

Genome imprinting regulated by the mouse Polycomb group protein Eed

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Epigenetic regulation is essential for temporal, tissue-specific and parent-of-origin-dependent gene expression. It has recently been found that the mouse Polycomb group (PcG) gene *Eed* (embryonic ectoderm development) acts to maintain repression of the imprinted X chromosome. Here, we investigated whether *Eed* is also required for regulation of autosomal imprinted loci. Expression analyses showed that transcripts from the silent alleles of a subset of paternally repressed genes were present in *Eed*^{-/-} embryos. Parent-of-origin methylation was preserved in these embryos, but we observed changes in the methylation status of specific CpGs in differentially methylated regions (DMRs) at affected but not at unaffected loci. These data identify *Eed* as a member of a new class of *trans*-acting factors that regulate parent-of-origin expression at imprinted loci.

A subset of the mouse and human genomes is expressed from only one allele in a parent-of-origin-specific manner. This subset includes imprinted X-chromosome inactivation and autosomal imprinted loci. It has been proposed that this epigenetic regulation is accomplished through covalent modifications of both the DNA and the N-terminal tails of core histones in nucleosomes^{1,2}. There are more than 60 identified autosomal imprinted genes, about half of which are paternally repressed and half maternally repressed. Most imprinted genes that have been examined contain at least one DMR located in the 5' promoter region or in the body of the gene itself³. Recently, several proteins (DNA methyltransferases, CpG methyl binding proteins, chromatin insulators) have been identified as *trans*-acting factors involved in the epigenetic regulation of these loci⁴. Many of these factors either possess or associate with proteins that possess DNA methyltransferase activity. Additionally, recent studies have shown correlations

between covalent histone modifications and the transcriptional status of imprinted alleles⁵. In particular, methylation of histone H3 has been associated with the inactive X chromosome⁶.

PcG protein complexes are thought to maintain long-term gene silencing during development through alterations of local chromatin structure⁷. In both *Drosophila* and mammals, recent reports have shown that the *Eed*/*Ezh2* PcG complex contains histone methyltransferase (HMT) activity, methylating histone H3, and that mutations in the SET domain of the *Ezh2* fly homolog, *E(Z)*, abolish the enzymatic activity of the complex *in vitro*⁸⁻¹⁰. The *Eed*/*Ezh2* complex has also been shown to interact with histone deacetylases (HDACs; ref. 11). These

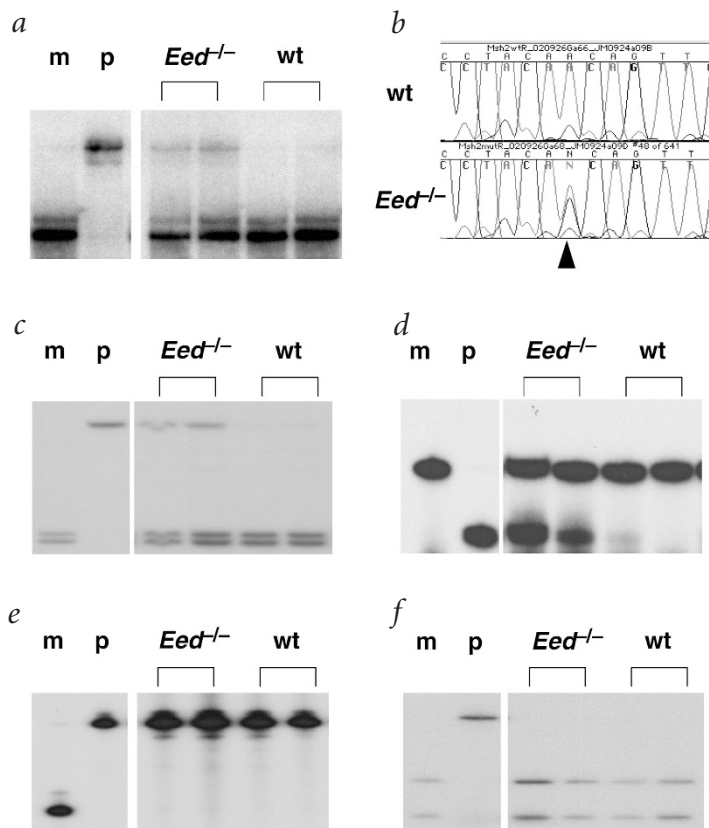


Fig. 1 Expression patterns of imprinted genes in wild-type (wt) and *Eed*^{-/-} embryos. Representative results from individual embryos (each lane) are shown for each assay. m, maternal control; p, paternal control. **a**, RFLP analysis of *Cdkn1c* at E7.5. **b**, Typical results for direct sequencing of *Ascl2* RT-PCR products. Arrowhead indicates base position of the expressed polymorphism (maternal = A, paternal = G). **c**, RFLP analysis of *Grb10* at E7.5. **d**, RFLP analysis of *Meg3* at E7.5. **e**, RFLP analysis of *Kcnq1ot1* at E7.5. **f**, RFLP analysis of *Kcnq1* at E7.5.

