

# DNA methylation divergence and tissue specialization in the developing mouse placenta

Benjamin E. Decato,<sup>1</sup> Jorge Lopez-Tello,<sup>2</sup> Amanda Sferruzzi-Perri,<sup>2</sup> Andrew D. Smith,<sup>1</sup> and Matthew D. Dean<sup>\*,1</sup>

<sup>1</sup>Molecular and Computational Biology, Department of Biological Sciences, University of Southern California, Los Angeles, California. <sup>2</sup>Centre for Trophoblast Research, Department of Physiology, Development, and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK

\*Corresponding author: E-mail: matthew.dean@usc.edu.

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All placental methylomes produced for this study are available under GEO accession GSE84350.

## Abstract

The placental epigenome plays a vital role in regulating mammalian growth and development. Aberrations in placental DNA methylation are linked to several disease states, including intrauterine growth restriction and preeclampsia. Studying the evolution and development of the placental epigenome is critical to understanding the origin and progression of such diseases. Although high resolution studies have found substantial variation between placental methylomes of different species, the nature of methylome variation has yet to be characterized within any individual species. We conducted a study of placental DNA methylation at high resolution in multiple strains and closely related species of house mice (*Mus musculus musculus*, *Mus m. domesticus*, and *M. spretus*), across developmental timepoints (embryonic days 15 to 18), and between two distinct layers (labyrinthine transport and junctional endocrine). We observed substantial genome-wide methylation heterogeneity in mouse placenta compared to other differentiated tissues. Species-specific methylation profiles were concentrated in retrotransposon subfamilies, specifically RLTR10 and RLTR20 subfamilies. Regulatory regions such as gene promoters and CpG islands displayed cross-species conservation, but showed strong differences between layers and developmental timepoints. Partially methylated domains exist in the mouse placenta and widen during development. Taken together, our results characterize the mouse placental methylome as a highly heterogeneous and deregulated landscape globally, intermixed with actively regulated promoter and retrotransposon sequences.

Key words: DNA Methylation, Placenta, Junctional Zone, Labyrinthine Zone, Epigenome

## Introduction

The placenta forms the crucial link between mother and developing offspring during mammalian pregnancy. It is responsible for

anchoring the fetus to the uterine wall, secretes hormones that adapt maternal physiology, prevents immunological rejection of the fetus, and exchanges substrates between fetal and maternal blood spaces. These functions are

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strongly conserved across mammals, despite extraembryonic tissues displaying remarkable morphological variation (Furukawa *et al.*, 2014) and placenta-specific genes accumulating a high rate of nonsynonymous mutations (Chuong *et al.*, 2010; Hughes *et al.*, 2000).

To effectively perform its wide array of functions, the placenta is composed of multiple trophoblast cell types which in the mouse are organized into specialized zones. The junctional zone lies proximal to the uterine wall and is composed of invasive endocrine trophoblast cells: the spongiotrophoblast, glycogen and giant cells. These cell types are important in promoting maternal immune tolerance, decidual vascularization and maternal metabolic adjustments that favour fetal nutrient delivery (Hu and Cross, 2010). The other layer of the mouse placenta is called the labyrinthine zone, and lies proximal to the developing embryo. It is composed of a dense network of fetal capillaries and maternal blood spaces that are lined with syncytiotrophoblast cells, which exchange nutrients, gases and waste between mother and fetus (Coan *et al.*, 2005; Sferruzzi-Perri *et al.*, 2009). Genomic studies have provided insight into placentation and diversification of placental morphology (Cross, 2000; Roberts and Cooper, 2001), and the recent maturation of assays designed to explore epigenetic features allows us to study these phenomena at unprecedented resolution.

DNA methylation occurs primarily on cytosines of CpG dinucleotides in mammals (Bird, 1985). Across the genome, most CpGs are methylated. In somatic cells, hypomethylated intervals have an average size of up to a few kilobases, although larger intervals exist. These regions co-locate with promoters and enhancers, and methylation through these intervals is associated with gene silencing and restriction of regulatory activity (Jones, 2012). Retrotransposons are methylated in most cell types, and this phenomenon is one form of genomic defense against their expression (Walsh *et al.*, 1998). DNA methylation changes in measurable and consistent ways as tissues differentiate (Seisenberger *et al.*, 2012) and can be compared across species (Molaro *et al.*, 2011; Pai *et al.*, 2011), enabling a precise characterization of cellular and species identity.

