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The genome-defence gene *Tex19.1* suppresses *LINE-1* retrotransposons in the placenta and prevents intra-uterine growth retardation in mice

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DNA methylation plays an important role in suppressing retrotransposon activity in mammalian genomes, yet there are stages of mammalian development where global hypomethylation puts the genome at risk of retrotransposition-mediated genetic instability. Hypomethylated primordial germ cells appear to limit this risk by expressing a cohort of retrotransposon-suppressing genome-defence genes whose silencing depends on promoter DNA methylation. Here, we investigate whether similar mechanisms operate in hypomethylated trophectoderm-derived components of the mammalian placenta to couple expression of genome-defence genes to the potential for retrotransposon activity. We show that the hypomethylated state of the mouse placenta results in activation of only one of the hypomethylation-sensitive germline genome-defence genes: *Tex19.1*. *Tex19.1* appears to play an important role in placenta function as *Tex19.1*$^{-/-}$ mouse embryos exhibit intra-uterine growth retardation and have small placentas due to a reduction in the number of spongiotrophoblast, glycogen trophoblast and sinusoidal trophoblast giant cells. Furthermore, we show that retrotransposon mRNAs are derepressed in *Tex19.1*$^{-/-}$ placentas and that protein encoded by the *LINE-1* retrotransposon is upregulated in hypomethylated trophectoderm-derived cells that normally express *Tex19.1*. This study suggests that post-transcriptional genome-defence mechanisms are operating in the placenta to protect the hypomethylated cells in this tissue from retrotransposons and suggests that imbalances between retrotransposon activity and genome-defence mechanisms could contribute to placenta dysfunction and disease.

INTRODUCTION

Retrotransposons are mobile genetic elements that amplify and integrate into new genomic locations through RNA intermediates. These parasitic DNA sequences are highly abundant in mammalian DNA and typically account for 40–70% of sequenced mammalian genomes (1–4). To limit the activity of these elements, mammals possess numerous genome-defence mechanisms that can suppress retrotransposon activity at transcriptional and post-transcriptional levels in different cell types (5–7). DNA methylation, histone modification and polycomb-mediated repression have all been implicated in transcriptional repression of retrotransposons in mouse embryonic stem (ES) cells (8–10). DNA methylation also appears to play an important role in repressing retrotransposons in differentiated embryonic tissue, particularly in the repression of intracisternal A particle (*IAP*) retrotransposons (9,11,12). Indeed, transcriptional repression of retrotransposons has been proposed to be the primary function of DNA methylation in mammalian genomes (13).

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At a genome-wide level, DNA methylation is high in most differentiating embryonic tissues, with the notable exception of the developing germline (14). Primordial germ cells undergo extensive loss of DNA methylation during their development as part of a wider epigenetic reprogramming event that resets imprints and other epigenetic marks (15,16). However, global DNA hypomethylation has the potential to induce retrotransposon activity and, therefore, genome instability, in the developing germline (17). We have previously shown that DNA hypomethylation in primordial germ cells induces expression of a group of hypomethylation-sensitive genome-defence genes (*Tex19.1*, *Piwil2*, *Mov10l1*, *Dazl* and *Asz1*) that function to protect the germline DNA from retrotransposon activity (17). Mutations in *Tex19.1*, *Piwil2*, *Mov10l1* or *Asz1* result in derepression of retrotransposons in testicular germ cells and male sterility (18–22), whereas *Dazl* is required for efficient translation of retrotransposon-suppressing genes *Tex19.1* and *Mvh* (23,24). The DNA methylation-dependent silencing of these genome-defence genes appears to represent a developmental mechanism that helps to suppress retrotransposon activity during periods of global DNA hypomethylation and epigenetic reprogramming in the developing germline when the potential for retrotransposon activation is high (17).

Like primordial germ cells, the placenta is globally hypomethylated relative to other mouse tissues, and the hypomethylated state of the placenta extends to retrotransposons (14,25–27). Approximately, 40–50% of cytosines in a CpG context are methylated within long terminal repeat (LTR), long interspersed nuclear element (LINE) and short interspersed nuclear element (SINE) classes of retrotransposon in mouse placentas, compared with ~75–80% in the embryo (27). Specific types of retrotransposon, such as *IAP* and *LINE-1* elements, have also been shown to be hypomethylated in the placenta relative to the embryo (25,28). The hypomethylated epigenetic state of placental DNA could potentially generate a transcriptionally permissive environment for retrotransposon expression (25), and numerous studies report expression of retrotransposons in this tissue (reviewed in 29). However, it is not known whether mechanisms operate in the placenta, as they do in the germline, to couple transcriptional activation of hypomethylation-sensitive genome-defence genes to the potential for retrotransposon activation (17).

In this study, we show that the hypomethylated epigenetic state of the placenta is associated with expression of specific retrotransposons, but does not result in widespread activation of retrotransposon expression in general. We find that only one of the hypomethylation-sensitive germline genome-defence genes, *Tex19.1*, is hypomethylated and expressed in mouse placenta and that *Tex19.1*$^{-/-}$ embryos exhibit intrauterine growth retardation. *Tex19.1*$^{-/-}$ placentas are small with thinner junctional zones and a reduced abundance of multiple trophectoderm-derived cell types when compared with littermate controls. Microarray expression profiling of *Tex19.1*$^{-/-}$ placentas shows that loss of *Tex19.1* results in increased expression of *LINE-1* retrotransposons in this tissue, and immunohistochemistry suggests that *LINE-1* derepression is occurring in the hypomethylated trophectoderm-derived cell types that normally express *Tex19.1*. Our data extend to the placenta the associations between DNA hypomethylation, *Tex19.1* and post-transcriptional genome-defence against retrotransposons that we have previously described in the developing germline (17) and suggest that imbalances between retrotransposon activity and host genome-defence mechanisms might be associated with impaired placenta function in mammals.

RESULTS

Retrotransposon expression in the mouse placenta

DNA methylation is associated with transcriptional repression of retrotransposons in embryos and embryo-derived cell lines (9,11,12,30,31). Therefore, we investigated whether the hypomethylated state of the mouse placenta (25–27) permits widespread retrotransposon expression in this tissue. We used our recently developed microarray repeat-annotation methodology (10) to extract information about placental expression of hundreds of different types of retrotransposon from mouse multiple-tissue gene expression data (32). Repeat annotation of gene expression microarray data from E16 placentas and E16 embryos from within this dataset showed that, in general, retrotransposon classes (LTR, LINE and SINE) of repeat probes appeared to be no more differentially expressed between placenta and embryo than non-repeat probes (Fig. 1A and B): although 51% of non-repeat probes are differentially expressed ($P < 0.01$) between placenta and embryo, only 39% of retrotransposon probes are differentially expressed ($P < 0.01$) between these tissues. Furthermore, similar numbers of retrotransposon probes are upregulated and downregulated in the placenta [1850 retrotransposon probes significantly ($P < 0.01$) upregulated, 1740 retrotransposon probes significantly ($P < 0.01$) downregulated, χ^2-test $P = 0.2$], suggesting that the placenta is not any more permissive than embryonic tissues for retrotransposon derepression, despite its hypomethylated epigenetic state. Multiple families of LTR retrotransposons (endogenous retroviruses) are reported to be hypomethylated in placenta (~40% DNA methylation) relative to the embryo (~75% DNA methylation) (27). Although LTR retrotransposons in general do not appear to be any more differentially expressed between placenta and embryo than non-repeat probes, we also investigated whether the five LTR retrotransposons that are upregulated in mouse ES cells in response to hypomethylation (9) might be specifically upregulated in the placenta. Interestingly, this group of methylation-sensitive LTR retrotransposons showed divergent behaviour in the placenta, with expression of the *IAPEz* subclass of *IAP* elements increased relative to embryonic tissues, *MMERGLN* and some *RLTR1B* elements decreased and *RLTR45* and some *RLTR1B* elements not changing (Fig. 1C). Thus, there does not appear to be a strong correlation between the retrotransposons upregulated in mouse ES cells in response to DNA hypomethylation and retrotransposons expressed in the hypomethylated placenta.

IAP and *LINE-1* elements are both strongly repressed by DNA methylation in the developing germline (11,33) and have each been shown to be hypomethylated in mouse placenta (25,28). Therefore, we examined whether the hypomethylated status of these elements in the placenta would

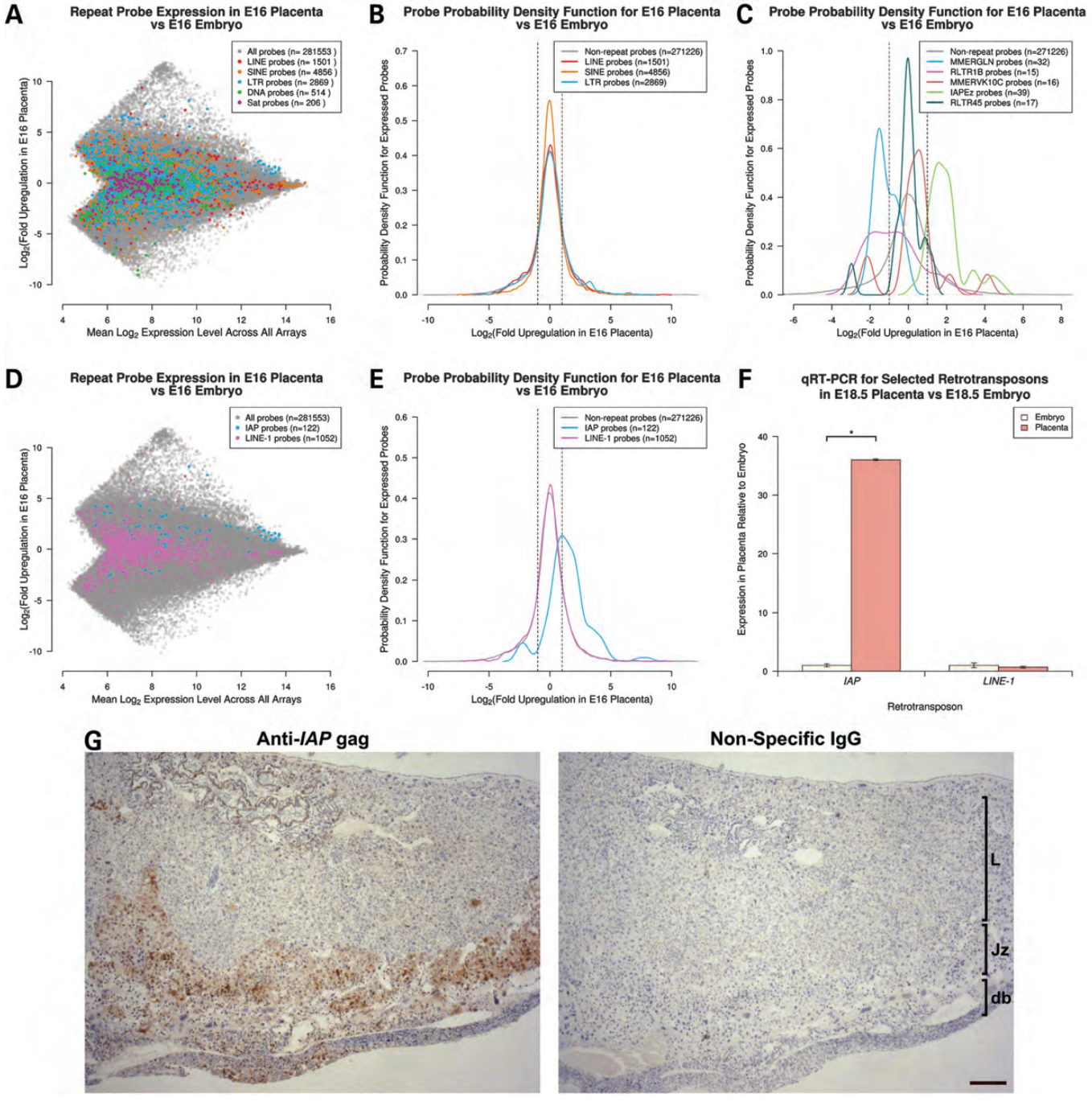

Figure 1. *IAP* retrotransposons are highly expressed in the placenta. (**A**–**E**) Repeat probe expression in gene expression microarray data from E16 placenta and E16 embryo. Repetitive element probes are coloured as shown in the legend. $n = 3$. (A and D) MA-plot showing the fold upregulation in the placenta versus the average expression in the dataset for each probe. (B, C and E) Probability density function plots showing the relative likelihood that any individual probe is upregulated by the indicated amount in the placenta. Dotted lines mark 2-fold changes in expression. (**F**) qRT-PCR analysis of *IAP* and *LINE-1* retrotransposon expression in placenta and embryo at E18.5. Error bars represent standard error, $n = 2$, asterisks indicate $P < 0.01$ (*t*-test). (**G**) Immunohistochemistry (brown precipitate) for *IAP* gag protein in E18.5 placenta. Sections were counterstained with haematoxylin, and non-specific IgG staining is shown as a negative control. Approximate positions of the decidua basalis (db), junctional zone (Jz) and labyrinth (L) are indicated. Scale bar, 500 μm.