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results provide biochemical mechanisms by which the Eed/Ezh2 complex may establish heritable epigenetic states leading to long-term gene repression *in vivo*. We recently showed that loss of function of *Eed* in the mouse results in reactivation of the imprinted X chromosome in extraembryonic lineages¹². Additionally, the Eed and Ezh2 proteins have been reported to co-localize with XIST on the imprinted X chromosome of mouse trophoblast stem cells¹³. Thus, we investigated whether Eed is also required for epigenetic regulation of autosomal imprinted loci.

We examined the expression patterns of 18 imprinted genes during peri-gastrulation stages (embryonic days (E) 5.5–8.5) using embryos derived either from an intersubspecific cross between *Mus musculus domesticus* (CD1) and *Mus musculus molossinus* (JF1) or from an intrasubspecific CD1 C57BL/6 cross. For each gene examined, we identified a polymorphism in the mRNA to distinguish between maternal and paternal transcripts after RT-PCR. At all stages examined, the paternally repressed genes *Cdkn1c*, *Ascl2*, *Grb10* and *Meg3* were expressed only from the maternal allele in wild-type mice (Fig. 1), showing that these four genes are imprinted during gastrulation. In

Eed^{17Rn5-3354SB} null homozygous (herein referred to as *Eed*^{-/-}) littermates, however, we found mRNA expression from both maternal and paternal alleles of these genes (Fig. 1a–d). Four other paternally repressed (*Kcnq1*, *Slc22a11*, *Tssc3* and *Igf2r*) and six maternally repressed genes (*Kcnq1ot1*, *Snrpn*, *Peg3*, *Dlk1*, *Nnat* and *Plagl1*) showed normal parent-of-origin expression patterns in *Eed*^{-/-} embryos (Figs. 1e,f and 2). These data indicate that loss of *Eed* function affects a subset of imprinted loci that are expressed early in development resulting in biallelic expression, herein referred to as loss of imprinting (LOI; ref. 14).

To determine whether LOI contributes to the early lethality of *Eed*^{-/-} embryos, we used a targeted null mutation of *Cdkn1c*¹⁵ to reduce *Cdkn1c* expression levels. Maternal inheritance of the *Cdkn1c* null allele resulted in *Eed*^{-/-} *Cdkn1c*^{+/-} embryos that had developed substantially further than had *Eed*^{-/-} *Cdkn1c*^{+/-} embryos, although they were smaller and developmentally delayed when compared with wild-type littermates (data not shown). Morphological analysis at E8.5–9.0 showed that these embryos produced neural folds, a beating heart and somites, tissues that *Eed*^{-/-} embryos do not develop¹⁶. These data indicate that the LOI at *Cdkn1c* is partially responsible for the *Eed*^{-/-} embryonic phenotype.

To address the molecular mechanisms leading to LOI at affected loci, we examined the methylation status of CpG islands at *Cdkn1c*, *Grb10*, *Snrpn* and *KvDMR1* by bisulfite sequencing of genomic DNA collected from E7.5 wild-type and *Eed*^{-/-} embryos. Analysis of a region 5' to the transcriptional start site of *Cdkn1c* (CpG2; Fig. 3a) showed that the active maternal allele was hypomethylated and the inactive paternal allele was hypermethylated in wild-type embryos (Fig. 3b,d). These data extend previous observations from methyl-sensitive restriction digests that showed parent-of-origin methylation at three sites in the *Cdkn1c* coding sequence¹⁷. In *Eed*^{-/-} embryos, parent-of-origin methylation was present, but there were specific changes in the methylation pattern when compared with that of their wild-type counterparts (Fig. 3c). *Eed*^{-/-} maternal *Cdkn1c* alleles were unmethylated except for two CpGs at the 3' end of the region analyzed. The *Eed*^{-/-} paternal *Cdkn1c* alleles also had a methylation pattern that was distinct from that of the wild-type paternal allele (Fig. 3c). Notably, the *Eed*^{-/-} paternal *Cdkn1c* allele was active even though it was hypermethylated. This result was not anticipated because the silent paternal allele is hypermethylated in wild-type embryos. These data indicate that methylation at this DMR does not silence paternal *Cdkn1c* gene expression in *Eed*^{-/-} embryos. The specific pattern of CpG methylation, however, could be essential to the epigenetic state (inactive) of the paternal *Cdkn1c* allele. It is also notable that whereas there is parent-of-origin methylation in wild-type embryos at this DMR, it is not differentially methylated in germ cells, indicating post-fertilization regulation of methylation at this CpG island.

