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Imprinting along the *Kcnq1* domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes

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Imprinted genes are clustered in domains, and their allelic repression is mediated by imprinting control regions[1–3]. These imprinting control regions are marked by DNA methylation, which is essential to maintain imprinting in the embryo[4]. To explore how imprinting is regulated in placenta, we studied the *Kcnq1* domain on mouse distal chromosome 7. This large domain is controlled by an intronic imprinting control region[5,6] and comprises multiple genes that are imprinted in placenta, without the involvement of promoter DNA methylation[7–10]. We found that the paternal repression along the domain involves acquisition of trimethylation at Lys27 and dimethylation at Lys9 of histone H3.

Eed-Ezh2 Polycomb complexes are recruited to the paternal chromosome and potentially regulate its repressive histone methylation. Studies on embryonic stem cells and early embryos support our proposal that chromatin repression is established early in development and is maintained in the placenta. In the embryo, however, imprinting is stably maintained only at genes that have promoter DNA methylation. These data underscore the importance of histone methylation in placental imprinting and identify mechanistic similarities with X-chromosome inactivation in extraembryonic tissues, suggesting that the two epigenetic mechanisms are evolutionarily linked.

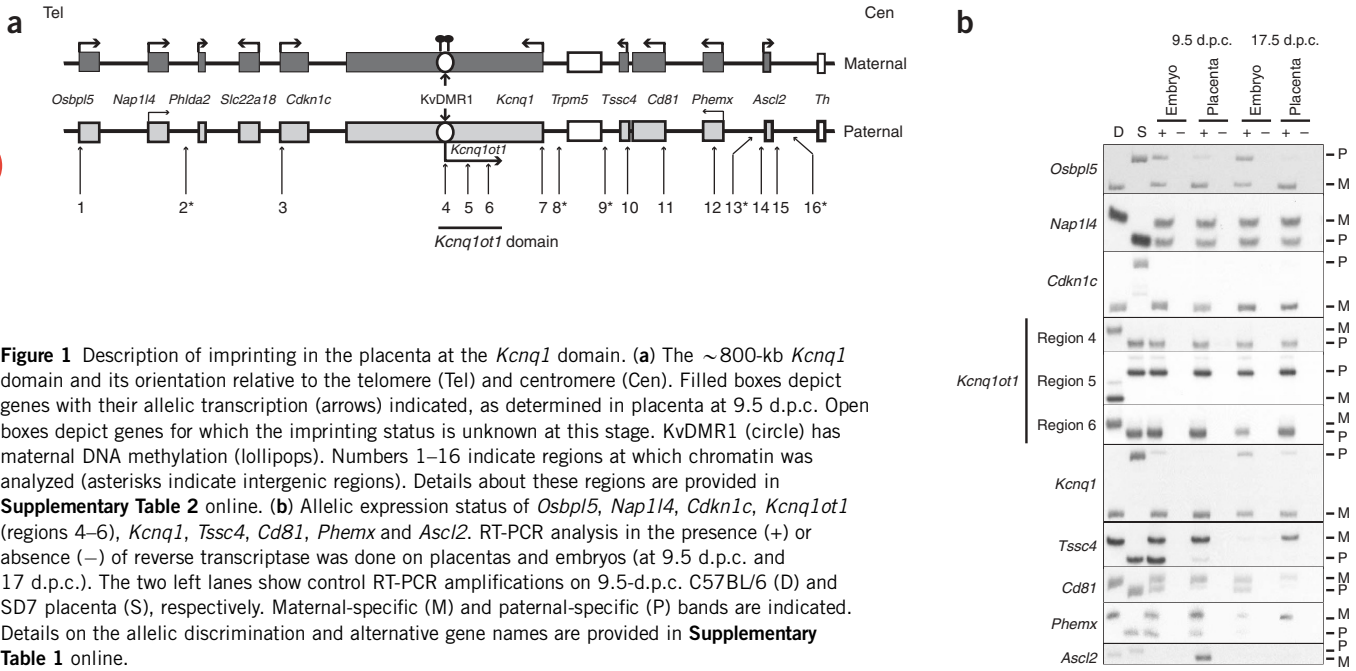

Figure 1 Description of imprinting in the placenta at the *Kcnq1* domain. (**a**) The ~800-kb *Kcnq1* domain and its orientation relative to the telomere (Tel) and centromere (Cen). Filled boxes depict genes with their allelic transcription (arrows) indicated, as determined in placenta at 9.5 d.p.c. Open boxes depict genes for which the imprinting status is unknown at this stage. KvDMR1 (circle) has maternal DNA methylation (lollipops). Numbers 1–16 indicate regions at which chromatin was analyzed (asterisks indicate intergenic regions). Details about these regions are provided in **Supplementary Table 2** online. (**b**) Allelic expression status of *Osbpl5*, *Nap1l4*, *Cdkn1c*, *Kcnq1ot1* (regions 4–6), *Kcnq1*, *Tssc4*, *Cd81*, *Phemx* and *Ascl2*. RT-PCR analysis in the presence (+) or absence (−) of reverse transcriptase was done on placentas and embryos (at 9.5 d.p.c. and 17 d.p.c.). The two left lanes show control RT-PCR amplifications on 9.5-d.p.c. C57BL/6 (D) and SD7 placenta (S), respectively. Maternal-specific (M) and paternal-specific (P) bands are indicated. Details on the allelic discrimination and alternative gene names are provided in **Supplementary Table 1** online.

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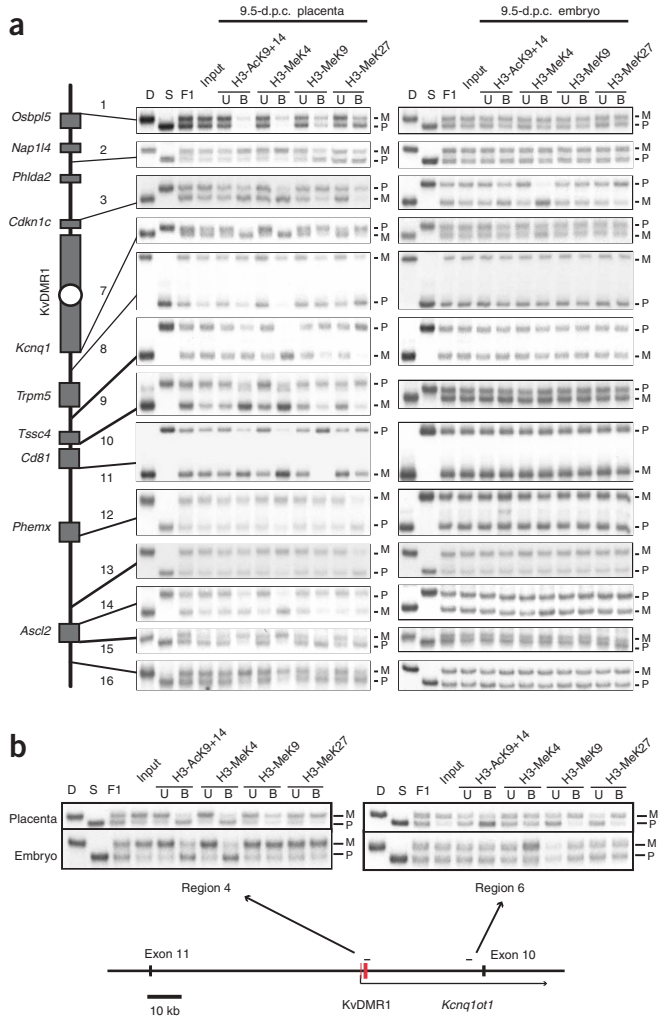