correlate with their increased expression in this tissue. Interestingly, although *LINE-1* elements do not show increased expression in the placenta, *IAP* elements (endogenous retrovirus type K family of LTR retrotransposons) do (Fig. 1D and E). Consistent with previous observations using hypomethylated ES cells (9,10), some *IAP* probes were more strongly upregulated than others in the hypomethylated placenta. The bulk *IAP* probe population is upregulated around 2-fold in the placenta; however, some groups of *IAP* probes are upregulated more than 10-fold (Fig. 1E). Quantitative reverse

transcription-polymerase chain reaction (qRT-PCR) for *IAP* and *LINE-1* expression in embryonic and placental tissue confirmed that *IAP* elements are upregulated in the placenta relative to the embryo, whereas *LINE-1* elements are expressed at a similar level in these tissues (Fig. 1F). Thus, expression of *IAP* elements is associated with their hypomethylated state in the placenta, but expression of *LINE-1* elements is not.

The placenta contains cells derived from the mother, and from the extraembryonic mesoderm and the trophectoderm of the embryo, but only trophectoderm-derived cells are expected to be hypomethylated (25,34,35). The trophectoderm-derived cells contribute to the decidua basalis, junctional zone and labyrinth layers of the mature placenta (34). To determine whether the hypomethylated trophectoderm-derived cell types in the placenta are responsible for the *IAP* expression in this tissue, we performed immunohistochemistry for *IAP* gag protein. Anti-*IAP* gag immunostaining showed that parietal trophoblast giant cells in the decidua basalis, and spongiotrophoblast cells in the junctional zone of the placenta, are expressing *IAP* (Fig. 1G). Low-level expression of *IAP* gag protein is also detectable in sinusoidal trophoblast giant cells in the labyrinth layer of the placenta (Fig. 1G). The distribution of *IAP* gag-expressing cells in the placenta correlates well with the distribution of trophectoderm-derived cells (34). Thus, as proposed in the original study on DNA methylation at repetitive sequences in extraembryonic tissues (25), the hypomethylated state of the placenta does appear to allow expression of certain retrotransposons such as *IAP*. However, hypomethylation does not appear to cause widespread upregulation of retrotransposon expression in general in this tissue. This suggests that mechanisms other than DNA methylation are contributing to transcriptional and/or post-transcriptional repression of *LINE-1* and other retrotransposons in the placenta.

DNA hypomethylation in the placenta is associated with expression of the hypomethylation-sensitive genome-defence gene *Tex19.1*

We have recently shown that expression of a cohort of germline genome-defence genes is activated by DNA hypomethylation in primordial germ cells as part of a mechanism that can protect germline DNA from the mutagenic activity of retrotransposons (17). The hypomethylated epigenetic state of placental DNA affects gene promoters in addition to retrotransposons (27); therefore, we investigated whether this cohort of hypomethylation-sensitive germline genome-defence genes is also expressed in the hypomethylated placenta. *Tex19.1* has been previously reported to be expressed in extraembryonic tissues (17,36), but analysis of multiple-tissue microarray data (32) suggests that no other known member of this group of genome-defence genes is expressed in the placenta (Fig. 2A). We verified by qRT-PCR that *Tex19.1* is the only known hypomethylation-sensitive germline genome-defence gene highly expressed in the placenta (Fig. 2B). The differential expression of the germline genome-defence genes in the placenta suggests that *Tex19.1* may be functioning independently of the remaining germline genome-defence genes in this tissue.

We next tested whether the differential expression of the genome-defence genes reflects differences in promoter DNA methylation. Bisulphite sequencing of placental and embryonic DNA showed that *Tex19.1* is more extensively hypomethylated in placental DNA than the other germline genome-defence genes (Fig. 2C). Interestingly, individual clones in the *Tex19.1* bisulphite sequencing data were either highly methylated or unmethylated suggesting that two epigenetically distinct populations of *Tex19.1* DNA molecules are present in the placenta. Furthermore, the proportion of hypomethylated *Tex19.1* clones (~60%) correlates reasonably well with the contribution of hypomethylated trophectoderm-derived cells in mid-gestation placentas (35). In contrast to *Tex19.1*, *Dazl* is highly methylated in both placenta and embryo, and although there is a small drop in methylation levels at *Mov10l1* and *Asz1* promoters in the placenta relative to the embryo, these genes are each highly methylated in both tissues (Fig. 2C). A small number of highly hypomethylated *Asz1* clones are present in the placenta samples (Fig. 2C), which could reflect some *Asz1* expression in a small proportion of cells in the placenta (Fig. 2B). *Piwil2* is not as highly methylated as any of the other genome defence genes in the embryo and also appears to be less methylated in the placenta (Fig. 2C). However, the placental hypomethylation of *Piwil2* is not as extensive as *Tex19.1*. The absence of robust *Piwil2* expression in the placenta (Fig. 2A and B) could reflect the activity of the residual ~40% methylation at this promoter, or the DNA methylation-independent component of *Piwil2* repression that is evident upon differentiation of *Dnmt3a*$^{-/-}$ *Dnmt3b*$^{-/-}$ ES cells (17). Regardless, with the exception of *Tex19.1*, the germline genome-defence genes do not appear to be as sensitive to placental DNA hypomethylation as they are to the DNA hypomethylation event that occurs in the developing germline (17). Thus, any methylation-sensitive feedback loop to protect the placenta from retrotransposons either involves only *Tex19.1* or a completely different set of genes altogether.

Dazl has been reported to be required for efficient translation of *Tex19.1* mRNA in germ cells (24); therefore, the absence of *Dazl* expression in the placenta (Fig. 2A and B) could impair TEX19.1 protein expression, despite the presence of *Tex19.1* mRNA in this tissue. Anti-TEX19.1 immunohistochemistry in E18.5 (Fig. 2D) and E9.5 (Supplementary Material, Fig. S1) placentas suggests that TEX19.1 protein is present in the placenta, despite the absence of *Dazl* expression. The anti-TEX19.1 immunostaining appears to be specific as it is not present in *Tex19.1*$^{-/-}$ placentas (Fig. 2D). In wild-type embryos, anti-TEX19.1 immunostaining is present in trophoblast cells in each of the decidua basalis, junctional zone and labyrinth layers of the placenta, but was not detected in the endothelial cells or the chorionic plate (Fig. 2D). This suggests that *Tex19.1* is expressed in the hypomethylated trophectoderm-derived components of the placenta, but not in methylated extraembryonic mesoderm-derived components (25,34) and is consistent with mRNA *in situ* hybridization data (36). There is also considerable overlap between IAP expression (Fig. 1G) and TEX19.1 expression (Fig. 2D) with parietal trophoblast giant cells, spongiotrophoblasts and sinusoidal trophoblast giant cells expressing both of these proteins. Taken together, these data suggest that *Tex19.1* is the only hypomethylation-sensitive germline genome-defence gene that is hypomethylated and expressed in the placenta.

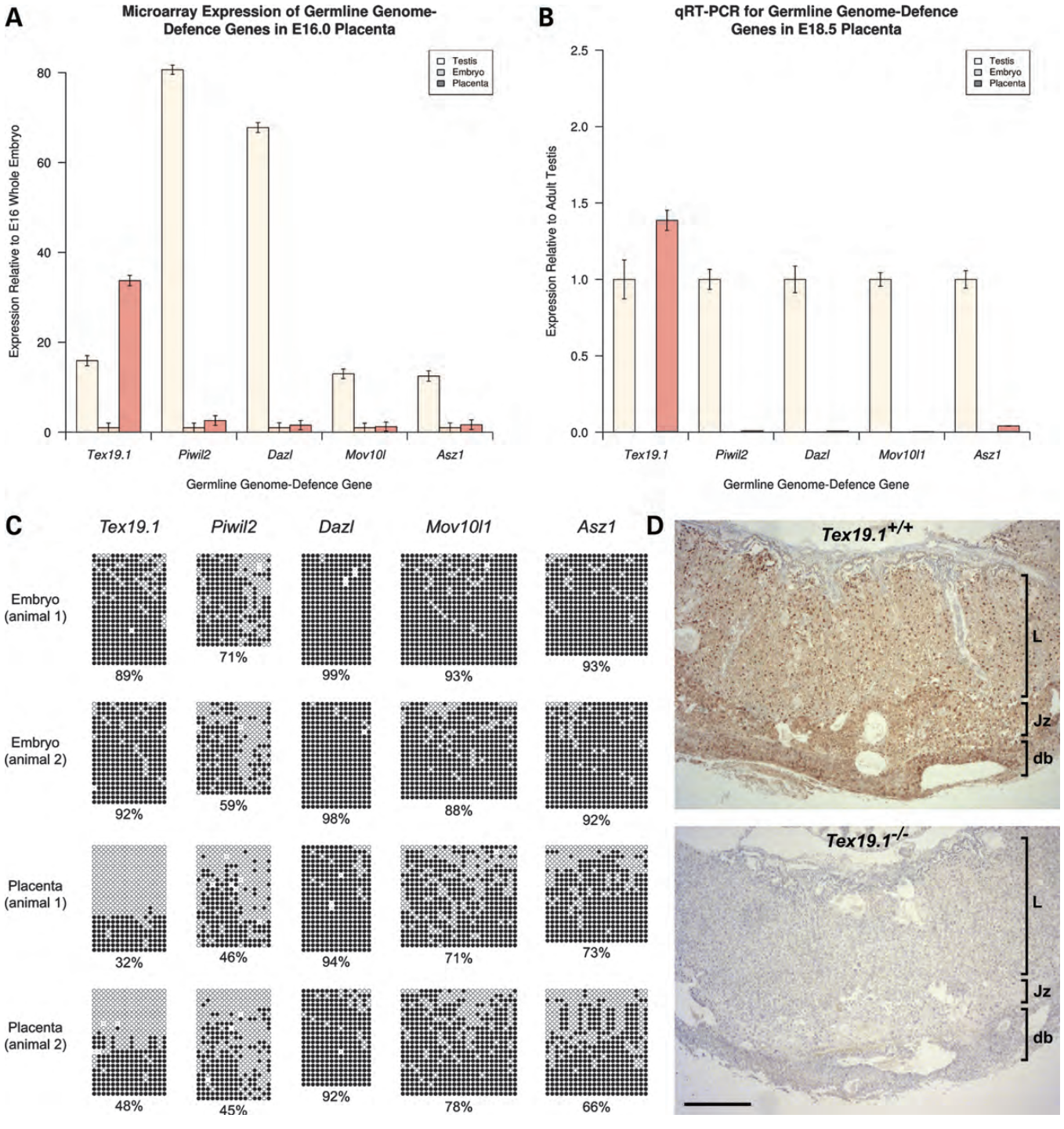

Figure 2. *Tex19.1* is the only known germline genome-defence gene that is hypomethylated and expressed in the placenta. (**A**) Microarray expression data showing expression of the hypomethylation-sensitive genome-defence genes in E16 placenta and adult testis relative to E16 embryo for each gene. Error bars indicate standard error. $n = 3$. (**B**) qRT-PCR for expression of the hypomethylation-sensitive genome-defence genes in E18.5 placenta and embryo relative to adult testis. Error bars indicate standard error. $n = 2$. (**C**) Bisulphite sequencing of the promoters of the hypomethylation-sensitive genome-defence genes in two independent placental and embryonic DNA samples. Each line represents a sequenced clone, filled circles represent methylated CpGs and open circles represent unmethylated CpGs. The percentage of total methylation in each sample is indicated. (**D**) Immunohistochemistry (brown precipitate) with anti-TEX19.1 antibody on wild-type and *Tex19.1*$^{-/-}$ E18.5 placenta. Sections were counterstained with haematoxylin. Approximate positions of the decidua basalis (db), junctional zone (Jz) and labyrinth (L) are indicated. Scale bar, 500 μm.