For *Grb10* on mouse chromosome 11, we identified a potential DMR containing 146 CpG dinucleotides in 1,200 bases located between exons 1 and 2. Bisulfite sequencing of 194 bases (Fig. 4a) containing 22 CpGs confirmed parent-of-origin methylation at this CpG island. The active maternal allele was hypermethylated and the silent paternal allele was hypomethylated in wild-type embryos (Fig. 4b). Three consecutive CpGs

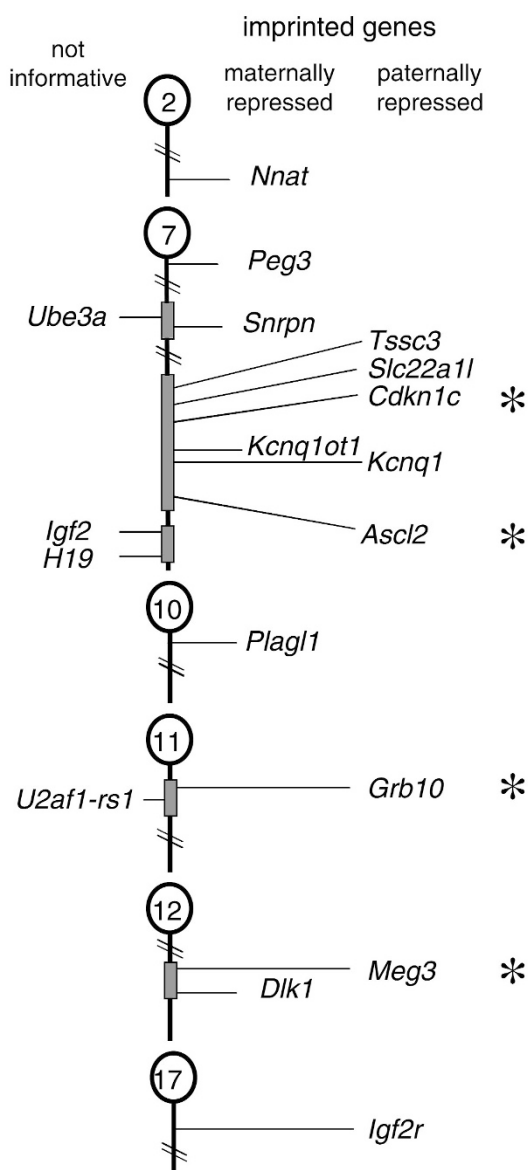


Fig. 2 Imprinted expression summary. Schematic representation indicating the chromosomal location of each of the 18 genes examined for imprinted expression in wild-type gastrulation-stage embryos. *Ube3a*, *Igf2* and *H19* showed biallelic expression, and *U2af1-rs1* was not detected by RT-PCR; therefore, these genes were not informative. Maternally repressed genes and paternally repressed genes are listed in separate columns on the right side of each chromosome (number in centromere denotes chromosome). Genes that showed LOI in *Eed*^{-/-} embryos are marked with an asterisk.

were always methylated (Fig. 4c) on the paternal allele in wild-type embryos, whereas all other CpGs were unmethylated. The *Eed*^{-/-} paternal *Grb10* allele showed a variable pattern of hypomethylation, and the three CpGs that were invariantly methylated in wild-type embryos were not methylated in mutant embryos (Fig. 4c). We observed no differences between the wild-type and *Eed*^{-/-} maternal *Grb10* alleles, both of which were methylated at every CpG in the region analyzed. Similar analyses of the *Snrpn* DMR¹⁸, whose imprinted expression is not affected in *Eed*^{-/-} embryos (Fig. 2), indicated no changes in parent-of-origin methylation patterns (Fig. 4d).

The imprinting control region (ICR) *KvDMR1* on distal chromosome 7 has been shown to regulate several closely linked, paternally repressed genes including *Cdkn1c*, *Tssc3*, *Slca221l*, *Kcnq1* and *Ascl2* (Fig. 5a), as paternal inheritance of a deletion of *KvDMR1* results in LOI at these imprinted loci¹⁹. The observed LOI at these loci may be due to removal of the CpG island at the ICR or to disruption of the *Kcnq1ot1* antisense transcript. To test the role of *Eed* at this ICR, we examined both parent-of-origin methylation at *KvDMR1* (Fig. 5) and expression of the antisense *Kcnq1ot1* transcript (Fig. 1f) in mutant embryos. Bisulfite sequencing in this ICR showed that parent-of-origin methylation was present in *Eed*^{-/-} embryos. Maternal *KvDMR1* alleles in both wild-type and *Eed*^{-/-} embryos were hypermethylated, but there were two CpGs that were never methylated in *Eed*^{-/-} maternal *KvDMR1* alleles but always methylated in wild-type maternal alleles (Fig. 5b,c). We recovered both methylated and unmethylated populations of paternal alleles from wild-type and *Eed*^{-/-} littermates. Preliminary tissue-specific analysis suggests that the paternally methylated populations are predominantly extraembryonic in origin (data not shown). In the methylated paternal *KvDMR1* alleles, there was one

CpG that was always methylated in wild-type embryos and never methylated in *Eed*^{-/-} embryos (Fig. 5c).