In mammals, around 70% of the cytosines in CpG dinucleotides are methylated in somatic cells (Song *et al.*, 2013), compared with closer to 50% methylated in the placenta (Ehrlich *et al.*, 1982; Razin *et al.*, 1984). A large body of evidence suggests that the placental methylome plays a critical functional role, and targeted assays have implicated aberrant DNA methylation in several placental disease phenotypes, including pre-eclampsia (Hogg *et al.*, 2013; Kulkarni *et al.*, 2011; Yuen *et al.*, 2010) and growth restriction (Banister *et al.*, 2011; Lambertini *et al.*, 2011). The recent advent of whole genome bisulfite sequencing (WGBS) has provided higher resolution maps

of DNA methylation in the placenta that have confirmed this lower methylation and detected the presence of partially methylated domains (PMDs), long stretches of the genome where methylation levels drop below the background, primarily hypermethylated state (Schroeder *et al.*, 2013). PMD presence is correlated with changes in gene expression, and outside of placenta they have only been observed in cancer and cultured cell lines (excluding ESCs and iPSCs). (Hansen *et al.*, 2011; Lister *et al.*, 2009).

A recent study found that despite widespread morphological changes, the placenta’s globally lowered methylation state is a common feature across mammalian species (Schroeder *et al.*, 2015). Reduced levels of methylation may allow for species-specific endogenous retroviral activity in the placenta, and could represent regulatory variation that is typically silenced in other tissues (Chuong *et al.*, 2013). Prior work also observed similarity between trophoblast methylomes and oocytes, suggesting minimal *de novo* methylation in extraembryonic-derived tissues following fertilization (Schroeder *et al.*, 2015). Knocking out *de novo* methyltransferases Dnmt3a/3b in trophoblasts resulted in few defects compared to wild type at embryonic day 9.5, further supporting a reduction or lack of *de novo* methylation in placenta to that timepoint (Branco *et al.*, 2016).

Little is known about how the placental epigenome varies within an individual species.

A full understanding of this within-species heterogeneity must precede the identification of meaningful between-species differences. In this study, we explored the placental methylome from 6 strains of 3 closely related mouse species and report a globally deregulated epigenome relative to other differentiated tissues. Species-specific methylation patterns primarily existed inside retrotransposon subfamilies, particularly in RLTR10 and RLTR20 subfamilies. Regulatory regions such as CpG islands and promoter regions displayed conservation across species. Promoter methylation levels showed a unique distribution wherein highly methylated promoters displayed intermediate methylation levels, rather than near-complete methylation as observed in other tissues. We used differential expression between placenta and other tissues to show that this intermediate methylation remains associated with gene repression.

Additionally, we produced the first purified methylomes of the functionally distinct placental junctional and labyrinthine zones at two developmental timepoints. We identified a subtle but consistent hypomethylation of the junctional zone globally, as well as many concentrated differences at gene promoters. Promoter differences were enriched on the X chromosome, and most differentially methylated promoters were hypermethylated in the junctional zone relative to the labyrinthine. Differential methylation between developmental timepoints uncovered evidence

for progressive PMD formation in the placenta as well as widespread *de novo* methylation, suggesting a role for the epigenome in mediating differentiation of the layers towards term. In spite of earlier studies suggesting the methylation in the placenta is static, our studies demonstrate a dynamic methylation program that varies by species, genetic strain, layer, and developmental timepoint.

### Results

Our study relied upon two datasets, which we will refer to as the “interspecific” and “intraspecific” datasets, respectively. The interspecific dataset included 12 WGBS methylomes from three species, including four samples from each of *Mus musculus musculus*, *M. m. domesticus*, and *M. spretus*. We reduced the potential confounds of inbreeding and litter effects by inter-crossing two strains per species. We sequenced to an average depth of 6.2x per covered CpG per sample, and surveyed an average of 77% of CpGs genome-wide per placenta.

The intraspecific dataset concentrated on a single genetic strain, C57BL/6J, the genome reference. We produced 24 WGBS methylomes from the two main placental layers (labyrinthine zone [LZ] and junctional zone [JZ]), from two developmental timepoints (embryonic days 15 [E15] and 18 [E18]), and from male and female siblings collected from three different litters. In this intraspecies dataset, each sample was sequenced to an average depth of 1.45x per

covered CpG, and surveyed on average 50% of CpGs genome-wide. Replicates for each factor allowed us to combine these methylomes for high coverage where necessary and increase statistical power to detect differences across factors. Due to poor quality one sample was thrown out.