Tex19.1 might therefore play an important role in suppressing retrotransposon activity in this tissue.

Tex19.1 is required for normal placental development

We next investigated if *Tex19.1* expression has any functional consequences for placental development. Defects in placental function would be expected to manifest as embryonic lethality and/or reduced embryo weight, depending on the severity of the defect. *Tex19.1*$^{-/-}$ embryos have been reported to be born at a sub-Mendelian frequency from heterozygous crosses (19,37), with lethality affecting females more strongly than males (37). The reduced viability of *Tex19.1*$^{-/-}$ embryos could reflect a function for *Tex19.1* in the placenta, or in pluripotent cells, where it is also expressed. In contrast to previously published data (37), although loss of *Tex19.1* was associated with embryonic lethality, this lethality did not appear to affect females more strongly than males, despite our analysis including almost 1000 genotyped pups (Supplementary Material, Table S1). However, we noticed that the requirement for *Tex19.1* during embryonic development was much stronger when mothers are lactating and nursing a pre-existing litter during pregnancy (20% of *Tex19.1*$^{-/-}$ embryos die without concurrent lactation, 66% die with concurrent lactation) and that loss of *Tex19.1* was affecting viability of female embryos more strongly than male embryos specifically in the concurrent pregnancies (*Tex19.1*$^{-/-}$ homozygotes born with a 1:1 male:female sex ratio without concurrent lactation, 4.7:1 male:female sex ratio with concurrent lactation) (Supplementary Material, Table S2). Concurrent lactation has been reported to affect the viability of embryos in a sex-specific manner in some mouse strains (38), and this phenomenon appears to be exacerbating the *Tex19.1*$^{-/-}$ embryonic lethality phenotype. Differences between breeding schedules, which significantly influence the magnitude and sex-specificity of the effects of *Tex19.1*$^{-/-}$ on embryonic lethality, are likely to account for differences between the data in Supplementary Material, Table S1 and previously published observations (37). Therefore, we performed subsequent analyses on pregnancies where mothers were not concurrently lactating to avoid this issue.

The reduced viability of *Tex19.1*$^{-/-}$ embryos could be indicative of defects in placenta function. Therefore, we assessed the size and weight of *Tex19.1*$^{-/-}$ placentas and embryos during development. *Tex19.1*$^{-/-}$ embryos dissected at E18.5 appeared visibly smaller than their heterozygous and wild-type littermates (Fig. 3A). We measured this effect by weighing E18.5 embryos and observed a 20% reduction ($P < 0.01$, Mann–Whitney *U*-test) in embryo weight in both male and female *Tex19.1*$^{-/-}$ embryos (Fig. 3C). *Tex19.1*$^{-/-}$ placentas are also smaller than wild-type and heterozygous littermates at E18.5: female and male *Tex19.1*$^{-/-}$ placentas are 26% ($P < 0.01$, Mann–Whitney *U*-test) and 30% ($P < 0.01$, Mann–Whitney *U*-test) lighter than littermate controls, respectively (Fig. 3A and D). Although *Tex19.1*$^{-/-}$ embryos are smaller than their littermates during embryonic development, male *Tex19.1*$^{-/-}$ animals reach normal weights during post-natal growth (Fig. 3E). In contrast, adult female *Tex19.1*$^{-/-}$ animals remain slightly smaller (8% reduction in weight, Mann–Whitney *U*-test, $P < 0.01$) than their littermate controls (Fig. 3B and E). However, the reduction in *Tex19.1*$^{-/-}$ female weight is less severe in adults than in embryos. Taken together, the reduction in placental weight and intra-uterine growth defect present in *Tex19.1*$^{-/-}$ embryos suggest that *Tex19.1* is required for normal placenta function during development.

Tex19.1$^{-/-}$ placentas have defects in the junctional zone and labyrinth

We next examined whether any defects in *Tex19.1*$^{-/-}$ placental function might be caused by abnormalities in placenta structure or development. Histology of E18.5 placenta sections revealed that *Tex19.1*$^{-/-}$ placentas possess the main structural layers: decidua basalis, junctional zone and labyrinth (Fig. 4A). However, the junctional zone appears to be thinner in *Tex19.1*$^{-/-}$ placenta sections when compared with littermate controls. To further investigate any potential defects in the junctional zone, we used periodic-acid Shiff's (PAS) stain to label the carbohydrate-rich junctional zone and decidua basalis layers (Fig. 4B) and measured the area of the junctional zone relative to the total placenta area in sections (Fig. 4E). This confirmed that there is a ~40% reduction in the amount of junctional zone in *Tex19.1*$^{-/-}$ placentas relative to littermate controls ($P < 0.05$, *t*-test).

The junctional zone comprises two main cell types, spongiotrophoblasts and glycogen trophoblasts, each of which is derived from the trophectoderm (34). To investigate whether loss of *Tex19.1* affects one or both of these cell types in the junctional zone, we counted the number of glycogen trophoblasts and spongiotrophoblasts in histological sections of E18.5 placentas (Fig. 4C and F). Interestingly, the abundance of both spongiotrophoblasts and glycogen trophoblasts was reduced by 40% ($P < 0.05$, *t*-test) and 80% ($P < 0.05$, *t*-test), respectively in *Tex19.1*$^{-/-}$ placentas. Thus, the reduction in the thickness of the junctional zone in *Tex19.1*$^{-/-}$ placentas is presumably caused by reduced numbers of spongiotrophoblast and glycogen trophoblast cells in this tissue.

In addition to the histological defects in the junctional zone of *Tex19.1*$^{-/-}$ placentas, we also noticed a difference in the labyrinth layer, which appeared to have fewer sinusoidal trophoblast giant cells, another trophectoderm-derived cell type (34), than their littermate controls (Fig. 4D). Cell counts confirmed that this cell type is 40% ($P < 0.05$, *t*-test) less abundant in *Tex19.1*$^{-/-}$ placentas (Fig. 4F). Thus, loss of *Tex19.1* affects trophectoderm-derived cells in both the junctional zone and labyrinth of the placenta.

As loss of *Tex19.1* appears to be affecting multiple cell types in the placenta, we performed qRT-PCR for cell type markers to confirm and extend our histological analysis. *Tek*, a marker of extraembryonic mesoderm-derived endothelial tissues (39), does not show any statistically significant change in mRNA abundance in *Tex19.1*$^{-/-}$ placentas when compared with littermate controls (Fig. 5A). Similarly, mRNAs encoding markers of chorion-derived trophoblast cells in the labyrinth (*Dlx3*, *Nr6a1*) (40), and markers of the two syncytiotrophoblast layers (*Syna* and *Synb*) (40), do not significantly change abundance in *Tex19.1*$^{-/-}$ placentas either (Fig. 5A). However, mRNA for the sinusoidal

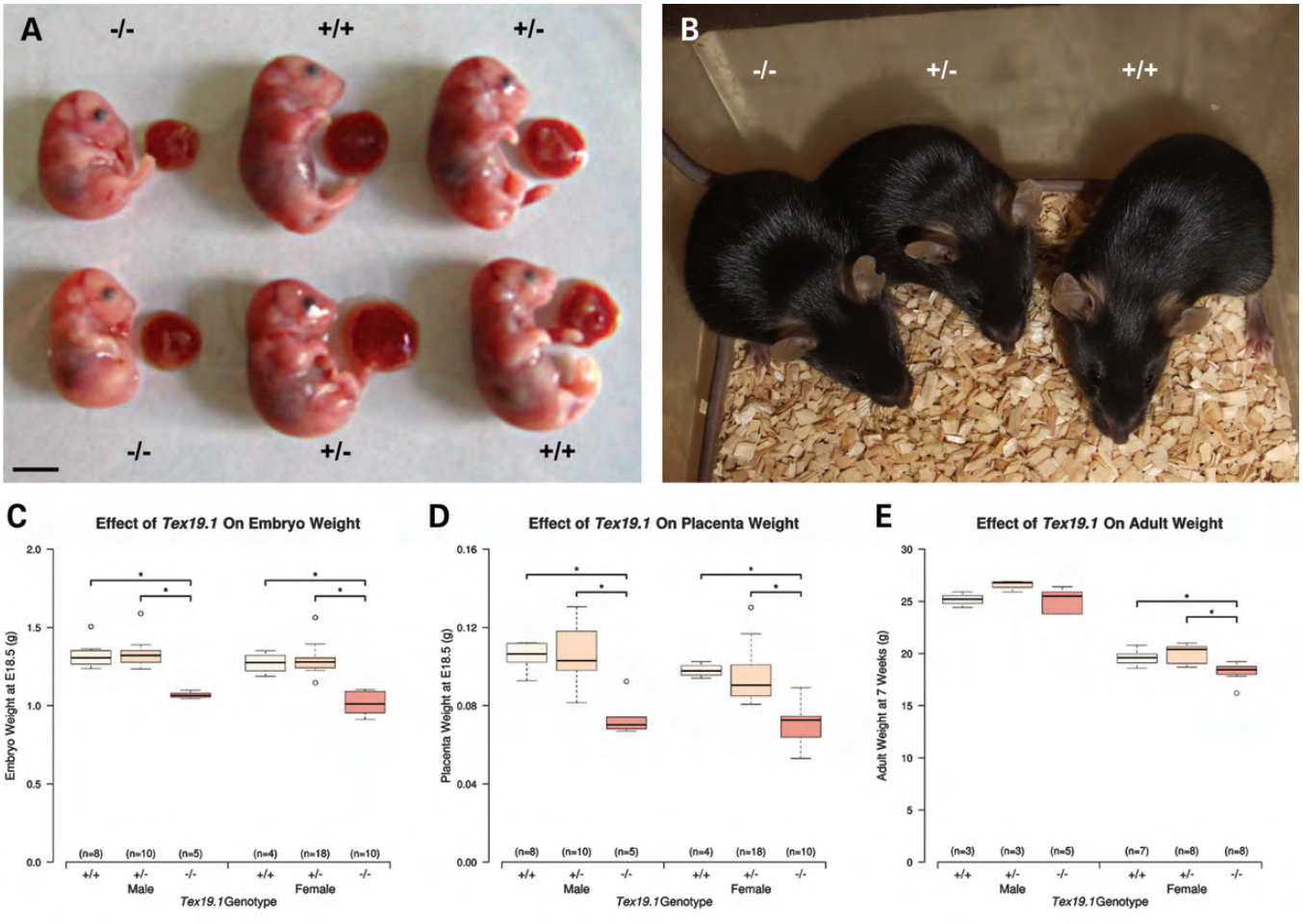

Figure 3. *Tex19.1*$^{-/-}$ embryos have small placentas and intrauterine growth retardation. (**A**) Photograph of E18.5 embryos and their placentas in a single litter from a cross between *Tex19.1*$^{+/-}$ heterozygotes. The *Tex19.1* genotype is indicated. Scale bar, 500 μm. (**B**) Photograph of 6–8 week old female adult mice. The *Tex19.1* genotype is indicated. (**C**–**E**) Boxplots showing the weights of littermate E18.5 embryos (C), E18.5 placentas (D) and adult animals (E) derived from heterozygous crosses, grouped according to sex and *Tex19.1* genotype. * indicates $P < 0.01$ (Mann–Whitney *U*-test).