An insulator model for imprinted gene expression at this cluster has been proposed, on the basis of *in vitro* reporter assays that show CTCF, a zinc-finger protein, binds to unmethylated sites in the *KvDMR1* ICR²⁰. Alternatively, imprinted expression at this cluster could be regulated by the *Kcnq1ot1* antisense transcript, similar to the model that has been proposed at the *Igf2r/Air* locus on chromosome 17 (ref. 21). Neither *Kcnq1ot1* expression nor the methylation status of the ICR were altered in *Eed*^{-/-} embryos. These data indicate that *Eed* represents an additional mechanism of epigenetic regulation that is required to maintain parent-of-origin expression at specific loci in this cluster. In *Eed*^{-/-} embryos, *Cdkn1c* and *Ascl2* had LOI but *Slca221l*, *Tssc3* and *Kcnq1* were properly imprinted, whereas all imprinted genes in the cluster were affected in the *KvDMR1/Kcnq1ot1* deletion¹⁹. Gene-specific LOI was also observed in the cluster of imprinted genes on chromosome 12 (ref. 22), where *Meg3* expression was subject to LOI but *Dlk1* expression was properly imprinted in *Eed*^{-/-} embryos.

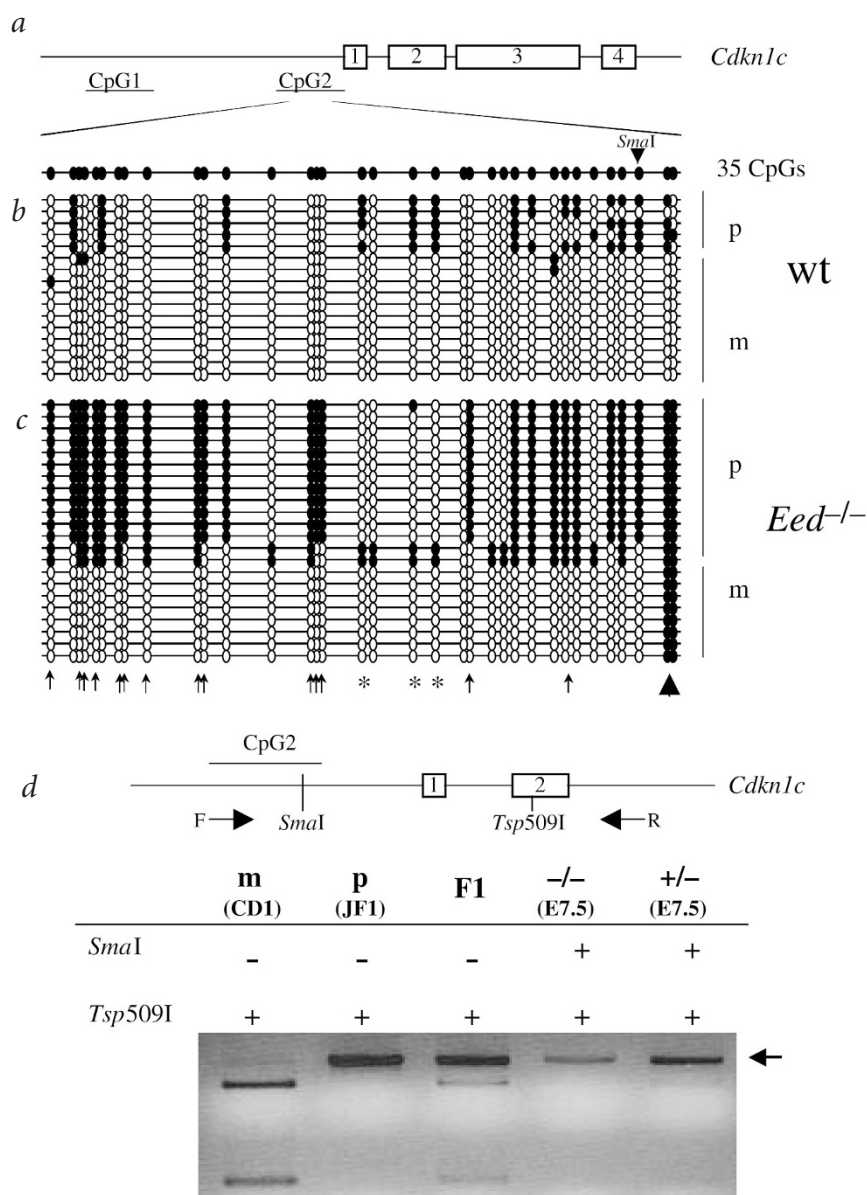


Fig. 3 *Cdkn1c* CpG methylation. **a**, Schematic representation of a 10-kb genomic fragment of the *Cdkn1c* locus, indicating the location of exons (boxes 1–4) and CpG islands in the region (CpG1 and CpG2). CpG2 is expanded to indicate all 35 CpG dinucleotides (filled ovals) in the region analyzed after bisulfite treatment. The *SmaI* restriction site used in **d** is indicated. **b**, **c**, Parent-of-origin methylation patterns in wild-type (wt; **b**) and *Eed*^{-/-} (**c**) E7.5 embryos. m, maternal; p, paternal. Filled ovals indicate methylated CpGs; open ovals indicate unmethylated CpGs. Wild-type includes both *Eed*^{+/+} and *Eed*^{+/-} embryos. Symbols denote changes in the *Eed*^{-/-} methylation patterns. Arrows, gain of paternal-allele methylation; asterisks, loss of paternal-allele methylation; arrowheads, gain of maternal-allele methylation. **d**, Genomic DNAs were digested with *SmaI* before PCR and then analyzed by *Tsp509I* RFLP to assign parent-of-origin to each of the alleles recovered through bisulfite sequencing, as there was no SNP in the region examined (**b**, **c**). In both wild-type and mutant embryos, only the paternal uncut PCR product (arrow) remained after *SmaI* digestion, indicating that the paternal allele was methylated at this site (arrowhead in **a**) whereas the maternal allele was not. m, maternal; p, paternal. F and R indicate locations of forward and reverse PCR primers, respectively; *SmaI* and *Tsp509I* restriction site locations are indicated in schematic (**a** and **d**).