The full experimental design and quality control statistics for all methylomes produced for this study can be found in Supplemental Figure 1 and Supplemental Tables 1 and 2, and descriptions of the mouse strains used can be found in the Methods.

Placenta DNA methylation is globally heterogeneous but highly conserved at regulatory regions

We observed global hypomethylation of the mouse placenta relative to other tissues: genome-wide methylation levels across interspecific samples ranged from 43.3% to 53.8%, and varied by species (ANOVA,  $p < 0.015$ ). Comparison of placental methylation levels in whole placental samples at single CpG resolution also revealed significantly higher within-tissue heterogeneity when compared with other fully differentiated tissues. To illustrate this, we computed the Pearson correlation and Euclidean distance between all pairs of whole placenta samples from the same species. We plotted these as a boxplot, together with boxplots of pairwise correlation and distance for three other tissues: brain (Lister *et al.*, 2013), intestine (Hon *et al.*, 2013; Kaaij *et al.*, 2013; Sheaffer *et al.*, 2014) and blood (Kieffer-Kwon

*et al.*, 2013) (Figure 1A). Despite this within-tissue variability, genome-wide methylation levels clustered reasonably well by strain and species in the placenta (Figure 1B), although they did not precisely capture the true species-level evolutionary relationship (Sarver *et al.*, in press; Tucker, 2006). Intra-species samples clustered well by layer, however both comparisons of single-CpG heterogeneity and pairwise binned correlations suffered from substantially lower sequencing depth (Supplemental Figure 2A,B). In the intraspecific dataset, the junctional zone was less methylated than the labyrinthine zone ( $p < 0.017$ ) (Figure 1C), but there was no significant difference in global levels of CpG methylation by developmental timepoint or sex.

DNA methylation is an important component of transcriptional regulation, with methylation of retrotransposons and gene promoters strongly correlated with their repression (Boyes and Bird, 1991). Figure 1D presents methylation levels in gene promoters in the placenta of each mouse species and high quality WGBS embryonic stem cell (ESC) methylomes from four separate projects curated in MethBase (Harten *et al.*, 2015; Li *et al.*, 2015; Lu *et al.*, 2014; Song *et al.*, 2013; Yearim *et al.*, 2015). In the placenta, promoter methylation remained bimodal, but with the high mode typically associated with transcriptional repression shifted from the near-complete methylation seen in other tissues to intermediate levels.

To explore how this reduced promoter methylation level might relate to transcriptional regulation, we identified differentially expressed (DE) genes between 5 E14.5 C57Bl6/J x FVB/n mouse placenta RNA-seq experiments (Mould *et al.*, 2013) and ESC RNA-seq data derived from two of the studies above with matched methylation and expression data (Lu *et al.*, 2014; Yearim *et al.*, 2015). We identified differentially expressed genes (see Methods) and plotted them by their normalized counts and log fold change between ESC and placenta (Supplemental Figure 3A). We filtered for DE genes with a log-fold change of greater than 5 and counts per million of less than 5, leaving us 797 and 733 DE genes expressed higher in placenta and ESC, respectively, and plotted the promoter methylation distributions for these genes (Figure 1E). For genes that were higher expressed in placenta, we observed nearly complete methylation of the promoter in ESCs and hypomethylation of the promoter in placenta. In genes higher expressed in ESCs however, the promoter methylation levels of placenta reached only intermediate levels. This pattern was conserved when considering the promoter methylation distributions of differentially expressed genes between placenta and other tissues, including brain (Lister *et al.*, 2013), intestine (Sheaffer *et al.*, 2014), and blood (Kieffer-Kwon *et al.*, 2013) (Supplemental Figures 3 and 4, all DE genes included in Supplemental

Table 3). These observations indicate that epigenomic repression of transcription in the placenta does not require methylation levels as high as seen in other tissues.