Figure 2 The *Kcnq1* domain is marked by paternal methylation at H3-Lys9 and H3-Lys27 in placenta, but not in the embryo. (**a**) Chromatin from 9.5-d.p.c. placentas (left) and embryos (right) was precipitated with antisera against acetylated H3-Lys9 and H3-Lys14 (H3-AcK9+14), dimethylated H3-Lys4 (H3-MeK4), dimethylated H3-Lys9 (H3-MeK9) and trimethylated H3-Lys27 (H3-MeK27). Sequence information on the regions analyzed is provided in **Supplementary Table 2** online. For each ChIP, PCR was done on the antibody-bound (B) and unbound (U) fractions, followed by SSCP analysis. Maternal-specific (M) and paternal-specific (P) bands are indicated. In all panels, the first four lanes show control amplifications from C57BL/6 (D), SD7 (S) and C57BL/6 × SD7 F_1 (F1) genomic DNAs and from the chromatin used for precipitation (Input). In placentas, the relative enrichment of the maternal allele in the precipitation of methylated H3-Lys4 was 10, 4.7, 4.3, 12, 6.0, 18, 6.0, 13, 17, 5.7, 7.0, 5.5 and 3.3 at regions 1–3 and 7–16, respectively. The paternal relative enrichment of methylated H3-Lys27 in placentas was 1.8, 1.7, 2.6, 5.0, 4.0, 7.2, 3.6, 1.5, 1.6, 1.9, 1.4, 3.4 and 1.8 at regions 1–3 and 7–16, respectively. In embryos, the maternal relative enrichment of methylated H3-Lys4 was >1.5 only at regions 3 (12) and 7 (1.8). The paternal relative enrichment of methylated H3-Lys27 was 3.0 and 1.3 at regions 3 and 7, respectively. (**b**) Allele-specific chromatin organization at the *Kcnq1ot1* domain. ChIP was done on placentas and embryos as in **a**. Maternal-specific (M) and paternal-specific (P) bands are indicated. The first four lanes show amplifications from C57BL/6 (D), SD7 (S) and C57BL/6 × SD7 F_1 (F1) genomic DNAs and the input chromatin used (Input). The map shows intron 10 of *Kcnq1* with the position of the KvDMR1 CpG island (in red) and regions 4 and 6 (small bars).

Paternal repression along the *Kcnq1* domain (**Fig. 1a**) is controlled by the KvDMR1 (ref. 5), a CpG island in *Kcnq1* that carries DNA methylation on the maternal allele[6,9,11]. The unmethylated paternal allele produces a noncoding RNA, *Kcnq1ot1* (also called *Lit1*)[5,6]. How KvDMR1 mediates paternal repression is unknown. *Ascl2* and other genes in the domain are imprinted in placenta only[7–12]. Notably, *Ascl2* imprinting is maintained in the absence of DNA methylation[7,8]. Its promoter is unmethylated in midgestation placenta and embryo; this was also reported for *Osbpl5* (also called *Obph1*), *Phlda2* (also called *Ipl*), *Kcnq1* (also called *Kvlqt1*), *Nap1l4* and *Tssc4* (refs. 8–10), suggesting that a DNA methylation-independent mechanism is involved.

To test this hypothesis, we first analyzed the imprinting status of genes along the domain at 9.5 and 17.5 d post coitum (d.p.c.). Almost all the genes (*Osbpl5*, *Tssc4*, *Cd81*, *Phemx* (also called *Tssc6*) and *Ascl2*) that we analyzed were paternally repressed in placenta, but not in the embryo, at 9.5 d.p.c. (**Fig. 1b**). *Nap1l4* was paternally repressed in placenta[11] and biallelically expressed in the embryo. *Kcnq1* was imprinted in both placenta and embryo at 9.5 d.p.c. and in the placenta but not in the embryo at 17.5 d.p.c. *Cdkn1c* was imprinted in placenta and embryo at both stages. Paternal expression of *Kcnq1ot1* was detected at KvDMR1 (region 4) and 23 kb (region 5) and 32 kb (region 6) farther downstream, both in placenta and embryo. Therefore, *Kcnq1ot1* alone can not explain the placenta-specific imprinting.