Our expression and methylation data identify Eed as a *trans*-acting factor that regulates imprinted gene silencing at a specific subset of autosomal loci during early development. Of the genes affected by mutations in *Eed* that have been identified thus far, no correlation with chromosomal location (Fig. 2) or methylation patterns was observed in wild-type embryos at ICRs or DMRs. For example, in normal littermates, the DMR of the active maternal *Cdkn1c* allele was unmethylated whereas at the DMR associated with *Grb10*, the active maternal allele was entirely methylated. The only consistent finding, including imprinted X-chromosome inactivation¹², is that in each case we observed biallelic expression of paternally repressed genes in mutant embryos (Fig. 2). Though these results are suggestive, more loci need to be examined to determine whether regulation by *Eed* is in fact restricted to paternally repressed genes.

DNA methyltransferase activity has not been associated with any PcG protein complexes. But it is possible that the alterations in DNA methylation patterns that we observed are intimately linked with altered covalent histone modifications.

Recent studies in both *Neurospora* and *Arabidopsis* have shown that cytosine methylation is dependent on histone methylation^{23,24}. Given the HMT activity of the Eed/Ezh2 complex and its potential for HDAC interactions, we propose that alterations to covalent histone modifications (methylation and possibly acetylation) are the primary defect resulting in LOI when the Eed/Ezh2 complex is disrupted and that the accompanying changes in DNA methylation patterns at *Cdkn1c*, *Grb10* and *KvDMR1* in *Eed*^{-/-} embryos are a secondary consequence.

The Eed/Ezh2 complex interacts through Eed with another PcG protein, Yy1, which has DNA-binding ability²⁵. One possible explanation for the specificity of genes that show LOI in *Eed*^{-/-} embryos could be that Yy1 targets the Eed/Ezh2 complex to specific recognition sites of imprinted genes, resulting in local histone modifications. In the absence of functional Eed, Yy1 may not be able to recruit the HMT activity of Ezh2, resulting in hypomethylation of histone H3 and a transcriptionally permissive environment leading to derepression of normally silent alleles. This model

also explains the observed biallelic expression (as opposed to loss of expression of the active allele at affected loci) and is consistent with the role of Eed in a PcG group complex for maintaining long-term repression¹¹.

At the affected loci examined, LOI cannot be the direct consequence of an inability of *Eed*^{-/-} cells to distinguish between the parental chromosomes, as parent-of-origin methylation patterns were present in *Eed*^{-/-} embryos. This observation is in contrast with LOI caused by loss of parental imprints in uniparental disomies, deletion of ICRs or ablation of DNA methyltransferases. Our findings suggest that the presence of parent-of-origin methylation is not sufficient to direct imprinted gene expression at specific loci. It is possible, however, that particular patterns of CpG methylation may be essential for silencing of the paternal allele at affected loci and that the changes observed in *Eed*^{-/-} embryos lead to LOI.