Another unique feature of the placental methylome is the hypomethylation of retrotransposons. Almost all retrotransposons are methylated in most other tissues, but show a relaxed methylation state in the placenta (Figure 1F). Of the 2,191,618 annotated retrotransposons overlapping at least one CpG, we sequenced to a depth of at least 5 observations per copy in 96.2%, 95.0%, and 81.9% of all retrotransposons in *M. m. domesticus*, *M. m. musculus*, and *M. spretus* respectively (see Methods). Comparisons of these interspecies samples revealed uniformly high correlation and low Euclidean distance for promoters and CpG islands, indicating conservation of epigenomic state at these regulatory regions. In contrast, we observed strong species-specific patterns in all classes of retrotransposons (Figures 1G,H). These patterns are consistent with an arms race hypothesis (Crespi and Nosil, 2013), where methylation divergence is driven by conflict with genomic parasites.

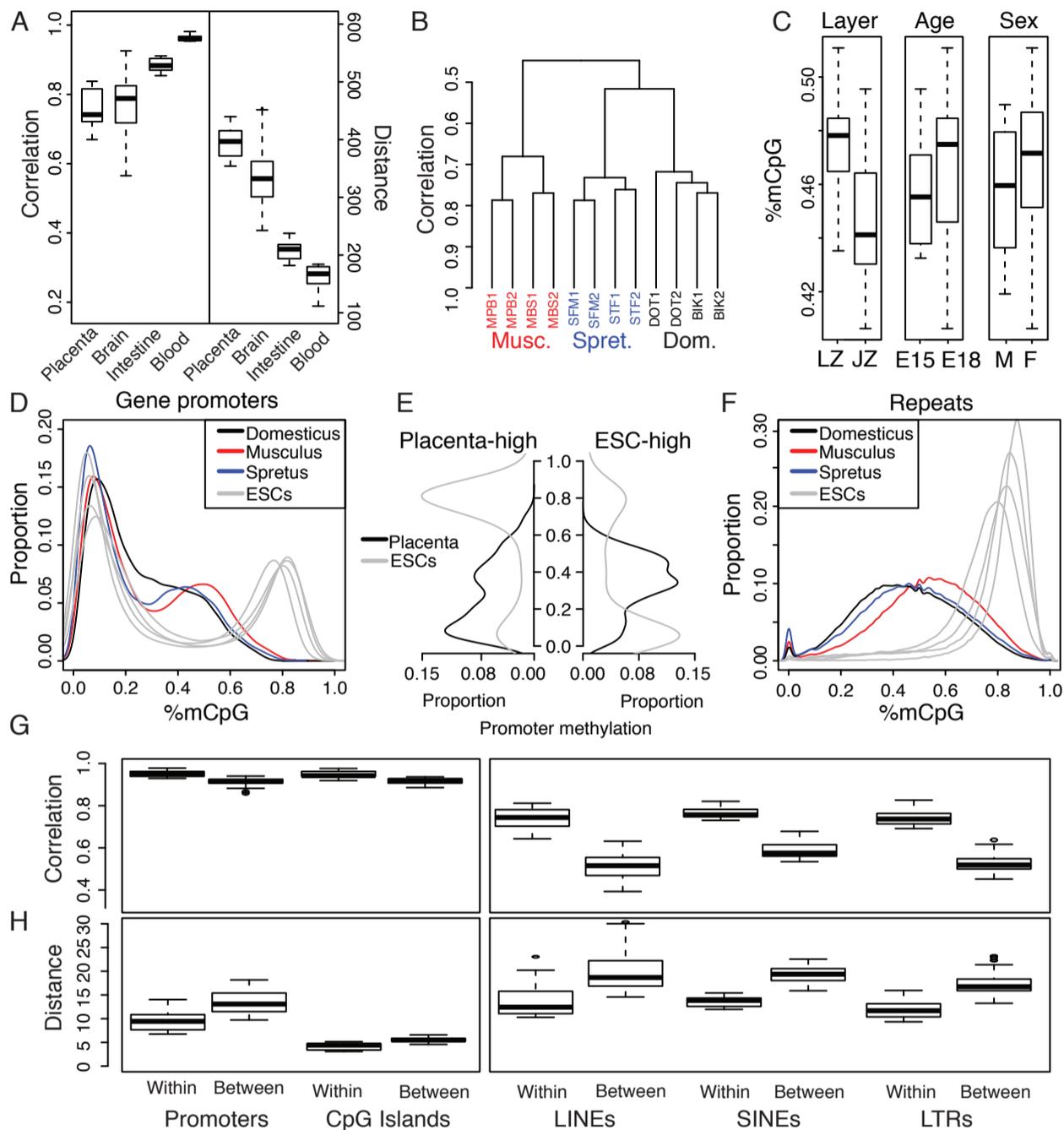
#### Quantifying species- and layer-specific methylation changes in the placenta