trophoblast giant cell marker *Ctsq* (40) is significantly depleted (∼4-fold, $P < 0.05$, *t*-test) in *Tex19.1*$^{-/-}$ placentas, and levels of *Hand1* mRNA, a marker for trophoblast giant cells (40), were also reduced (∼2-fold, $P < 0.05$, *t*-test) (Fig. 5A). This is consistent with our histology data showing that *Tex19.1*$^{-/-}$ placentas have fewer sinusoidal trophoblast giant cells. Similarly, ∼2-fold reductions ($P < 0.05$) in the levels of junctional zone markers [*Tpbpa*, glycogen trophoblasts and spongiotrophoblasts (41); *Pcdh12* and *Gjb3*, glycogen trophoblasts (41,42); *Prl8a8* and *Prl3a1*, spongiotrophoblasts (41)] in *Tex19.1*$^{-/-}$ placentas are consistent with the reduced thickness of this layer and the reduction in the number of glycogen trophoblasts and spongiotrophoblasts we observed histologically.

We performed a more objective analysis of gene expression changes in *Tex19.1*$^{-/-}$ placentas using Illumina WG-6v2.0 Beadchip microarrays to profile gene expression at a genome-wide level. Microarray analysis shows that mRNAs for only 2 genes increase in abundance by more than 3-fold in *Tex19.1*$^{-/-}$ placentas ($P < 0.01$) and that mRNAs for 22 genes decrease in abundance by the same amount ($P < 0.01$) (Fig. 5B). A number of placenta-associated gene families, such as placental lactogen genes (*Prl*), pregnancy-specific glycoprotein genes (*Psg*), cathepsin genes (*Cts*) and caudal homeobox genes (*Cdx*) (41,43–45), had multiple genes whose mRNAs were at least 1.5-fold lower in *Tex19.1*$^{-/-}$ placentas ($P < 0.05$) [microarray data are available in GEO repository (46) accession GSE41823]. Expression levels of seven placental lactogen genes (*Prl7c1*, *Prl4a1*, *Prl3c1*, *Prl5a1*, *Prl7b1*, *Prl8a6* and *Prl8a9*), six pregnancy-specific glycoprotein genes (*Psg17*, *Psg18*, *Psg19*, *Psg23*, *Psg25* and *Psg29*), three cathepsin genes (*Ctsm*, *Cts3*, *Cts6*) and two caudal homeobox genes (*Cdx1* and *Cdx2*) were all reduced in the *Tex19.1*$^{-/-}$ placentas by these criteria. We verified by qRT-PCR that mRNAs for candidate genes belonging to each of these families are less abundant in *Tex19.1*$^{-/-}$ placentas than littermate controls ($P < 0.05$, *t*-test) (Fig. 5C). Taken together, the microarray and qRT-PCR data are consistent with our histological analyses and show that the amount of junctional zone in the placenta and the abundance of specific trophoblast cell types are reduced in the absence of *Tex19.1*.

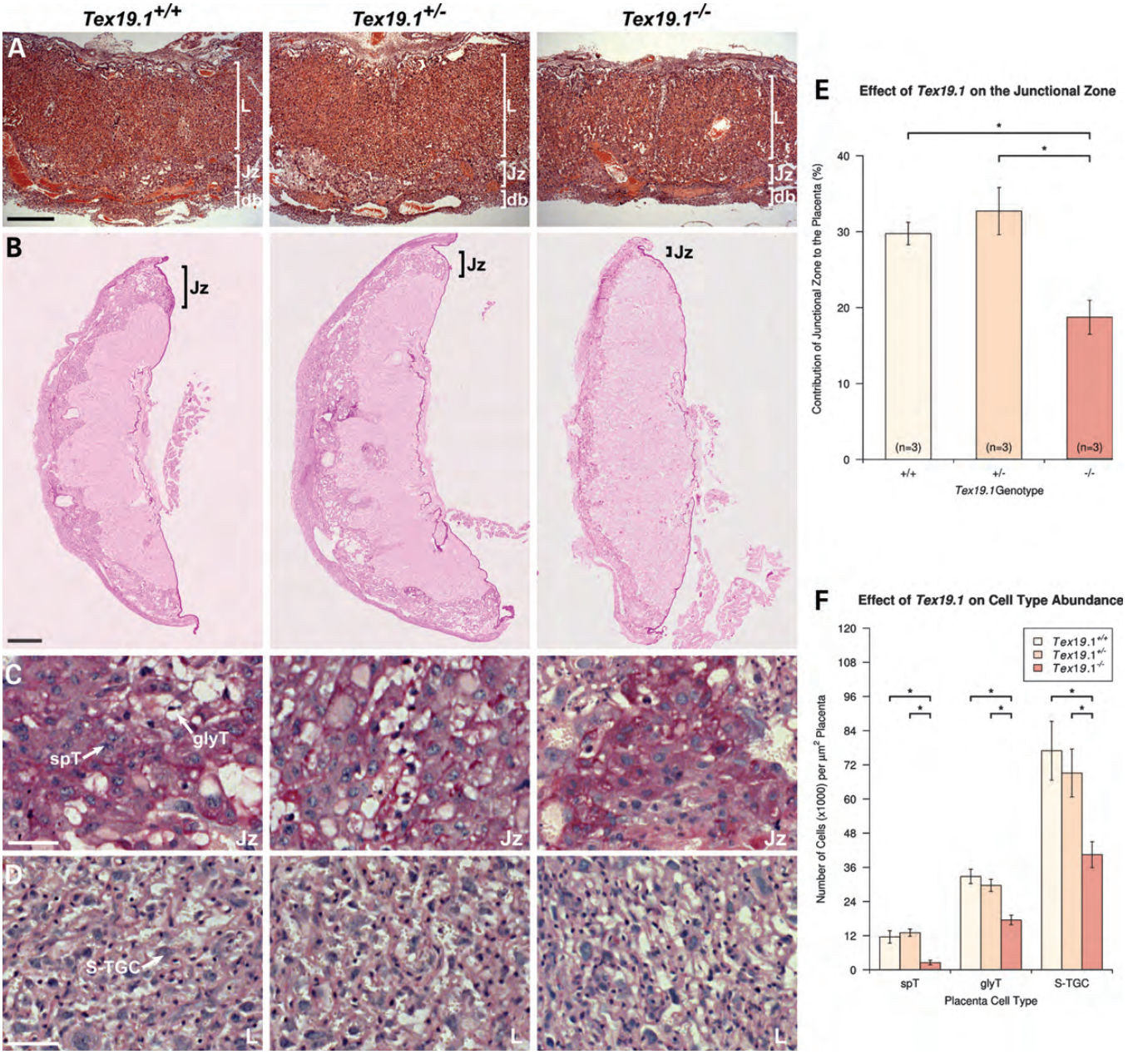

Figure 4. *Tex19.1*$^{-/-}$ placentas have reduced numbers of spongiotrophoblasts, glycogen trophoblasts and sinusoidal trophoblast giant cells. (**A**) Haematoxylin and eosin staining of E18.5 *Tex19.1*$^{+/+}$, *Tex19.1*$^{+/-}$ and *Tex19.1*$^{-/-}$ placenta sections. The approximate positions of the decidua basalis (db), junctional zone (Jz) and labyrinth (L) layers are indicated. Scale bar, 500 μm. (**B**) PAS staining of E18.5 placenta sections highlighting the carbohydrate-rich decidua basalis and junctional zone layers in the placenta. Scale bar, 500 μm. (**C** and **D**) High magnification images of placenta sections stained with PAS and haematoxylin. Regions of the junctional zone (C) and labyrinth (D) are shown. Scale bars, 50 μm. (**E**) Contribution of the junctional zone to the placenta in PAS-stained sections. Error bars indicate standard error, * indicates $P < 0.05$ (*t*-test). (**F**) Abundance of trophoblast cell types in *Tex19.1*$^{-/-}$ and control littermate placenta sections. $n = 3$, error bars indicate standard error, * indicates $P < 0.05$ (*t*-test). spT, spongiotrophoblast; glyT, glycogen trophoblast; S-TGC, sinusoidal trophoblast giant cell.

A number of imprinted genes are important for placenta development and function, and some imprinted genes have different requirements for their silencing in hypomethylated placental tissue than in the embryo-derived somatic cells (47,48). Defective silencing of the imprinted allele of imprinted genes would result in a gene expression change that falls below the 3-fold cutoff used in the microarray analysis in Figure 5B, but these changes can be sufficient to cause a placenta phenotype (49,50). However, even in the absence of a fold-change threshold, imprinted genes are no more likely to be differentially expressed in *Tex19.1*$^{-/-}$ placentas than non-imprinted genes (Supplementary Material, Fig. S2). Although we cannot exclude the possibility that differences in cell composition between *Tex19.1*$^{-/-}$ and control placentas are masking the detection of derepression of imprinted genes, loss of *Tex19.1* does not appear to be causing widespread defects in imprinted gene silencing in the placenta.