We next analyzed chromatin on both sides of the *Kcnq1ot1*-expressing domain (regions 1–3 and 7–16; **Fig. 1a**). Our analysis focused on trimethylation at Lys27 on histone H3 (H3-Lys27) and dimethylation at Lys9 on histone H3 (H3-Lys9) as markers of repressive chromatin at domains other than pericentric heterochromatin[13,14], and on acetylation at Lys9 and Lys14 on histone H3 (H3-Lys9 and H3-Lys14) and dimethylation at Lys4 on histone H3 (H3-Lys4) as markers of active chromatin[15]. We carried out chromatin immunoprecipitation (ChIP) on unfixed chromatin from 9.5-d.p.c. placentas and embryos (C57BL/6 × *Mus spretus* F_1 for distal chromosome 7). In placenta, methylated H3-Lys4 and acetylated H3-Lys9 and H3-Lys14 were precipitated on the maternal chromosome predominantly, at all regions (**Fig. 2a**). Methylated H3-Lys27 and H3-Lys9 were mainly precipitated on the repressed paternal chromosome. In 9.5-d.p.c. embryos, in contrast, there was no allelic precipitation along the domain, except for the promoters of *Cdkn1c* and *Kcnq1*, which are paternally repressed at this stage. *Cdkn1c* had paternal methylation at H3-Lys27 and maternal methylation at H3-Lys4 and acetylation at H3-Lys9 and H3-Lys14. *Kcnq1* also had maternal methylation at H3-Lys4, but no allelic methylation at H3-Lys27 or H3-Lys9.

At the *Kcnq1ot1* domain (**Fig. 2b**) we detected the opposite allelic pattern, with maternal methylation at H3-Lys27 and H3-Lys9 and paternal methylation at H3-Lys4 and acetylation at H3-Lys9 and H3-Lys14. This pattern was present at KvDMR1 (region 4) and at regions 5 and 6 in placenta, but only at KvDMR1 in the embryo.

In placenta, levels of methylated H3-Lys27 and H3-Lys9, quantified by real-time PCR on precipitated chromatin (**Supplementary Fig. 1** online), varied between different regions and were highest at *Ascl2* and region 8. Levels of methylated H3-Lys4 (marking the active maternal chromosome) were extremely high (20–40%) at all the regions that we analyzed in placenta. In the embryo, the levels of methylated H3-Lys4 were highest at *Tssc4*, which is biallelically expressed.

Our data thus far indicated that repressive histone methylation correlates with imprinting of genes along the domain. To determine