To identify the biologically meaningful and statistically significant epigenetic differences driven by species, layer, and developmental timepoints, we used RADMeth to find

differentially methylated (DM) CpGs, combine the p-values of neighboring (within 100bp) CpGs, and perform false discovery rate correction according to Benjamini and Hochberg (1995).

To compare species, we identified DM CpGs for each species relative to the other two species combined. This allows us to identify features specific to each species.

To compare layers and developmental timepoints, we accounted for the full experimental design to avoid confounding by other factors. Importantly, we removed one female sample from the junctional zone (M1043-F5-F15-JZ) from the same developmental timepoint as the missing LZ sample to ensure equal numbers of male and female samples while calling differential methylation. Principle component analysis (Wold *et al.*, 1987) using our identified DM CpGs as the feature set for each factor showed a clear segregation by factor status (Supplemental Figure 5). Most of the significantly DM CpGs between layers are hypomethylated in the junctional zone relative to the labyrinthine zone (Figure 2A). We also detected a sizeable number of CpG sites whose methylation level increased between E15 and E18 (Figure 2B), suggesting that while previous studies on Dnmt3a/b knockout mice revealed normal trophoblast formation during early development, *de novo* methylation likely plays a role in the late stage development and differentiation of the placental layers.



**FIG. 1.** DNA methylation is variable in the placenta, except at regulatory regions. (A) Pearson correlation and Euclidean distance between pairs of whole placenta samples, compared with pairwise correlation and distance between samples of other tissues at single CpG resolution. (B) Hierarchical clustering of pairwise binned correlation for whole placental samples in 1kb bins. Three-letter codes indicate genetic strain, number indicates individual. (C) Global methylation between layers, timepoints, and sex. (D) Promoter methylation density plot comparing inter-species placenta and ESCs. (E) Promoter methylation distributions in placenta and ESC for genes upregulated in placenta (left) and ESC (right). (F) Retrotransposon methylation density plot. (G, H) Within- and between-species pairwise correlation and distance by genomic feature.

To identify the sources of species-, layer-, and age-specific variation, we investigated DM CpG occupancy inside various genomic regions (Figure 2C). We observed an order of magnitude more differences by species than by layer or age, and

DM CpGs seem to be uniformly distributed throughout the genome. To identify the DM CpGs that are most likely to drive meaningful differences in transcriptional regulation, we focused on those located in gene promoters. For each promoter, we

