger sequencing. All deletion and non-deletion primer pairs are listed here.

Primer pair used to screen CRISPR mediated biallelic deletion clones:

| Deleted genomic regions | Primer pair for deletion screen | Primer names | Sequences |
|-------------------------|----------------------------------|--------------|-----------|
| IG-DMR                  | Deletion primer pair             | IGDMR/Enh1_DEL_F | GTCAATCTGAAAAACTGGGAAAAAA |
|                         |                                  | IGDMR/Enh1_DEL_R | TCCCCAGAAACTCGTTCTGTTTT |
|                         | Non-deletion primer pair         | IGDMR/Enh1_5flank_F | GTCAATCTGAAAAACTGGGAAAAAA |
|                         |                                  | IGDMR/Enh1_5flank_R | GGGAGGAGAGAAGGAGAACTA |
| Enh2                    | Deletion primer pair             | Enh2_DEL_F | CTCAGATGTGTGTTCACCTCCT |
|                         |                                  | Enh2_DEL_R | TATCTTCTTCACAGCCTTCTC |
|                         | Non-deletion primer pair         | Enh2_5flank_F | ATTTCCCATCTCCTGTCTTC |
|                         |                                  | Enh2_5flank_R | TCCTCAAGCCTGCATAAACTG |
| Non-Enh2                | Deletion primer pair             | Non-Enh2_DEL_F | TAGAATCCACAGACCCCTGAC |
|                         |                                  | Non-Enh2_DEL_R | ACTGTTTACCAACTGAGTCGAA |
|                         | Non-deletion primer pair         | Non-Enh2_5flank_F | AGGCAAGTGCTTTTTACCAC |
|                         |                                  | Non-Enh2_5flank_R | TGGTGAGATGTGTTCACGAGC |
| Dlk1                    | Deletion primer pair             | Dlk1 prom_DEL_F | CCCATTACCAAGGAGCTATG |
|                         |                                  | Dlk1 prom_DEL_R | CTCTCCTCTGTACCCTCCTTC |
|                         | Non-deletion primer pair         | Dlk1 prom_5flank_F | TGTGAGAGAGAGAGAGAGAGAGAGAGAGAGA |
|                         |                                  | Dlk1 prom_5flank_R | TCACATTAGGCGTTTCAACCAG |
| Gtl2                    | Deletion primer pair             | Gtl2_prom_Del_F | AAGCTGAAAACAACATTTAACAG |
|                         |                                  | Gtl2_prom_Del_R | GCTGTAAGAAAGAAGACAGACACT |
|                         | Non-deletion primer pair         | Gtl2_prom_5flank_F | CAGACAGAAACAGATCCCCATC |
|                         |                                  | Gtl2_prom_5flank_R | ATCTGAGAAAGGAGAGAGTTGG |

**Enhancer reporter assay**

Dual-luciferase assays in mESCs were performed using the Dual-Luciferase Reporter Assay System following the manufacturer’s instructions (Promega). Genomic DNA fragments containing the putative enhancers (Enh-1, Enh-2 and Nanog Enh) and control non-enhancer regions (Non-Enh1, Non-Enh2) were cloned into the pGL3-basic vector.
Constructs were transfected into mESCs cells by nucleofection, and luciferase activities were measured following standard protocols.

Primers used for cloning enhancer regions for reporter assay:

| Primer names       | Sequences                                                                 |
|--------------------|---------------------------------------------------------------------------|
| PPD_IGDMR/Enh1_F   | ACGCGTCGACGTCGGCCATAGCGGCCGCGAATTGGGCTCAAGCTCCACAT                      |
| PPD_IGDMR/Enh1_R   | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAAAGGGCCCTCTCCAGAGCAGG                   |
| PPD_Enh2_F         | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAACCCTCCAGTGAGGACTCAGA                   |
| PPD_Enh2_R         | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAAGACATTGGACAACAAGGAGG                   |
| PPD_Nanog Enh_F    | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAACTATTTATTATATCTGCGCTTG                 |
| PPD_Nanog Enh_R    | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAAATGGAGTTAAGTACTCCGACACT                |
| PPD_Non-Enh1_F     | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAAAGCTGATGGGCCTTACAAAG                  |
| PPD_Non-Enh1_R     | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAATTGGTTGATACGAAGATGTG                   |
| PPD_Non-Enh2_F     | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAAAGGACAATAGAAAAGTAACA                   |
| PPD_Non-Enh2_R     | ACGCGTCGACGTCGGCCATAGCGGCCGCGGAAAGGACAATAGAAAAGTAACA                   |