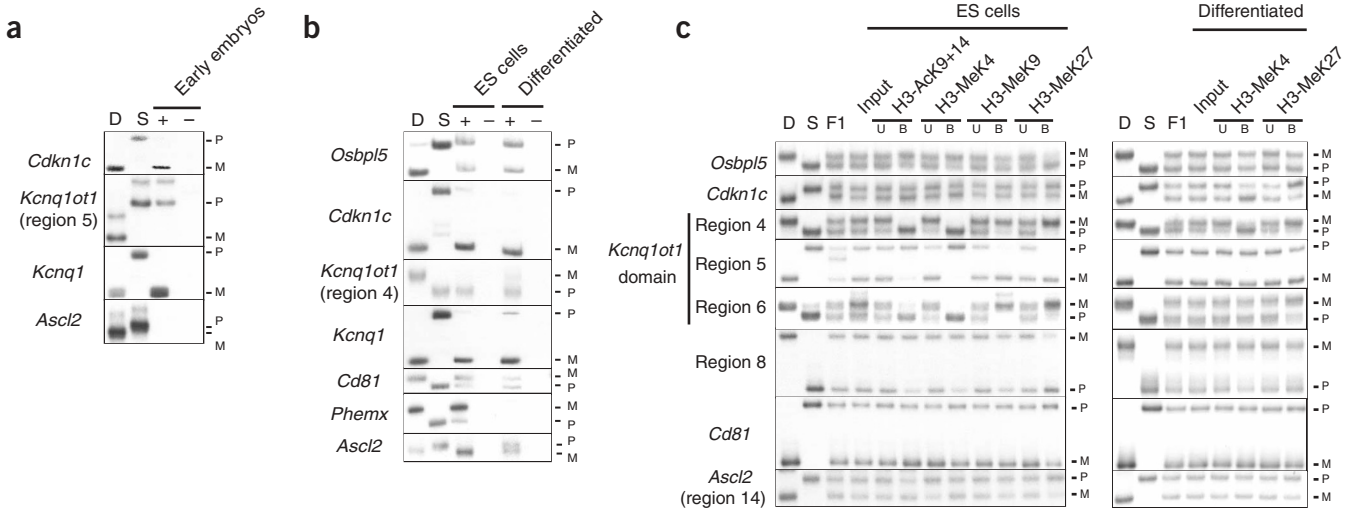

Figure 3 The *Kcnq1* domain is imprinted in ES cells and early embryos. (**a**) Imprinted gene expression in C57BL/6 × SD7 F_1 morulas and early blastocysts. RT-PCR was done as in **Figure 1b** using the same control cDNAs. Maternal-specific (M) and paternal-specific (P) bands are indicated. (**b**) Imprinted gene expression in ES cells and differentiated ES cells (line SF1-1). RT-PCR analysis was done as in **Figure 1b** using the same control cDNAs. Maternal-specific (M) and paternal-specific (P) bands are indicated. (**c**) ChIP on undifferentiated (left panels) and differentiated (right panels) ES cells (line SF1-1). Maternal-specific (M) and paternal-specific (P) are indicated. In all panels, the first four lanes show control amplifications from C57BL/6 (D), SD7 (S) and C57BL/6 × SD7 F_1 (F1) genomic DNAs and from the chromatin used for precipitation (Input). B, antibody-bound fraction; U, unbound fraction; H3-AcK9+14, acetylated H3-Lys9 and H3-Lys14; H3-MeK4, dimethylated H3-Lys4; H3-MeK9, dimethylated H3-Lys9; H3-MeK27, trimethylated H3-Lys27.

when the paternal silencing is established, we analyzed morulas and early blastocysts (**Fig. 3a**). *Kcnq1ot1* was paternally expressed, whereas *Cdkn1c* and *Kcnq1* were maternally expressed. Because the expression of *Ascl2* and other genes was too low for allelic analysis, we also studied two embryonic stem (ES) cell lines with unaltered differential DNA methylation at KvDMR1 (ref. 11; data not shown) and other imprinting control regions[16]. In ES cells, *Kcnq1ot1* was paternally expressed, and *Cdkn1c*, *Kcnq1*, *Cd81*, *Phemx* and *Ascl2* were maternally expressed (**Fig. 3b**). As in placenta, paternal silencing correlated with methylation at H3-Lys27 (**Fig. 3c**). There was no paternal methylation at H3-Lys9 along the domain, indicating that methylation at H3-Lys9 is regulated differently than methylation of H3-Lys27. *Osbpl5* had paternal methylation at H3-Lys27, but this was insufficient to repress its promoter, as we detected biallelic expression. As in placenta, the *Kcnq1ot1* domain had maternal methylation at H3-Lys27 and H3-Lys9. We also analyzed undifferentiated ES cells trimethylation at H3-Lys9 and at H3-Lys4. We detected trimethylation at H3-Lys9 on the maternal chromosome at KvDMR1 and on the paternal allele of *Cdkn1c*. We observed paternal enrichment of trimethylation at H3Lys4 at KvDMR1 and regions 5 and 6 (data not shown).

As a model for embryonic differentiation, we differentiated ES cells *in vitro*. Notably, at *Ascl2*, *Cd81*, *Osbpl5*, region 8 and the *Kcnq1ot1* domain (regions 5 and 6), the allelic methylation at H3-Lys27 and H3-Lys4 was lost during differentiation (**Fig. 3c**), whereas at KvDMR1 (region 4) and *Cdkn1c*, the allelic methylation at H3-Lys27 and H3-Lys4 was maintained. Concordantly, at *Kcnq1ot1* and *Cdkn1c*, imprinting was fully maintained, whereas at *Cd81*, *Ascl2* and *Kcnq1*, we observed a partial loss of imprinting (**Fig. 3b**). Furthermore, ChIP showed that levels of histone acetylation and methylation in trophectoderm stem cells were similar to those in ES cells at the regions analyzed (**Supplementary Fig. 2** online). This suggests that chromatin along the domain is similarly organized in the two types of stem cells derived from blastocysts.