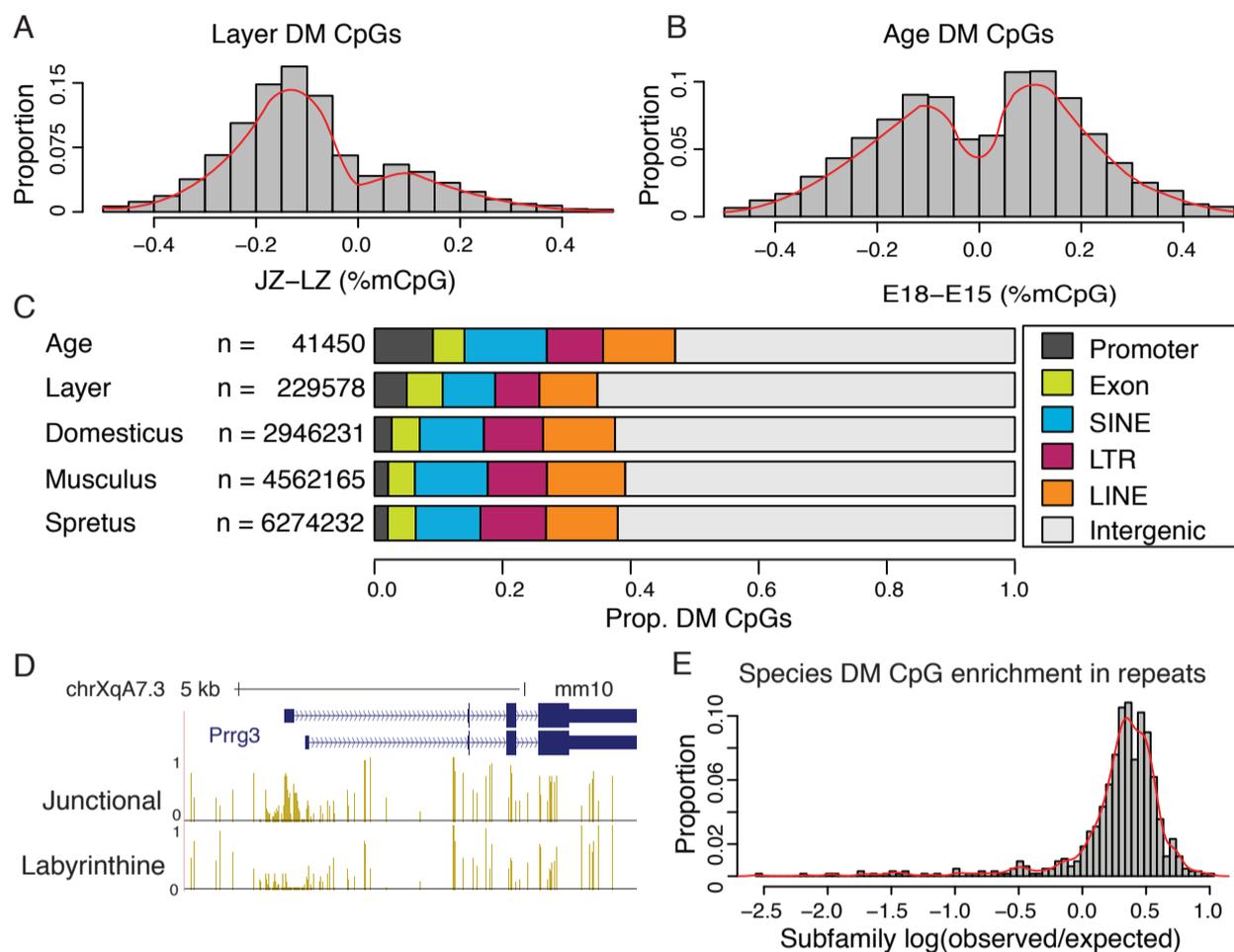
counted the number of significantly DM CpGs with at least 30% methylation difference between levels for each factor. Between layers, this yielded 5 genes with at least 10 DM promoter CpGs, with the top two (*Srrt* and *Zmym3*) having 45 and 25 DM CpGs respectively. Three of these genes were on the X-chromosome, and analysis of male and female samples separately show conservation of magnitude and directionality of this differential methylation between sexes (Supplemental figure 6A-C). Subject to the same analysis, differences between ages yielded only 2 genes with at least 10 DM CpGs (Table 1). Of note, the next highest difference between ages (8 DM CpGs) was *Tjp1*, a human ortholog of which was previously associated with trophoblast cell differentiation and whose promoter was methylated in E18 samples (Pidoux *et al.*, 2010). No gene set was enriched for placenta-related gene ontology terms.

Interestingly, differentially methylated gene promoters between layers were enriched on the X-chromosome and primarily hypermethylated in the junctional zone, despite most DM CpGs showing junctional hypomethylation. An example of junctional zone promoter hypermethylation is shown in Figure 2D.

An analysis of the X-chromosome revealed global hypomethylation relative to autosomes ( $p < 3.56e-05$ , Supplemental Figure 7A) in females but similar methylation levels in males. We also observed elevated methylation levels in CpG islands of both male and female X

chromosomes relative to autosomes, but with greater levels in female placentas ( $p < 2.21e-10$ , Supplemental Figure 7B), suggesting CpG islands have elevated methylation levels on the inactive X. The male CpG island methylation increase is slightly enriched in the junctional layer ( $p < 0.03$ , Supplemental Figure 7C).