**Chromosome Conformation Capture (3C)**

3C assay was performed as described previously (Hagège et al., 2007; Xu et al., 2012) with some modifications. Briefly, mESCs were harvested and crosslinked with 2% formaldehyde for 10 min at room temperature. Crosslinked cells were lysed with ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.2% NP-40, 1 mM dithiothreitol) for 10 min. The nuclei were harvested and resuspended in appropriate restriction enzyme buffer containing 0.3% SDS and incubated at 37°C for 1 hour with vigorous shaking. Triton X-100 was then added to 2% final concentration to sequester the SDS, and samples were incubated at 37°C for another 1 hour. Samples were digested with NheI (for IG-DMR/Enh1, Gtl2 promoter, Enh2 and Dio3 promoter) or Scal (for Nanog) overnight at 37°C. DNA ligation was performed at 16°C for 4 hours and 30 min at room temperature. Crosslinks were reversed, and DNA was then purified by phenol extraction.
and ethanol precipitation. To correct for the PCR amplification efficiency of different primer sets, BAC clones containing the complete mouse IG-DMR/Enh1, Gtl2 promoter (RP23-117C15), Enh2 (RP23-409I23), Dio3 promoter (RP24-316E16) and Nanog promoter (RP23-474F18) were used as control templates. Equimolar amounts of BAC DNA were mixed, digested, and ligated. Quantification of the data was performed by quantitative real-time PCR using the SYBR Green Supermix (Bio-Rad). 3C product detection was done by RT-qPCR, and the average signal was corrected by dividing the average signal in the BAC control template.

**RNA immunoprecipitation (RIP)**

Mouse ES Cells were grown on gelatin coated 15 cm dishes, ~10 million cells were harvested and resuspended in 2ml of 1X PBS. Nuclear Isolation Buffer (2ml) (1.28M sucrose, 40mM Tris-Cl pH 7.4, 20mM MgCl2 and 4% Triton X-100 with freshly added 1:1000 Protease inhibitor (Sigma), 1mM DTT) and water (6ml) were added to the resuspended cells and incubated for 20-30 min on gentle rotation at 4ºC. Nuclei were pelleted by centrifugation at 2500g for 15 min at 4ºC. Then, nuclei pellet were resuspended in 1ml of RNA immunoprecipitation buffer (RIP buffer-150mM KCl, 25mM Tris-Cl pH 7.4, 5mM EDTA, 0.5% NP40 with freshly added 1:200 Protease inhibitor (Sigma), 1mM DTT and 200 units of RNaseOUT (Invitrogen/ Life tech)). Resuspended nuclei were divided into two fractions of 500µl each (one for mock and one for IP), and passed through 26 G syringe 5 times. Nuclear membrane and debris were pelleted by centrifugation at 13000 rpm for 15 min at 4ºC; supernatant was collected and 5µg of antibody against protein of interest (IP sample) and normal IgG (as control) (mock
sample) were added and incubated for overnight at 4°C with gentle rotation. Next day, Protein A/G Dynabeads (Invitrogen/ Life tech) (75µl) were added to this and incubated for 2 hours at 4°C with gentle rotation to capture the antibodies that bound to protein of interest. Beads were pelleted using magnetic stand, and washed twice with 1 ml of 150mM of RIP buffer, followed by 3 times wash with 1ml of 1M of RIP buffer. Immunoprecipitated protein was resuspended in 1ml of Trizol (Invitrogen) and RNAs that are bound to protein of interest were extracted according to the manufacture’s instructions. RNA was eluted in 15µl of water and treated with TURBO DNA-free kit (Ambion/ Life tech) to remove the DNA contamination as per as manufacture’s instructions.