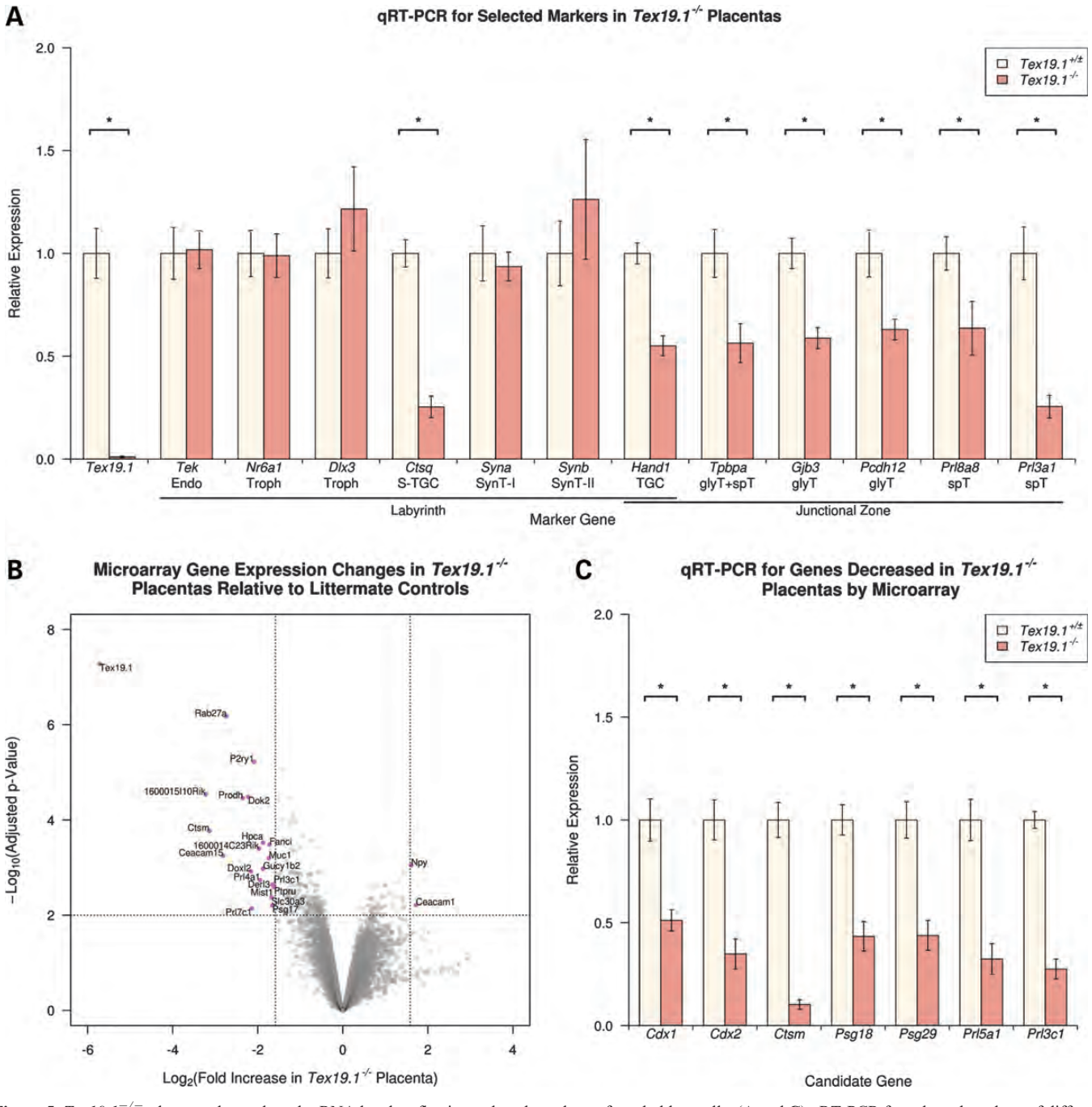

Figure 5. *Tex19.1*$^{-/-}$ placentas have altered mRNA levels reflecting reduced numbers of trophoblast cells. (**A** and **C**) qRT-PCR for selected markers of different placental cell types (A) and gene families identified by microarray (C) in E18.5 *Tex19.1*$^{-/-}$ placentas. Expression relative to *Tex19.1*$^{+/\pm}$ placentas is indicated. $n = 6$, error bars indicate standard error, * indicates $P < 0.05$ (*t*-test). Endo, endothelial cell; Troph, trophoblast; S-TGC, sinusoidal trophoblast giant cell; SynT-I, syncytial trophoblast type I; SynT-II, syncytial trophoblast type II, TGC, trophoblast giant cell; spT, spongiotrophoblast; glyT, glycogen trophoblast. (**B**) Volcano plot of gene expression microarray data showing the probability that each gene on the microarray is differentially expressed and its level of upregulation in *Tex19.1*$^{-/-}$ placentas. Genes changing more than 3-fold ($P < 0.01$) are highlighted in pink and annotated with their gene names.

Tex19.1$^{-/-}$ placentas derepress retrotransposons

We have previously shown that *Tex19.1* represses the LTR retrotransposon *MMERVK10C* in spermatocytes (19). However, any derepression of retrotransposon expression in *Tex19.1*$^{-/-}$ placentas would not be detected in the gene expression microarray analysis as any retrotransposon probes in the microarray were excluded from the gene expression analysis. Therefore, we re-analyzed the *Tex19.1*$^{-/-}$ placenta microarray data using our recently developed microarray repeat-annotation technique (10) and found that a number of microarray probes corresponding to the LINE and LTR classes of retrotransposon are upregulated between 2- and

4-fold in *Tex19.1*$^{-/-}$ placentas relative to littermate controls. Interestingly, 11 of the 13 retrotransposon probes that are upregulated more than 2-fold in *Tex19.1*$^{-/-}$ placentas ($P < 0.01$) correspond to *LINE-1* elements (Fig. 6A), one of the most abundant retrotransposons in the mouse genome (2). An LTR retrotransposon probe corresponding to *MMVL30* LTR retrotransposons is also among the upregulated retrotransposon probes in *Tex19.1*$^{-/-}$ placentas (Fig. 6A).

The large number of significantly upregulated *LINE-1* probes in the *Tex19.1*$^{-/-}$ placenta microarray data prompted us to look more closely at *LINE-1* expression in this tissue. Overall, *LINE-1* probes were differentially distributed relative to non-repeat probes within the microarray dataset (Mann–Whitney *U*-test, $P < 0.01$), and a subset of *LINE-1* probes showed a ~2-fold upregulation in *Tex19.1*$^{-/-}$ placentas relative to littermate controls (Fig. 6B). However, some *LINE-1* probes appear to be more strongly affected by loss of *Tex19.1* than others (Fig. 6B). We verified the microarray repeat-annotation data by performing qRT-PCR on *Tex19.1*$^{-/-}$ placentas and littermate controls. Consistent with the microarray data, *MMERVK10C* and *IAP* retrotransposons are not upregulated in *Tex19.1*$^{-/-}$ placentas by qRT-PCR (Fig. 6C); however, *MMVL30* retrotransposons are modestly upregulated ~1.7-fold (*t*-test, $P < 0.05$). Generic primers to the ORF2 region of *LINE-1* and primers designed to detect the copies of *LINE-1* that correspond to the upregulated microarray probes, all show a modest but statistically significant ~1.6–2-fold ($P < 0.05$) upregulation in *Tex19.1*$^{-/-}$ placentas by qRT-PCR (Fig. 6C). We confirmed that the upregulation of *LINE-1* mRNA in *Tex19.1*$^{-/-}$ placentas that we detected by microarray and qRT-PCR represents a bona fide increase in *LINE-1* element expression by western blotting for *LINE-1* ORF1p (Fig. 6D). *Tex19.1*$^{-/-}$ placentas showed a modest, but consistent increase in *LINE-1* ORF1p protein abundance commensurate with the ~2-fold increase in *LINE-1* mRNA levels detected by qRT-PCR and microarray (Fig. 6D).

The increase in *LINE-1* mRNA and protein abundance that occurs in *Tex19.1*$^{-/-}$ placentas could be caused by the difference in cell composition between wild-type and knockout placentas, or by upregulation of *LINE-1* in the trophoblast cell types that normally express *Tex19.1*. Immunohistochemistry for *LINE-1* ORF1p protein showed that *LINE-1* is primarily expressed in the junctional zone trophoblast cells in both *Tex19.1*$^{-/-}$ and control littermate placentas, with some low level expression also detectable in the trophoblast giant cells in the labyrinth (Fig. 6E). The cell types primarily expressing *LINE-1* in *Tex19.1*$^{-/-}$ placentas are, therefore, the trophectoderm-derived cell types that normally express *Tex19.1*. Furthermore, as there are fewer junctional zone trophoblast cells in *Tex19.1*$^{-/-}$ placentas (Fig. 4), the ~2-fold increases in *LINE-1* expression observed by qRT-PCR and western blotting likely underestimate the increase in *LINE-1* expression that occurs in the junctional zone cells that remain in *Tex19.1*$^{-/-}$ placentas (Fig. 6D and E). Taken together, these data suggest that expression of the genome-defence gene *Tex19.1* is linked to the epigenetic state of the placenta and suppresses *LINE-1* retrotransposons in the hypomethylated trophectoderm-derived cells in this tissue.

DISCUSSION

Suppressing retrotransposons in the hypomethylated placenta

The mammalian placenta has been associated with retrotransposon expression in a number of studies (reviewed in 29), and retrotransposon-derived proteins have even been co-opted to fulfil placental functions on multiple occasions during mammalian evolution (51–54). However, our microarray analysis of retrotransposon expression suggests that, although a number of retrotransposons are differentially expressed between placenta and embryo, the murine placenta is in general no more permissive for retrotransposon expression than the rest of the embryo. This contrasts strongly with, for example, ES cells knocked down for the histone methyltransferase *Eset* that show widespread upregulation of many different types of retrotransposon in microarray repeat annotation (10). Studies assessing the behaviour of specific types of retrotransposons in the human placenta also suggest that the association between retrotransposon expression and the placenta reflects expression of a subset of hypomethylated genomic copies of these elements (55). Thus, the strong associations between retrotransposon expression and the placenta might represent expression of specific types of retrotransposon, or even expression of specific genomic copies of a retrotransposon, rather than a widespread general activation of retrotransposon expression in this tissue.

Multiple retrotransposon families and classes have been reported to be DNA hypomethylated in the placenta relative to the embryo (25,27,28). At least for *LINE-1*, this hypomethylation is present in trophectoderm-derived rather than extraembryonic mesoderm-derived components of the placenta (25). *IAP* elements appear to be repressed by DNA methylation in multiple embryo-derived cell types (11,30,31) and is one of the hypomethylation-sensitive genes strongly activated in mouse somatic cells in multiple models of DNA hypomethylation (17). Thus, although the hypomethylated state of the placenta does not appear to cause widespread derepression of retrotransposons in general, the derepression of *IAP* elements in the placenta likely represents a direct consequence of its hypomethylated state. A number of other retrotransposons that have been shown to be repressed by DNA methylation in ES cells or germ cells, including *LINE-1* (9,10,33), do not appear to be strongly upregulated in the placenta relative to the embryo. However, it is still the hypomethylated derivatives of the trophectoderm that are the primary source of *LINE-1* ORF1p expression in this tissue. Thus, although differences in transcription factor expression, and/or differences in additional retrotransposon suppression mechanisms, could all contribute to the differential sensitivity of retrotransposons to DNA hypomethylation between placenta and embryo, the hypomethylated epigenetic state of the trophectoderm derivatives within the placenta does appear to make these cell types vulnerable to retrotransposon activity.