Enhancer-of-zeste homolog-2 (Ezh2) is a histone methyltransferase with specificity for H3-Lys27 (refs. 14,17,18). It forms part of a Polycomb repressive complex, PRC2/3, that also includes suppressor-of-zeste-12 (Suz12) and Eed (embryonic ectoderm development)[18,19]. ChIP on fixed chromatin showed that Ezh2, Eed and Suz12 associate with the paternal allele at *Ascl2* (regions 14 and 15), *Cdkn1c*, *Cd81* and region 8 (**Fig. 4**). Levels of Ezh2 and Eed precipitated at *Cd81* and region 8 were close to background levels, suggesting that Eed-Ezh2

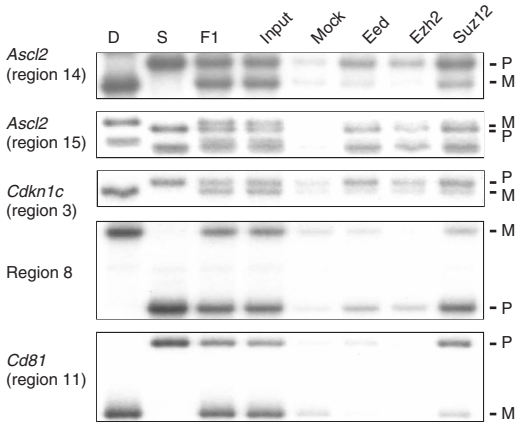

Figure 4 Polycomb group proteins Ezh2, Suz12 and Eed are associated with the repressed paternal chromosome. After ChIP on fixed ES cell chromatin, PCR was done using equal amounts of template DNA extracted from the antibody-bound fractions. Results are shown for *Ascl2* (regions 14 and 15), *Cdkn1c*, intergenic region 8 and *Cd81*. The first four lanes show control amplifications from C57BL/6 (D), SD7 (S) and C57BL/6 × SD7 F_1 (F1) genomic DNAs and from the chromatin used for precipitation (Input). Mock indicates a control precipitation with a neutral, unrelated IgG antiserum. Maternal-specific (M) and paternal-specific (P) bands are indicated.

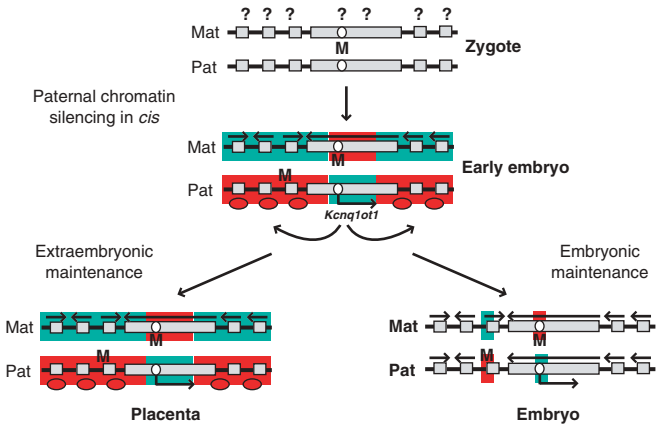

Figure 5 A working model for imprinting along the *Kcnq1* domain. In the zygote, KvDMR1 (open circles) is marked by maternally-derived DNA methylation (M). Early in development, KvDMR1 brings about repressive chromatin (in red) along the paternal chromosome characterized by methylation at H3-Lys27. This silencing process potentially involves the noncoding *Kcnq1ot1* RNA and repressive complexes (red ovals) including Eed-Ezh2 complexes. The repressive chromatin inhibits transcription at most genes. Along the maternal chromosome, on both sides of the *Kcnq1ot1* domain there is transcriptionally permissive chromatin (in green) characterized by methylation at H3-Lys4. Possibly, this originates from the female germ line. The silenced paternal chromatin is stably maintained in placenta. In the developing embryo, in contrast, there is gradual loss of the repressive chromatin at most regions along the domain. As at KvDMR1, chromatin repression is faithfully maintained throughout embryonic development at one gene (*Cdkn1c*) only, presumably because its promoter acquires DNA methylation on the repressed allele early in development.

complexes associate preferentially with certain regions. This agrees with the genetic demonstration that *Eed* is essential for imprinting at *Cdkn1c* and *Ascl2* (ref. 20). We detected no association of Ezh2, Suz12 or Eed at KvDMR1 (data not shown), possibly because of inaccessible chromatin conformation or because other complexes are involved in its maternal methylation at H3-Lys27 and H3-Lys9.