DNA-free RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad), followed by qRT-PCR using iQ SYBR Green supermix (Bio-Rad) to detect the specific RNAs that are bound with protein of interest.

ChIPAb+ Ezh2, clone AC22 (17-662, Millipore), RIPAb+ Eed (03-196, Millipore), Suz12 (39357, Active motif) and Dnmt3a (IMG-268A, Imgenex) were used for RIP.

**Gene expression microarray analyses**

Affymetrix GeneChip mouse genome 430A 2.0 arrays were used for gene expression profiles. Total RNAs were extracted using RNeasy plus mini kit (Qiagen) and subsequent cDNA synthesis, labeling, hybridization, washing and scanning were performed by the Microarray Core Facility at the Dana Farber Cancer Institutes (DFCI). Expression data
were normalized using dChip software. The raw data set used in this study is available at Gene Expression Omnibus (GEO Accession: GSE58414).

**Overexpression of Ezh2 and Dnmt3a**

Adeno-Ezh2-GFP virus was infected in wild-type and *Dnmt3a*/*- mESCs, and GFP+ve cells were grown after FACS sorting.

Single copy of Dnmt3a cDNA transgene was integrated at the ROSA26 locus. Dnmt3a expressed through induction of Doxycycline (unpublished). This cell line was a gift from Alex Meissner.
SUPPLEMENTAL REFERENCES

Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V.B., Wong, E., Orlov, Y.L., Zhang, W., Jiang, J., et al. (2008). Integration of External Signaling Pathways with the Core Transcriptional Network in Embryonic Stem Cells. Cell 133, 1106–1117.

Das, P.P., Shao, Z., Beyaz, S., Apostolou, E., Pinello, L., De Los Angeles, A., O’Brien, K., Atsma, J.M., Fujiwara, Y., Nguyen, M., et al. (2014). Distinct and Combinatorial Functions of Jmjd2b/Kdm4b and Jmjd2c/Kdm4c in Mouse Embryonic Stem Cell Identity. Molecular Cell 53, 32–48.

Hagège, H., Klous, P., Braem, C., Splinter, E., Dekker, J., Cathala, G., de Laat, W., and Forné, T. (2007). Quantitative analysis of chromosome conformation capture assays (3C-qPCR). Nat Protoc 2, 1722–1733.

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.

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. Nature Publishing Group 20, 1258–1264.

Koehler, R., Issac, H., Cloonan, N., and Grimmond, S.M. (2011). The uniqueome: a mappability resource for short-tag sequencing. Bioinformatics 27, 272–274.

Marson, A., Levine, S.S., Cole, M.F., Frampton, G.M., Brambrink, T., Johnstone, S., Guenther, M.G., Johnston, W.K., Wernig, M., Newman, J., et al. (2008). Connecting microRNA Genes to the Core Transcriptional Regulatory Circuitry of Embryonic Stem Cells. Cell 134, 521–533.

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.

Shen, Y., Yue, F., McCleary, D.F., Ye, Z., Edsall, L., Kuan, S., Wagner, U., Dixon, J., Lee, L., Lobanenkov, V.V., et al. (2012). A map of the cis-regulatory sequences in the mouse genome. Nature 488, 116–120.

Whyte, W.A., Bilodeau, S., Orlando, D.A., Hoke, H.A., Frampton, G.M., Foster, C.T., Cowley, S.M., and Young, R.A. (2012). Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. Nature 482, 221–225.

Xu, J., Shao, Z., Glass, K., Bauer, D.E., Pinello, L., Ben Van Handel, Hou, S., Stamatoyannopoulos, J.A., Mikkola, H.K.A., Yuan, G.-C., et al. (2012). Combinatorial Assembly of Developmental Stage-Specific Enhancers Controls Gene Expression Programs during Human Erythropoiesis. Developmental Cell 23, 796–811.
Zang, C., Schones, D.E., Zeng, C., Cui, K., Zhao, K., and Peng, W. (2009). A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Bioinformatics 25, 1952–1958.