We have recently shown that the DNA hypomethylation that occurs in developing primordial germ cells induces expression of a cohort of germline genome-defence genes that can act to protect the genome from the activity of any retrotransposons that might be derepressed during this period of

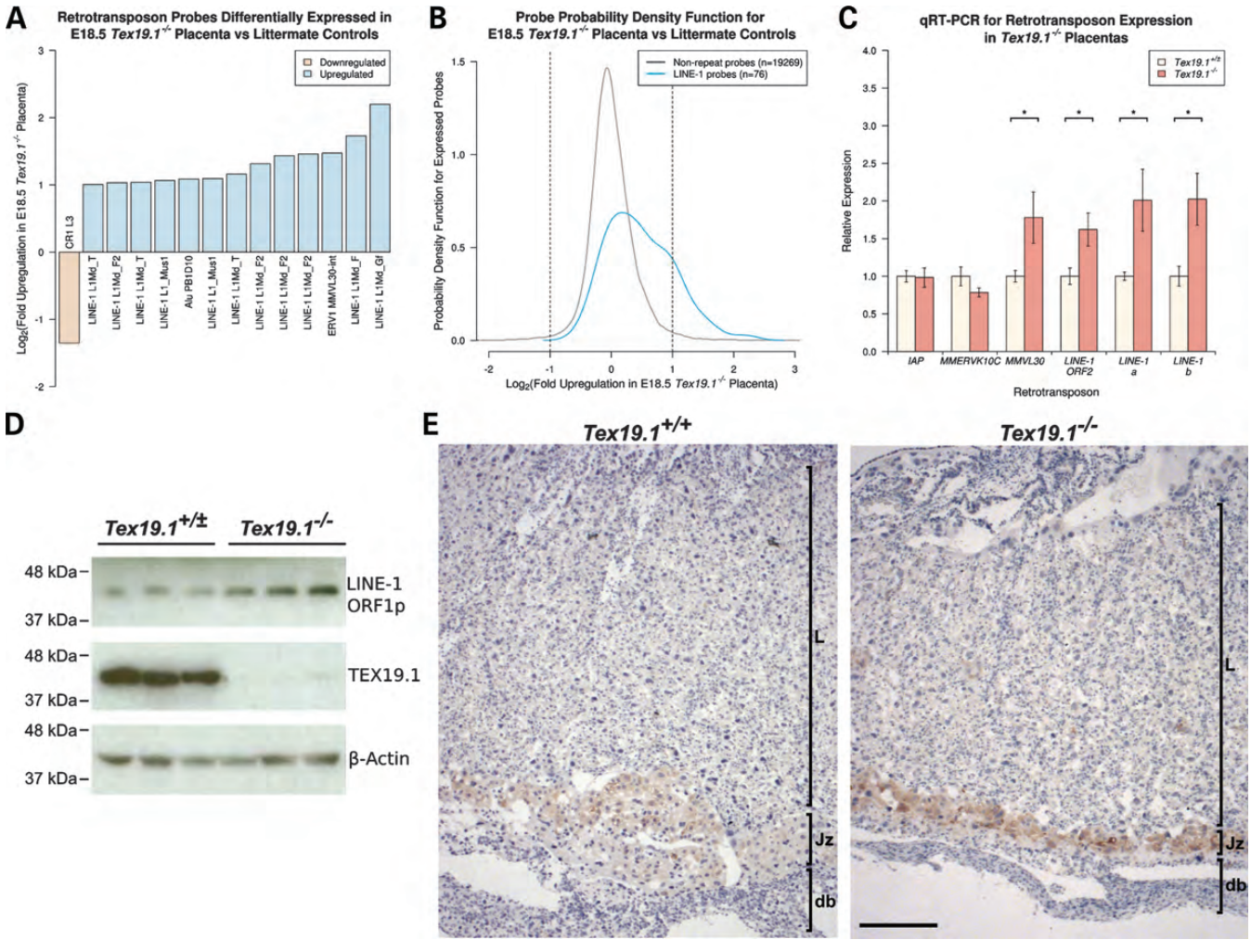

Figure 6. *LINE-1* retrotransposons are de-repressed in *Tex19.1*$^{-/-}$ placentas. (**A**) Bar chart showing all retrotransposon probes that change expression more than 2-fold ($P < 0.01$) in *Tex19.1*$^{-/-}$ placentas. (**B**) Probability density function plots showing the relative likelihood that the microarray signal of any individual *LINE-1* probe is increased by the indicated amount in *Tex19.1*$^{-/-}$ placentas. Dotted lines mark 2-fold changes in expression. (**C**) qRT-PCR analysis of expression of selected retrotransposons in *Tex19.1*$^{-/-}$ placentas at E18.5. Error bars represent standard error, $n = 6$, asterisks indicate $P < 0.05$ (*t*-test). (**D**) Western blot for *LINE-1* ORF1p, TEX19.1 and β-actin in *Tex19.1*$^{+/\pm}$ and *Tex19.1*$^{-/-}$ placentas. (**E**) Immunohistochemistry (brown precipitate) for *LINE-1* ORF1p in E18.5 *Tex19.1*$^{+/+}$ and *Tex19.1*$^{-/-}$ placentas. Sections are counterstained with haematoxylin. Approximate positions of the decidua basalis (db), junctional zone (Jz) and labyrinth (L) are indicated. No specific staining was detected with non-specific IgG controls. Scale bar, 200 μm.

epigenetic reprogramming (17). Interestingly, although this cohort of genes all become hypomethylated and expressed during the extensive epigenetic reprogramming event that occurs in the developing germline, only *Tex19.1* is hypomethylated and expressed in the placenta. DNA hypomethylation in developing germ cells has been proposed to occur through multiple active mechanisms (27,56), whereas DNA hypomethylation in the placenta might be a consequence of reduced *de novo* DNA methylation in trophectoderm-derived cells (57,58), possibly in combination with additional events. Differences between the molecular mechanisms responsible for DNA hypomethylation could potentially account for the differences in genome-defence gene promoter methylation between the placenta and the germline. However, our finding that *Tex19.1* functions to repress retrotransposons in the placenta, where the remaining genome-defence genes are not expressed, suggests that at least some genome-defence genes are able to function independently of each other, possibly in a modular manner. Deploying a group of genome-defence genes in hypomethylated germ cells that are each independently able to target multiple retrotransposons at different stages of the retrotransposon life cycle would be expected to provide an effective multi-layered defence against these elements.

Tex19.1 function in the placenta

Our study shows that *Tex19.1* is required for normal placenta development and function. In the absence of *Tex19.1*, trophectoderm-derived spongiotrophoblasts, glycogen trophoblasts and sinusoidal trophoblast giant cells are all reduced in abundance, or have altered gene expression patterns in the placenta. TEX19.1 protein is expressed in the trophoblast giant cells, extraembryonic ectoderm and chorion at E6.5 and E7.5 (17), and the defects that we describe here

in E18.5 placenta may arise from defects at much earlier stages of placenta development. We have previously shown that *Tex19.1* plays a role in suppressing a specific LTR retrotransposon, *MMERVK10C*, in the developing germline, but has no effect on the abundance of *LINE-1* mRNA in that tissue (10,19). Our findings here extend the range of retrotransposons that this genome-defence mechanism is targeting and highlight the complex interplay that is operating between genome-defence mechanisms in different tissues. The insensitivity of *LINE-1* to the loss of *Tex19.1* in developing germ cells presumably reflects the existence of multiple overlapping defence mechanisms operating against this element in the germline. Analysis of retrotransposon mRNAs might be a relatively indirect approach to detect the activity of *Tex19.1*, and the increases in retrotransposon mRNA that we have identified in the placenta (this study) and in the testis (19) could reflect loss of a genome-defence mechanism operating at any point in a retrotransposon life cycle as increased flux through a retrotransposon life cycle will generate an increase in the genomic copy number of that element that can then produce more retrotransposon mRNA. Differences in the types of retrotransposon derepressed in *Tex19.1*$^{-/-}$ testes and *Tex19.1*$^{-/-}$ placentas may simply reflect the presence or absence of mechanisms that are operating alongside *Tex19.1* to suppress different retrotransposons at different stages of their life cycle in each of these tissues.

The increase in *LINE-1* retrotransposon mRNA that is present in *Tex19.1*$^{-/-}$ placentas has the potential to cause increased retrotransposition and could also reflect increased *LINE-1* retrotransposition that has already occurred in this tissue. Emerging technologies that identify and map *de novo LINE-1* retrotransposition events in human tissue (59,60) might allow the extent of any de novo *LINE-1* retrotransposition in these mouse tissues to be assessed in future studies. As *Tex19.1* is expressed from early stages of placental development, there is plenty of opportunity for any increased *LINE-1* expression in these mutant tissues to cause *de novo* retrotransposition and for these *de novo* retrotransposition events to accumulate and cause genetic instability. We have not yet determined whether the reduction in placental cell types and changes in mRNA abundance that we describe in *Tex19.1*$^{-/-}$ placentas are caused by increased apoptosis, decreased proliferation or impaired differentiation. In addition, although *Tex19.1* has a role in suppressing retrotransposons in the placenta, we cannot exclude the possibility that *Tex19.1* also has a role in mediating other aspects of cell function and that the placental defects in *Tex19.1*$^{-/-}$ mice are not directly caused by an increase in retrotransposition. However, it is also possible that the defects that we observe in *Tex19.1*$^{-/-}$ placentas are a direct consequence of increased retrotransposon activity causing high levels of insertional mutagenesis. As some combinations of *de novo LINE-1* integration events are likely to be more deleterious than others, the somatic variation between cells in *Tex19.1*$^{-/-}$ placentas could result in sporadic occurrences of cell death, proliferation defects or developmental abnormality in trophectoderm-derived cell types throughout placenta development. *LINE-1* retrotransposition has been proposed to generate somatic variation in human neural tissue (61) and to drive tumourigenesis in some cases of human colorectal cancer (60). Thus, although a small amount of *LINE-1* retrotransposition has been proposed to be important for generating somatic variation and normal brain function, too much retrotransposition is likely to be deleterious. It would appear that mutating *Tex19.1* might be sufficient to this balance in the developing placenta.

The exacerbating sex-biased effect that concurrent lactation has on *Tex19.1*$^{-/-}$ embryonic lethality is intriguing, but the molecular basis for this phenomenon is not clear at present. The preferential loss of XX *Tex19.1*$^{-/-}$ embryos during concurrent lactation could indicate defects in X-inactivation under these conditions. However, the *Tex19.1*$^{-/-}$ placental phenotype that we describe in this work occurs in both XX and XY placentas suggesting that *Tex19.1* is not required for X-inactivation in the absence of concurrent lactation and that X-inactivation defects are not causing the placental phenotype characterized here. In addition, there is no evidence for activation of X-linked genes in the microarray gene expression profiles of XX *Tex19.1*$^{-/-}$ placentas (Supplementary Material, Fig. S3). Interestingly, there are differences in gene expression between XX and XY placentas, and gene expression in XX placentas is more sensitive to changes in maternal diet than gene expression in XY placentas (62,63). Perhaps the increased nutritional demand of concurrent pregnancy and lactation is similarly altering placenta gene expression, and these changes could be increasing the requirement for *Tex19.1* function in this tissue. Given the findings presented here, it would be of interest to determine whether retrotransposon expression or activity is altered in the placenta during concurrent lactation and pregnancy.

Although placenta development is compromised in the absence of *Tex19.1*, *Tex19.1* does not appear to be absolutely required for development or survival of any specific terminally differentiated placenta cell type. Nevertheless, *Tex19.1*$^{-/-}$ embryos exhibit intra-uterine growth retardation suggesting that loss of *Tex19.1* impairs placenta function. The reduction in the number of trophoblast cells in *Tex19.1*$^{-/-}$ placentas is likely to result in lower amounts of some placenta-derived hormones entering the maternal circulatory system and reduced communication across the maternal–fetal interface. mRNAs encoding a number of secreted hormones such as placental lactogens, which are secreted into the maternal blood to influence maternal metabolism (41), and pregnancy-specific glycoproteins, one of the most abundant group of fetal-derived proteins present in the maternal blood during pregnancy (44), are all reduced in abundance in *Tex19.1*$^{-/-}$ placentas. Pregnancy-specific glycoproteins have been proposed to have a role in modulating maternal immune responses during pregnancy, possibly through inducing expression of anti-inflammatory cytokines (44). It is, therefore, noteworthy that the two genes whose mRNAs were more than 3-fold higher in *Tex19.1*$^{-/-}$ placentas, *Npy* and *Ceacam1*, have been reported to be upregulated in response to inflammation and to promote angiogenesis (64,65). Reduced levels of placental lactogens and pregnancy-specific glycoproteins in the maternal blood have both been reported to be associated with impaired placenta function and conditions such as intrauterine growth retardation and pre-eclampsia in humans (66). The placental defects in *Tex19.1*$^{-/-}$ mice, therefore, raise the intriguing possibility that deregulation of retrotransposons could contribute to impaired placenta function in humans. In

this respect, the generally hypomethylated epigenetic state of the placenta might make retrotransposons in this tissue particularly sensitive to any additional epigenetic disruption or sequence variation that would remove another layer of suppression from these elements. Although there are a number of differences between the retrotransposons present in human and mouse genomes (1,2), and presumably also between genome-defence mechanisms operating between these species, it is possible that imbalances between retrotransposon suppression and genome-defence mechanisms might contribute to placenta dysfunction and disease in both mice and humans.