Our data lead to a working model for imprinting along the *Kcnq1* domain (**Fig. 5**). KvDMR1 has maternal DNA methylation. Histone methylation could be linked to its somatic maintenance[21]. In ES cells and placenta, we detected a domain of maternal methylation at H3-Lys27 and H3-Lys9 comprising KvDMR1. In the embryo, maternal methylation at H3-Lys27 and H3-Lys9 is confined to KvDMR1. On the paternal chromosome, possibly involving the noncoding *Kcnq1ot1* RNA, KvDMR1 induces repressed chromatin in *cis* characterized by methylation at H3-Lys27 and H3-Lys9 (and hypomethylation at H3-Lys4 and hypoacetylation of H3-Lys9 and H3-Lys14; this study and ref. 22). The repressive chromatin is established early in development, at promoters, genes and intergenic regions. This involves recruitment of Eed-Ezh2 complexes. Chromatin repression is maintained in the trophoblast, presumably because Eed-Ezh2 and other repressive complexes are expressed sufficiently[17,23]. Paternal repression is gradually lost in differentiating embryos, however, despite continued *Kcnq1ot1* expression. It is unclear why there is acquisition of DNA methylation on the repressed allele seemingly along only one promoter or CpG island (*Cdkn1c*[9,22]). Because of this methylation, however, the allelic chromatin repression is stably maintained in the embryo at this gene only, as at KvDMR1.

Future work should determine whether this model is relevant to placental imprinting at other domains as well. For instance, the *Igf2r* domain on chromosome 17 comprises several genes that are paternally repressed in the placenta without the involvement of promoter DNA methylation[24]. But Eed does not seem to be involved in *Igf2r* imprinting[20]. Generally, its dependence on histone rather than DNA methylation could explain why imprinting in the placenta is less firmly fixed than in the embryo and is particularly susceptible to *in vitro* culture effects[25]. Finally, our study identifies substantial similarities with imprinted X-chromosome inactivation (of the paternal X), which is established early in development and maintained in extraembryonic tissues, involves acquisition of trimethylation at H3-Lys27 and di-methylation at H3-Lys9, and requires Eed-Ezh2 complexes[17,22,26–30]. Random X inactivation in the embryo proper, however, also involves repressive histone methylation and recruitment of Eed-Ezh2 complexes[23]. The parallels between placental imprinting and imprinted X inactivation suggest that the two epigenetic mechanisms could be evolutionarily linked.

METHODS

Mouse embryos and stem cell lines. We produced placentas and embryos (C57BL/6 × *M. spretus* F_1 for distal chromosome 7) by crossing C57BL/6 females with male SD7 mice (C57BL/6, congenic for distal chromosome 7 from *M. spretus*[16]). The opposite cross generated embryos that were SD7 × C57BL/6 F_1 for distal chromosome 7. Animal husbandry and breeding were licensed by Direction Départementale des Services Vétérinaires. For ChIP and expression studies on ES cells (passages 11–13), we grew lines SF1-1 and SF1-G as described[16]. These lines are of the C57BL/6 × *M. spretus* F_1 genotype[16]. We differentiated them by plating ES cells at low density (100 cells per cm^2) on gelatine-coated plates and then growing them in ES medium[16] without leukemia inhibitory factor. We changed the medium daily throughout 7 d of differentiation, after which expression of *Oct4* was strongly reduced and most cells had morphologically differentiated into parietal endoderm and ectoderm-like cells (data not shown). Trophectoderm stem cell line A2 was a gift from N. Brockdorff (MRC Clinical Sciences Centre, London); these female cells are of the (C57BL/6 × CBA) × PGK F_1 genotype. We obtained preimplantation embryos from natural matings between C57BL/6 × CBA F_1 females and SD7 males. For RT-PCR, we selected 3.5-d.p.c. embryos ($n = 100$) that were at late-morula to early-blastocyst stage.