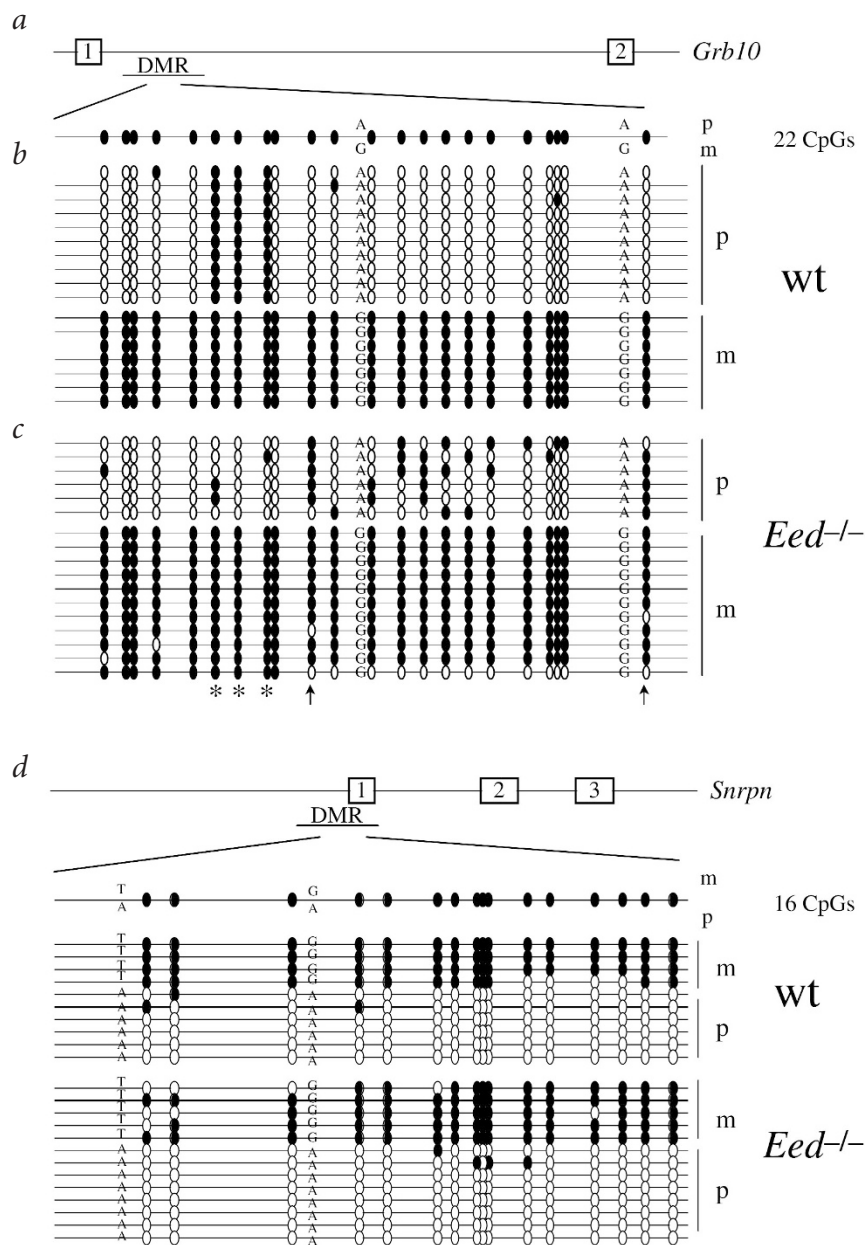
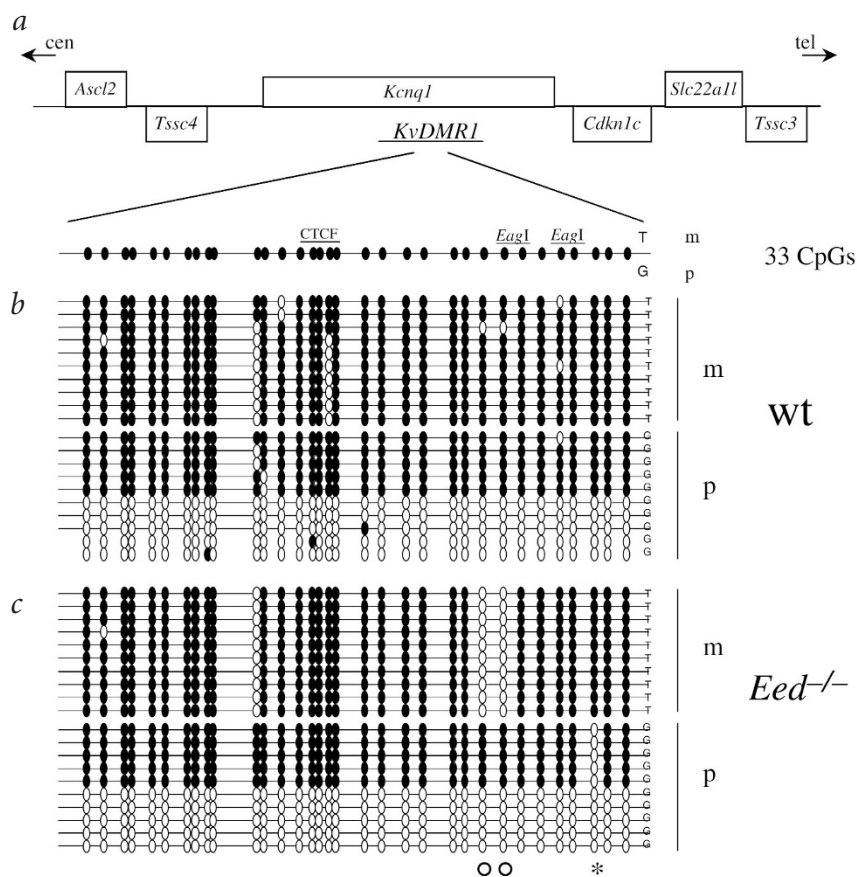