MATERIALS AND METHODS

Animals

The *Tex19.1* mutation used in this study is a complete replacement of the *Tex19.1* open reading frame (19), back-crossed three times to C57BL/6 mice (Charles River). For timed matings, noon on the day the vaginal plug was found was termed E0.5. Genotyping was performed essentially as described (19). All animal experiments were performed in concordance with local ethical guidelines and national regulations under the authority of UK Home Office Project Licence PPL 60/3785.

qRT-PCR

RNA was extracted from tissues using TRIzol (Invitrogen), DNAse-treated and used as a template for random-primed cDNA synthesis with Superscript III (Invitrogen). Quantitative PCR was performed using this cDNA as a template, primers listed in Supplementary Material, Table S3, Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) and a CFX96 Real-Time PCR Detection System (Bio-Rad). Quantification was performed relative to a standard curve, and β-actin was used to normalize gene expression between samples. Data were tested for statistical significance using a two-tailed *t*-test. Six $Tex19.1^{-/-}$ placentas and six $Tex19.1^{+/-}$ or $Tex19.1^{+/+}$ control littermate placentas were used for qRT-PCR assays to investigate changes in mRNA abundance in $Tex19.1^{-/-}$ placentas (four XX and two XY for each group).

Bisulphite sequencing

Genomic DNA was extracted from tissues by SDS/proteinase K lysis and ethanol precipitation. Five hundred nanograms of genomic DNA were bisulphite treated with the EZ DNA Methylation Gold kit (Zymo Research), then used as a template for nested PCR using primers listed in Supplementary Material, Table S3. PCR products were gel purified using a Nucleospin Extract II kit (Macherey-Nagel) and cloned into pGEM-T Easy (Promega). Plasmid DNA from individual colonies was isolated, sequenced using the SP6 sequencing primer, and the methylation status of each CpG in the plasmid insert was determined and plotted using QUMA (67). Sequences were excluded from the analysis, if the CpH conversion rate was less than 95%, or if identical sequences were already present in the sample.

Microarray

RNA was isolated from six $Tex19.1^{-/-}$ and six $Tex19.1^{+/-}$ or $Tex19.1^{+/+}$ control littermate placentas (four XX and two XY for each group) using TRIzol (Invitrogen) and biotin-labelled using the Illumina TotalPrep RNA Amplification Kit (Ambion). RNA quality was assessed using an Agilent Bioanalyzer (RNA Integrity Number ≥8) and labelled RNA hybridized to Illumina Mouse WG-6 v2.0 Expression Beadchips. For analysis of gene expression in this dataset, the raw sample probe and control probe data from Illumina Beadstudio were read into *R* (68), background-adjusted (force Positive method), log-transformed and quantile-normalized using *lumi* (69). Poorly performing probes were removed from the dataset as described (70), and probe level data summarized to gene level using *limma*. Differences between $Tex19.1^{-/-}$ and control littermate datasets were identified by linear modelling with *limma* (71), using false discovery rate-adjusted *P*-values to determine statistical significance. The $Tex19.1^{-/-}$ placenta microarray data are available in the GEO repository (46), accession GSE41823. Gene expression analysis of publicly available multiple-tissue microarray data (32) (GEO accession GSE9954) was performed similarly, except that the rma method in *affy* (72) rather than *lumi* was used for pre-processing. Probe level repeat-annotation of microarray data (GEO accessions GSE9954 and GSE41823) was performed as previously described (10).

Histology and immunohistochemistry

Tissue was fixed with freshly depolymerized 4% paraformaldehyde in PBS at 4°C overnight, washed with PBS, then dehydrated through ethanol, xylene and embedded in paraffin wax. Microtome sections (6 μm) were collected on glass slides, dewaxed with xylene and re-hydrated through ethanol to dH_2O. For histology, sections were then stained with either haematoxylin and eosin, PAS or PAS and haematoxylin. For immunohistochemistry, antigen retrieval was performed by boiling the slides in 0.1 M citrate buffer pH 6.0 in a microwave for 15–20 min. Sections were treated with 3% H_2O_2 in methanol for 30 min, blocked with PBS containing 10% goat serum and 0.1% Tween, then incubated with primary antibodies [rabbit anti-Tex19.1 (19), 1:300; rabbit anti-*IAP* gag, 1:300 (73); rabbit anti-*LINE-1* ORF1p, 1:500 (74)] diluted in blocking buffer at 4°C overnight. Sections were washed with PBS, bound primary antibody was detected using the Envision HRP Rabbit DAB+ system (Dako) and sections were counterstained with haematoxylin. Stained sections were dehydrated through ethanol and xylene, mounted with DPX mounting media (CellPath) and photographed using an Olympus BX51 upright or Zeiss Axioplan 2 microscope equipped with a digital camera.

For measurement of the contribution of junctional zone to the placenta, shapes were drawn around the junctional zone and the entire placenta on digital images of PAS-stained sections for each placenta, and areas calculated using the Olympus dotSlide system. For measurement of cell type

abundance, three separate rectangular segments were drawn on digital images of PAS and haematoxylin-stained sections of each placenta, with each segment spanning the entire width of the placenta section. The numbers of spongiotrophoblast, glycogen trophoblast and sinusoidal trophoblast cells in each segment were counted, and the area of the placenta within each segment was calculated using the Olympus dotSlide system.

Western blotting

Placenta was homogenized in Laemmli sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.1 M dithiothreitol and 0.01% bromophenol blue), boiled for 5 min and then sonicated to disrupt genomic DNA. Western blotting was performed using an Invitrogen precast gel system according to the manufacturer's instructions. Rabbit anti-TEX19.1 polyclonal antibodies (19) were used at a 1:100 dilution, mouse anti-β-actin antibodies (Abcam) at 1:5000 and rabbit anti-*LINE-1* ORF1p (74) at 1:2000. Bound primary antibodies were detected with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature*, **409**, 860–921.
2. Waterston, R.H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J.F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P. *et al.* (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature*, **420**, 520–562.
3. Goodier, J.L. and Kazazian, H.H. (2008) Retrotransposons revisited: the restraint and rehabilitation of parasites. *Cell*, **135**, 23–35.
4. de Koning, A.P.J., Gu, W., Castoe, T.A., Batzer, M.A. and Pollock, D.D. (2011) Repetitive elements may comprise over two-thirds of the human genome. *PLoS Genet.*, **7**, e1002384.
5. Maksakova, I.A., Romanish, M.T., Gagnier, L., Dunn, C.A., van de Lagemaat, L.N. and Mager, D.L. (2006) Retroviral elements and their hosts: insertional mutagenesis in the mouse germ line. *PLoS Genet.*, **2**, e2.
6. Öllinger, R., Reichmann, J. and Adams, I.R. (2010) Meiosis and retrotransposon silencing during germ cell development in mice. *Differentiation*, **79**, 147–158.
7. Rowe, H.M. and Trono, D. (2011) Dynamic control of endogenous retroviruses during development. *Virology*, **411**, 273–287.
8. Leeb, M., Pasini, D., Novatchkova, M., Jaritz, M., Helin, K. and Wutz, A. (2010) Polycomb complexes act redundantly to repress genomic repeats and genes. *Genes Dev.*, **24**, 265–276.
9. Karimi, M.M., Goyal, P., Maksakova, I.A., Bilenky, M., Leung, D., Tang, J.X., Shinkai, Y., Mager, D.L., Jones, S., Hirst, M. *et al.* (2011) DNA methylation and SETDB1/H3K9me3 regulate predominantly distinct sets of genes, retroelements, and chimeric transcripts in mESCs. *Cell Stem Cell*, **8**, 676–687.
10. Reichmann, J., Crichton, J.H., Madej, M.J., Taggart, M., Gautier, P., Garcia-Perez, J.L., Meehan, R.R. and Adams, I.R. (2012) Microarray analysis of LTR retrotransposon silencing identifies Hdac1 as a regulator of retrotransposon expression in mouse embryonic stem cells. *PLoS Comput. Biol.*, **8**, e1002486.
11. Walsh, C.P., Chaillet, J.R. and Bestor, T.H. (1998) Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat. Genet.*, **20**, 116–117.
12. Hutnick, L.K., Huang, X., Loo, T.-C., Ma, Z. and Fan, G. (2010) Repression of retrotransposal elements in mouse embryonic stem cells is primarily mediated by a DNA methylation-independent mechanism. *J. Biol. Chem.*, **285**, 21082–21091.
13. Yoder, J.A., Walsh, C.P. and Bestor, T.H. (1997) Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.*, **13**, 335–340.
14. Reik, W. (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature*, **447**, 425–432.
15. Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J. and Surani, M.A. (2002) Epigenetic reprogramming in mouse primordial germ cells. *Mech. Dev.*, **117**, 15–23.
16. Hajkova, P., Ancelin, K., Waldmann, T., Lacoste, N., Lange, U.C., Cesari, F., Lee, C., Almouzni, G., Schneider, R. and Surani, M.A. (2008) Chromatin dynamics during epigenetic reprogramming in the mouse germ line. *Nature*, **452**, 877–881.
17. Hackett, J.A., Reddington, J.P., Nestor, C.E., Dunican, D.S., Branco, M.R., Reichmann, J., Reik, W., Surani, M.A., Adams, I.R. and Meehan, R.R. (2012) Promoter DNA methylation couples genome-defence mechanisms to epigenetic reprogramming in the mouse germline. *Development*, **139**, 3623–3632.
18. Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K. and Hannon, G.J. (2007) Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science*, **316**, 744–747.
19. Öllinger, R., Childs, A.J., Burgess, H.M., Speed, R.M., Lundegaard, P.R., Reynolds, N., Gray, N.K., Cooke, H.J. and Adams, I.R. (2008) Deletion of the pluripotency-associated Tex19.1 gene causes activation of endogenous retroviruses and defective spermatogenesis in mice. *PLoS Genet.*, **4**, e1000199.
20. Ma, L., Buchold, G.M., Greenbaum, M.P., Roy, A., Burns, K.H., Zhu, H., Han, D.Y., Harris, R.A., Coarfa, C., Gunaratne, P.H. *et al.* (2009) GASZ is essential for male meiosis and suppression of retrotransposon expression in the male germline. *PLoS Genet.*, **5**, e1000635.
21. Frost, R.J.A., Hamra, F.K., Richardson, J.A., Qi, X., Bassel-Duby, R. and Olson, E.N. (2010) MOV10L1 is necessary for protection of spermatocytes against retrotransposons by Piwi-interacting RNAs. *Proc. Natl. Acad. Sci. USA*, **107**, 11847–11852.
22. Zheng, K., Xiol, J., Reuter, M., Eckardt, S., Leu, N.A., McLaughlin, K.J., Stark, A., Sachidanandam, R., Pillai, R.S. and Wang, P.J. (2010) Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway. *Proc. Natl. Acad. Sci. USA*, **107**, 11841–11846.
23. Reynolds, N., Collier, B., Maratou, K., Bingham, V., Speed, R.M., Taggart, M., Semple, C.A., Gray, N.K. and Cooke, H.J. (2005) Dazl binds