Analysis of allelic expression. We extracted total RNAs from tissues and cells using Trizol Reagent (Invitrogen). After digestion with RNase-free DNase-I, we generated first-strand cDNA with Superscript-II (Invitrogen) using randomized primers. We used previously published and new single-nucleotide and restriction fragment length polymorphisms between C57BL/6 and *M. spretus* to assay the allelic expression status of genes (**Supplementary Table 1** online). Maternal and paternal RT-PCR products were distinguished by applying single-strand conformation polymorphisms (SSCPs)[21] or by enzymatic digestion of polymorphic restriction sites. For the latter, we used hot-stop PCR to prevent formation of nondigestible heteroduplexes[10]. All RT-PCR amplifications were done in the presence of ^{32}P-dCTP (1% of total dCTP), during all cycles (for SSCP analysis) or during a last cycle of reamplification (hot-stop PCR).

ChIP and PCR-SSCP. We carried out immunoprecipitations in triplicate on carefully dissected 9.5-d.p.c. placentas (to exclude contamination with maternal decidua) and embryos (80–100 for each ChIP assay) and on ES and trophectoderm stem cells. We prepared unfixed chromatin fragments of one to six nucleosomes in length as described[21]. For ChIP, we used antisera directed against dimethylated H3-Lys4 (Upstate Biotechnology), trimethylated H3-Lys4 (Abcam), dimethylated H3-Lys9 (Upstate), acetylated H3-Lys9 (Upstate) and acetylated H3-Lys9 and H3-Lys14 (Upstate), and an affinity-purified antiserum against trimethylated H3-Lys27 (ref. 27). ChIP on ES cells also included antisera against trimethylated H3-Lys4 (Abcam) and trimethylated H3-Lys9 (ref. 27). In the antibody-bound and unbound fractions, the parental alleles were distinguished by radioactive PCR followed by electrophoretic detection of SSCP polymorphisms in the amplification products (for sequence and oligonucleotide information, see **Supplementary Table 2** online). We determined the

relative intensities of the maternal and paternal bands using Aida-2 software. We analyzed precipitated chromatin (DNA) fractions by real-time PCR as well, using Quantitect SYBR Green PCR kit (Qiagen). Each PCR was run in triplicate, and results are presented as the percentage of precipitation, obtained by dividing the average value of the precipitated material by the average value of the corresponding input chromatin. We carried out ChIP on fixed chromatin by cross-linking semiconfluently-grown ES cells with 1% formaldehyde (15 min at room temperature). We stopped cross-linking by adding glycine (to 125 mM) and washed cells three times in phosphate-buffered saline (2×), buffer I (100 mM Tris-HCl (pH 8.0) and 10 mM dithiothreitol), buffer II (5 mM PIPES buffer (pH 8.0), 85 mM KCl, 0.5% Nonidet-P40 and 1× protease inhibitor from Roche) and buffer III (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0) and 1× protease inhibitor from Roche), respectively. Subsequent sonication yielded chromatin fragments of 0.1–1 kb in length. We precleared chromatin for 4 h with protein-A sepharose beads (Amersham BioScience) and sheared salmon sperm DNA, and carried out ChIP overnight at 4 °C in 0.01% SDS, 1% Triton-X100, 1.2 mM EDTA, 16 mM Tris-HCl (pH 8.0), 165 mM NaCl and 1× protease inhibitor from Roche. We used affinity-purified rabbit antisera against Eed, Ezh2 and Suz12 (refs. 14,19). We obtained comparable results (not shown) with commercial antisera (Abcam antiserum to Ezh2 and Upstate antiserum to Eed). As a negative control (mock precipitation), we used a rabbit antiserum to chicken IgG (Sigma). After precipitation, we incubated samples for 4 h with protein-A sepharose and sheared salmon sperm DNA and, after centrifugation, washed the protein-A sepharose beads three times with 0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1x protease inhibitor from Roche; three times with 0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl and 1× protease inhibitor from Roche; and three times with 1 mM EDTA and 10 mM Tris-HCl (pH 8.0). We eluted samples with 1% SDS and 100 mM $NaHCO_3$ at 50 °C for 20 min. Cross-links were reversed with 200 mM NaCl at 65 °C overnight. We carried out PCR-SSCP on extracted DNAs, in precisely the same way as for ChIP on unfixed chromatin.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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