Fig. 4 *Grb10* and *Snrpn* CpG methylation. **a**, Schematic representation of the genomic *Grb10* locus (roughly 40 kb shown), indicating exons (boxes 1,2) and the location of the CpG island 4 kb 3' of exon 1. The CpG island is expanded to show all 22 CpG dinucleotides in the region analyzed after bisulfite treatment. Two SNPs in the region allowed for assignment of alleles recovered, and these are indicated by the base-pair changes (paternal (JF1) = A, maternal (CD1) = G at each SNP). **b,c**, Parent-of-origin methylation patterns in wild-type (wt; **b**) and *Eed*^{-/-} (**c**) E7.5 embryos. Filled ovals indicate methylated CpGs; open ovals indicate unmethylated CpGs. Wild-type includes both *Eed*^{+/+} and *Eed*^{-/-} embryos. Symbols denote changes in the *Eed*^{-/-} methylation patterns. Arrows, gain of paternal-allele methylation; asterisks, loss of paternal-allele methylation; arrowheads, gain of maternal-allele methylation. m, maternal; p, paternal. **d**, Methylation analyses at the *Snrpn* promoter region. Filled ovals indicate methylated CpGs; open ovals indicate unmethylated CpGs. Wild-type includes both *Eed*^{+/+} and *Eed*^{-/-} embryos. m, maternal; p, paternal.

Fig. 5 *KvDMR1* CpG methylation. **a**, Schematic representation of genomic *KvDMR1* locus indicating the six paternally repressed genes (boxes) that have been shown to be regulated by this ICR, located in intron 10 of *Kcnq1* (ref. 28). *KvDMR1* is expanded to indicate all 33 CpG dinucleotides in the region analyzed after bisulfite treatment. One SNP in the region allowed for assignment of alleles recovered, and these are indicated by the base-pair change (paternal (JF1) = G, maternal (CD1) = T). One putative CTCF binding site was identified containing 11 of 16 bases identical to conserved CTCF binding sites²⁹. Two *EagI* sites previously examined for parent-of-origin methylation are shown³⁰. **b,c**, Parent-of-origin methylation at the ICR in wild-type (wt; **b**) and *Eed*^{-/-} (**c**) embryos. Note the presence of two populations of paternal alleles in both wild-type and *Eed*^{-/-} embryos. Filled ovals indicate methylated CpGs; open ovals indicate unmethylated CpGs. Wild-type includes both *Eed*^{+/+} and *Eed*^{+/-} embryos. Symbols denote changes in the *Eed*^{-/-} methylation patterns. Asterisks, loss of paternal-allele methylation; arrowheads, gain of maternal-allele methylation. m, maternal; p, paternal. Open circles, loss of maternal methylation.



Incorrect gene expression at imprinted loci has been reported in many human disorders, including Beckwith-Wiedemann syndrome, Prader-Willi syndrome, Angelman syndrome and several types of cancer²⁶. In a substantial fraction of individuals with these diseases, imprinting defects are observed in a subset of imprinted genes in the absence of any detectable mutations in either the affected genes or known *cis*-acting regulatory elements¹⁴. Because *Eed* is required to maintain the epigenetic state leading to imprinted gene expression, we propose that mutations in *EED* or other *trans*-acting regulatory factors may account for the imprinting defects in these diseases. Here we showed that *Eed* is one such *trans*-acting factor that is necessary for parent-of-origin silencing of a subset of imprinted genes, providing the first link between a mammalian PcG gene and genome imprinting.