in vivo to specific transcripts and can regulate the pre-meiotic translation of Mvh in germ cells. *Hum. Mol. Genet.*, **14**, 3899–3909.
24. Chen, J., Melton, C., Suh, N., Oh, J.S., Horner, K., Xie, F., Sette, C., Blelloch, R. and Conti, M. (2011) Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition. *Genes Dev.*, **25**, 755–766.
25. Chapman, V., Forrester, L., Sanford, J., Hastie, N. and Rossant, J. (1984) Cell lineage-specific undermethylation of mouse repetitive DNA. *Nature*, **307**, 284–286.
26. Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N. and Cedar, H. (1984) Variations in DNA methylation during mouse cell differentiation *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. USA*, **81**, 2275–2279.
27. Popp, C., Dean, W., Feng, S., Cokus, S.J., Andrews, S., Pellegrini, M., Jacobsen, S.E. and Reik, W. (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature*, **463**, 1101–1105.
28. Schütt, S., Florl, A.R., Shi, W., Hemberger, M., Orth, A., Otto, S., Schulz, W.A. and Fundele, R.H. (2003) DNA methylation in placentas of interspecies mouse hybrids. *Genetics*, **165**, 223–228.
29. Haig, D. (2012) Retroviruses and the placenta. *Curr. Biol.*, **22**, R609–R613.
30. Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E. *et al.* (2001) Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. *Nat. Genet.*, **27**, 31–39.
31. Tsumura, A., Hayakawa, T., Kumaki, Y., Takebayashi, S., Sakaue, M., Matsuoka, C., Shimotohno, K., Ishikawa, F., Li, E., Ueda, H.R. *et al.* (2006) Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b. *Genes Cells*, **11**, 805–814.
32. Thorrez, L., Van Deun, K., Tranchevent, L.-C., Van Lommel, L., Engelen, K., Marchal, K., Moreau, Y., Van Mechelen, I. and Schuit, F. (2008) Using ribosomal protein genes as reference: a tale of caution. *PLoS One*, **3**, e1854.
33. Bourc'his, D. and Bestor, T.H. (2004) Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature*, **431**, 96–99.
34. Simmons, D.G. and Cross, J.C. (2005) Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. *Dev. Biol.*, **284**, 12–24.
35. Rossant, J. and Croy, B.A. (1985) Genetic identification of tissue of origin of cellular populations within the mouse placenta. *J. Embryol. Exp. Morphol.*, **86**, 177–189.
36. Celebi, C., van Montfoort, A., Skory, V., Kieffer, E., Kuntz, S., Mark, M. and Viville, S. (2012) Tex 19 paralogs exhibit a gonad and placenta-specific expression in the mouse. *J. Reprod. Dev.*, **58**, 360–365.
37. Yang, F., Cheng, Y., An, J.Y., Kwon, Y.T., Eckardt, S., Leu, N.A., McLaughlin, K.J. and Wang, P.J. (2010) The ubiquitin ligase Ubr2, a recognition E3 component of the N-end rule pathway, stabilizes Tex19.1 during spermatogenesis. *PLoS One*, **5**, e14017.
38. Krackow, S. and Gruber, F. (1990) Sex ratio and litter size in relation to parity and mode of conception in three inbred strains of mice. Lab Anim. (NY), **24**, 345–352.
39. Shaut, C.A.E., Keene, D.R., Sorensen, L.K., Li, D.Y. and Stadler, H.S. (2008) HOXA13 is essential for placental vascular patterning and labyrinth endothelial specification. *PLoS Genet.*, **4**, e1000073.
40. Simmons, D.G., Natale, D.R.C., Begay, V., Hughes, M., Leutz, A. and Cross, J.C. (2008) Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. *Development*, **135**, 2083–2091.
41. Simmons, D.G., Rawn, S., Davies, A., Hughes, M. and Cross, J.C. (2008) Spatial and temporal expression of the 23 murine prolactin/placental lactogen-related genes is not associated with their position in the locus. *BMC Genomics*, **9**, 352.
42. Coan, P.M., Conroy, N., Burton, G.J. and Ferguson-Smith, A.C. (2006) Origin and characteristics of glycogen cells in the developing murine placenta. *Dev. Dyn.*, **235**, 3280–3294.
43. Beck, F., Erler, T., Russell, A. and James, R. (1995) Expression of Cdx-2 in the mouse embryo and placenta: Possible role in patterning of the extra-embryonic membranes. *Dev. Dyn.*, **204**, 219–227.
44. Finkenzeller, D., Fischer, B., Lutz, S., Schrewe, H., Shimizu, T. and Zimmermann, W. (2003) Carcinoembryonic antigen-related cell adhesion molecule 10 expressed specifically early in pregnancy in the decidua is dispensable for normal murine development. *Mol. Cell. Biol.*, **23**, 272–279.
45. Bode, S., Peters, C. and Deussing, J.M. (2005) Placental cathepsin M is alternatively spliced and exclusively expressed in the spongiotrophoblast layer. *Biochim. Biophys. Acta*, **1731**, 160–167.
46. Barrett, T., Troup, D.B., Wilhite, S.E., Ledoux, P., Evangelista, C., Kim, I.F., Tomashevsky, M., Marshall, K.A., Phillippy, K.H., Sherman, P.M. *et al.* (2010) NCBI GEO: archive for functional genomics data sets–10 years on. *Nucleic Acids Res.*, **39**, D1005–D1010.
47. Hudson, Q.J., Kulinski, T.M., Huetter, S.P. and Barlow, D.P. (2010) Genomic imprinting mechanisms in embryonic and extraembryonic mouse tissues. *Heredity*, **105**, 45–56.
48. Fowden, A.L., Coan, P.M., Angiolini, E., Burton, G.J. and Constancia, M. (2011) Imprinted genes and the epigenetic regulation of placental phenotype. *Prog. Biophys. Mol. Biol.*, **106**, 281–288.
49. Salas, M., John, R., Saxena, A., Barton, S., Frank, D., Fitzpatrick, G., Higgins, M.J. and Tycko, B. (2004) Placental growth retardation due to loss of imprinting of Phlda2. *Mech. Dev.*, **121**, 1199–1210.
50. Tunster, S.J., Tycko, B. and John, R.M. (2010) The imprinted Phlda2 gene regulates extraembryonic energy stores. *Mol. Cell. Biol.*, **30**, 295–306.
51. Mi, S., Lee, X., Li, X., Veldman, G.M., Finnerty, H., Racie, L., LaVallie, E., Tang, X.-Y., Edouard, P., Howes, S. *et al.* (2000) Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature*, **403**, 785–789.
52. Dupressoir, A., Vernochet, C., Harper, F., Guégan, J., Dessen, P., Pierron, G. and Heidmann, T. (2011) A pair of co-opted retroviral envelope syncytin genes is required for formation of the two-layered murine placental syncytiotrophoblast. *Proc. Natl. Acad. Sci. USA*, **108**, E1164–E1173.
53. Ono, R., Nakamura, K., Inoue, K., Naruse, M., Usami, T., Wakisaka-Saito, N., Hino, T., Suzuki-Migishima, R., Ogonuki, N., Miki, H. *et al.* (2006) Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. *Nat. Genet.*, **38**, 101–106.
54. Sekita, Y., Wagatsuma, H., Nakamura, K., Ono, R., Kagami, M., Wakisaka, N., Hino, T., Suzuki-Migishima, R., Kohda, T., Ogura, A. *et al.* (2008) Role of retrotransposon-derived imprinted gene, Rtl1, in the feto-maternal interface of mouse placenta. *Nat. Genet.*, **40**, 243–248.
55. Reiss, D., Zhang, Y. and Mager, D.L. (2007) Widely variable endogenous retroviral methylation levels in human placenta. *Nucleic Acids Res.*, **35**, 4743–4754.
56. Hackett, J.A., Zylicz, J.J. and Surani, M.A. (2012) Parallel mechanisms of epigenetic reprogramming in the germline. *Trends Genet.*, **28**, 164–174.
57. Santos, F., Hendrich, B., Reik, W. and Dean, W. (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev. Biol.*, **241**, 172–182.
58. Watanabe, D., Suetake, I., Tada, T. and Tajima, S. (2002) Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. *Mech. Dev.*, **118**, 187–190.
59. Baillie, J.K., Barnett, M.W., Upton, K.R., Gerhardt, D.J., Richmond, T.A., De Sapio, F., Brennan, P., Rizzu, P., Smith, S., Fell, M. *et al.* (2011) Somatic retrotransposition alters the genetic landscape of the human brain. *Nature*, **479**, 534–537.
60. Solyom, S., Ewing, A.D., Rahrmann, E.P., Doucet, T.T., Nelson, H.H., Burns, M.B., Harris, R.S., Sigmon, D.F., Casella, A., Erlanger, B. *et al.* (2012) Extensive somatic L1 retrotransposition in colorectal tumors. *Genome Res*, **22**, 2328–2338.
61. Singer, T., McConnell, M.J., Marchetto, M.C.N., Coufal, N.G. and Gage, F.H. (2010) LINE-1 retrotransposons: mediators of somatic variation in neuronal genomes? *Trends Neurosci.*, **33**, 345–354.
62. Mao, J., Zhang, X., Sieli, P.T., Falduto, M.T., Torres, K.E. and Rosenfeld, C.S. (2010) Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. *Proc. Natl. Acad. Sci. USA*, **107**, 5557–5562.
63. Gabory, A., Ferry, L., Fajardy, I., Jouneau, L., Gothié, J.-D., Vigé, A., Fleur, C., Mayeur, S., Gallou-Kabani, C., Gross, M.-S. *et al.* (2012) Maternal diets trigger sex-specific divergent trajectories of gene expression and epigenetic systems in mouse placenta. *PLoS One*, **7**, e47986.
64. Ji, R.R., Zhang, X., Wiesenfeld-Hallin, Z. and Hökfelt, T. (1994) Expression of neuropeptide Y and neuropeptide Y (Y1) receptor mRNA in

rat spinal cord and dorsal root ganglia following peripheral tissue inflammation. *J. Neurosci.*, **14**, 6423–6434.
65. Horst, A.K., Bickert, T., Brewig, N., Ludewig, P., van Rooijen, N., Schumacher, U., Beauchemin, N., Ito, W.D., Fleischer, B., Wagener, C. *et al.* (2009) CEACAM1+ myeloid cells control angiogenesis in inflammation. *Blood*, **113**, 6726–6736.
66. Bersinger, N.A. and Ødegård, R.A. (2004) Second- and third-trimester serum levels of placental proteins in preeclampsia and small-for-gestational age pregnancies. *Acta Obstet. Gynecol. Scand.*, **83**, 37–45.
67. Kumaki, Y., Oda, M. and Okano, M. (2008) QUMA: quantification tool for methylation analysis. *Nucleic Acids Res.*, **36**, W170–W175.
68. R Development Core Team (2012) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/. (date last accessed February 2013).
69. Du, P., Kibbe, W.A. and Lin, S.M. (2008) Lumi: a pipeline for processing illumina microarray. *Bioinformatics*, **24**, 1547–1548.
70. Barbosa-Morais, N.L., Dunning, M.J., Samarajiwa, S.A., Darot, J.F.J., Ritchie, M.E., Lynch, A.G. and Tavaré, S. (2010) A re-annotation pipeline for illumina beadarrays: improving the interpretation of gene expression data. *Nucleic Acids Res.*, **38**, e17.
71. Smyth, G.K. (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.*, **3**, Article 3.
72. Gautier, L., Cope, L., Bolstad, B.M. and Irizarry, R.A. (2004) Affy–analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics*, **20**, 307–315.
73. Mietz, J.A., Grossman, Z., Lueders, K.K. and Kuff, E.L. (1987) Nucleotide sequence of a complete mouse intracisternal A-particle genome: relationship to known aspects of particle assembly and function. *J. Virol.*, **61**, 3020–3029.
74. Martin, S.L. and Branciforte, D. (1993) Synchronous expression of LINE-1 RNA and protein in mouse embryonal carcinoma cells. *Mol. Cell. Biol.*, **13**, 5383–5392.