Methods

Expression analyses. We examined imprinted expression patterns by intron-spanning RT-PCR from freshly dissected gastrulation-stage embryos. We crossed *Eed*^{+/+} CD1 dams with *Eed*^{-/-} JF1 sires to produce embryos that were polymorphic at all loci examined except for *Meg3*. We used CD1 C57BL/6 embryos to examine *Meg3*. For all expression analyses, we extracted mRNA from individual gastrulation-stage embryos using Invitrogen Tri-Reagent. We synthesized cDNA in 20-1 reactions at 37 °C for 1 h using Invitrogen SuperscriptII RT and then amplified it by 32 rounds of PCR with 1 mM MgCl₂ at an annealing temperature of 60 °C. Polymorphism position (bp), SNP (CD1/JF1) and type of analysis used (enzyme) for each gene were as follows: *Cdkn1c*, bp 35,253 A/G, RFLP (*Tsp509I*); *Dlk1*, undetermined, bp 7,670–8,062, SSCP; *Grb10*, bp 897 G/A, RFLP (*Cac8I*); *H19*, bp 846 G/A, RFLP (*BclI*); *Igf2*, undetermined, bp 2,202–2,471, SSCP. *Igf2r*, 13-bp length polymorphism, bp 4,486–4,725; *Kcnq1*, bp 1,018 T/C, RFLP (*AluI*); *Kcnq1ot1*, bp 2,883 T/G, RFLP (*HpyCH4IV*); *Ascl2*, bp 1,315 T/C, direct sequencing; *Meg3*, bp 1,835 CD1 = G, B6 = A, RFLP (*BsrDI*); *Nnat*, undetermined bp 442–666, SSCP; *Peg3*, bp 5,329 A/C, RFLP (*TaqI*); *Slc22a11*, bp 680 C/T, RFLP (*NdeI*); *Snrpn*, bp 391 (A/C), RFLP (*NlaIII*); *Tssc3*, bp 122 A/C, SSCP; *Ube3a*, bp 2,366 T/C, RFLP (*Tsp509I*); *U2af1-rs1*, undetermined, bp 4,966–5,381, SSCP; *Plagl1*, bp 941 A/G, RFLP (*TaqI*). Primer sequences are available on request. All mice used were treated according to the recommendations of the Insti-

tutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Bisulfite sequencing. After bisulfite treatment (as described in ref. 27), we carried out nested PCR reactions to amplify the following regions for methylation analysis: *Cdkn1c*, bp 34,033–34,328; *Grb10*, bp 65,194–65,398, SNPs at bp 65,297 and bp 65,230 (at both SNPs CD1 = C, JF1 = T); *Snrpn*, bp 67,729–68,105, SNP at bp 67,875 (CD1 = G, JF1 = A); *KvDMR1*, bp 2,542–2,934, SNP at bp 2,883 (CD1 = T, JF1 = G). Primer sequences are available on request. The conditions for first-round PCR were 3 min at 95 °C and then 36 cycles of 45 s at 95 °C, 56 °C and 72 °C. The conditions for second-round PCR were 3 min at 95 °C and then 36 cycles of 45 s at 95 °C, 60 °C and 72 °C. All reactions included 10% dimethylsulfoxide and 1.5 mM MgCl₂. We cloned PCR products from at least two independent reactions and sequenced individual clones from each sample. Bisulfite conversion efficiency ranged from 94.6% to 99.1% for independently treated samples. Between 59% and 81% of individual clones sequenced (range represents independent PCR reactions) showed unique patterns of bisulfite conversion (considering all cytosine residues).

Methylation-sensitive restriction digest at *Cdkn1c*. We first digested genomic DNAs from both mutant and heterozygous E7.5 embryos with the methyl-sensitive restriction enzyme *SmaI* and then carried out PCR analysis of a 1.4-kb segment (containing no additional *SmaI* sites; primer sequences available on request). We then digested the PCR products with *Tsp509I* for RFLP analysis and resolved them on a 0.5% agarose gel (*Tsp509I* polymorphism at position 35,253; CD1 = A, JF1 = G).

Accession numbers. *Dlk1*, AB047760; *Grb10*, NM010345; *H19*, NM023123; *Igf2*, 6754309; *Igf2r*, 20897376; *Kcnq1*, U70068; *Kcnq1ot1*, AF119385; *Ascl2*, BC019520; *Meg3*, Y13832; *Nnat*, AK003004; *Peg3*, AB003040; *Slc22a11*, 6679179; *Snrpn*, 7705508; *Tssc3*, NM_009434; *Ube3a*, 27804320; *U2af1-rs1*, AF309654; *Plagl1*, AF147785; *Cdkn1c*, AP001293; *Grb10*, AL663087; *Snrpn*, AF332579; *KvDMR1*, AF119385.

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Competing interests statement

The authors declare that they have no competing financial interests.

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