To better understand the strong species-specific retrotransposon signals we observed, we utilized the RepeatMasker annotation of retrotransposons in the LINE, SINE, and LTR classes, which was downloaded via the UCSC Table Browser tool (Karolchik *et al.*, 2004). We computed the enrichment of species-specific DM CpGs in each retrotransposon subfamily given each subfamily's total CpG density. The distribution of observed over expected (O/E) ratios of DM CpG occupancy inside retrotransposon subfamilies is notably shifted to the right, indicating that almost all retrotransposon subfamilies are more differentially methylated between species than expected by chance (Figure 3E). By filtering for subfamilies with at least 50 DM CpGs and O/E ratio of at least 2x, we saw almost exclusive enrichment in RLTR10 and RLTR20 subfamilies (Supplemental Table 4), notably in the same broad group of ERVs (ERV2) as the retrotransposons bearing species-specific enhancers identified previously (Chuong *et al.*, 2013).



**FIG. 2.** Methylomes differ between layers, developmental timepoints, and species. (A, B) Directional DM CpG methylation distributions between layer and age. (C) DM CpG quantity and location by genomic region, with  $N$  equal to the total number of DM CpGs for each factor. (D) Example promoter that is differentially methylated between layers. (E) Distribution of enrichment of *M. musculus*-specific DM CpGs in retrotransposon subfamilies, showing enrichment ( $\log O/E > 0$ ) of differential methylation in almost every subfamily.

Rank	Layer	# DM CpGs	Age	# DM CpGs
1	Srrt	45	Cdc42	16
2	Zmym3*	25	Picalm	12
3	Stag2*	25	Tjp1	8
4	Prrg3*	11		
5	1810009A15Rik	10		

**Table 1.** Top differentially methylated promoters between placental layers and developmental timepoints. Between layers, there is an enrichment for X-chromosome genes (asterisk).

Progressive PMD formation in the mouse placenta

PMDs are megabase-scale stretches of the genome with consistently low methylation relative to the background of genome-wide equilibrium

methylation level. They were first observed in human immortalized cell lines (Lister *et al.*, 2009) and later found to be present in cancer (Berman *et al.*, 2012; Hansen *et al.*, 2011) and

then observed in human placenta methylomes (Schroeder *et al.*, 2013). In contrast to a prior study that reported the absence of PMDs in mouse placenta (Schroeder *et al.*, 2015), we identified a highly reproducible segmentation of the methylome in all three mouse species into background and PMD regions using the HMM approach described in Song *et al.* (2013) (Figure 3A). This method segments the methylome based on consecutive observations of weighted average methylation levels inside 1kb bins, reducing the effect of local hypermethylation introduced by regulatory regions such as gene promoters or enhancers. We compared the previously reported mouse placenta methylome to our own data and found PMDs covering 26.5% of the genome and reaching similar in-PMD methylation levels to our own using the same identification technique, with a bin size of 20kb to compensate for substantially lower coverage than our own whole placental samples (Supplemental Figure 8A).

Placental PMDs are located in gene poor regions and exist in both layers of the placenta (Figure 3A). Taking the union of PMDs across all inter-specific samples, we observed an overlap with 8,828 gene promoters compared with an expected 12,025, given the size of the genome and assuming a hypergeometric distribution of the overlaps. While PMD locations stayed generally constant, the overall fraction of the genome inside PMDs varied substantially, from nearly absent in the MPB strain to extremely

prevalent in the DOT strain (Figure 3B). For this interspecific data, the collection method for the whole placenta samples allowed the age of the embryo at dissection to vary up to 5 days (see Methods), and we observed an order-of-magnitude decrease in embryonic weight in the MPB strain that coincided with an earlier developmental stage and absent PMDs (Supplemental Table 1). Therefore we hypothesized that PMDs gradually appear over developmental time in the placenta. In the intraspecific data, analysis of PMD size between developmental timepoints in late gestation revealed an increase in the size of PMDs between E15 and E18 ( $p < 0.032$ ) (Figure 3C). While PMDs widened over time, the methylation level inside PMDs did not show significant differences between timepoints ( $p < 0.47$ ). The methylation levels outside of PMDs increased slightly but not significantly, consistent with the observed global methylation levels of the two timepoints. Corroborating the observations reviewed in Novakovic and Saffery (2013), the *Dnmt1* promoter is hypomethylated in mouse placenta at all timepoints, in all layers, and in all species, suggesting that PMD formation in the mouse placenta is likely not driven by differential expression of *Dnmt1*.

The globally lower methylation level in *M. spretus* compared to the other two species led to lower average methylation inside PMDs ( $p < 0.0006$ ) (Figure 3D) but similar PMD depth. Interestingly, despite the lack of PMDs in MPB

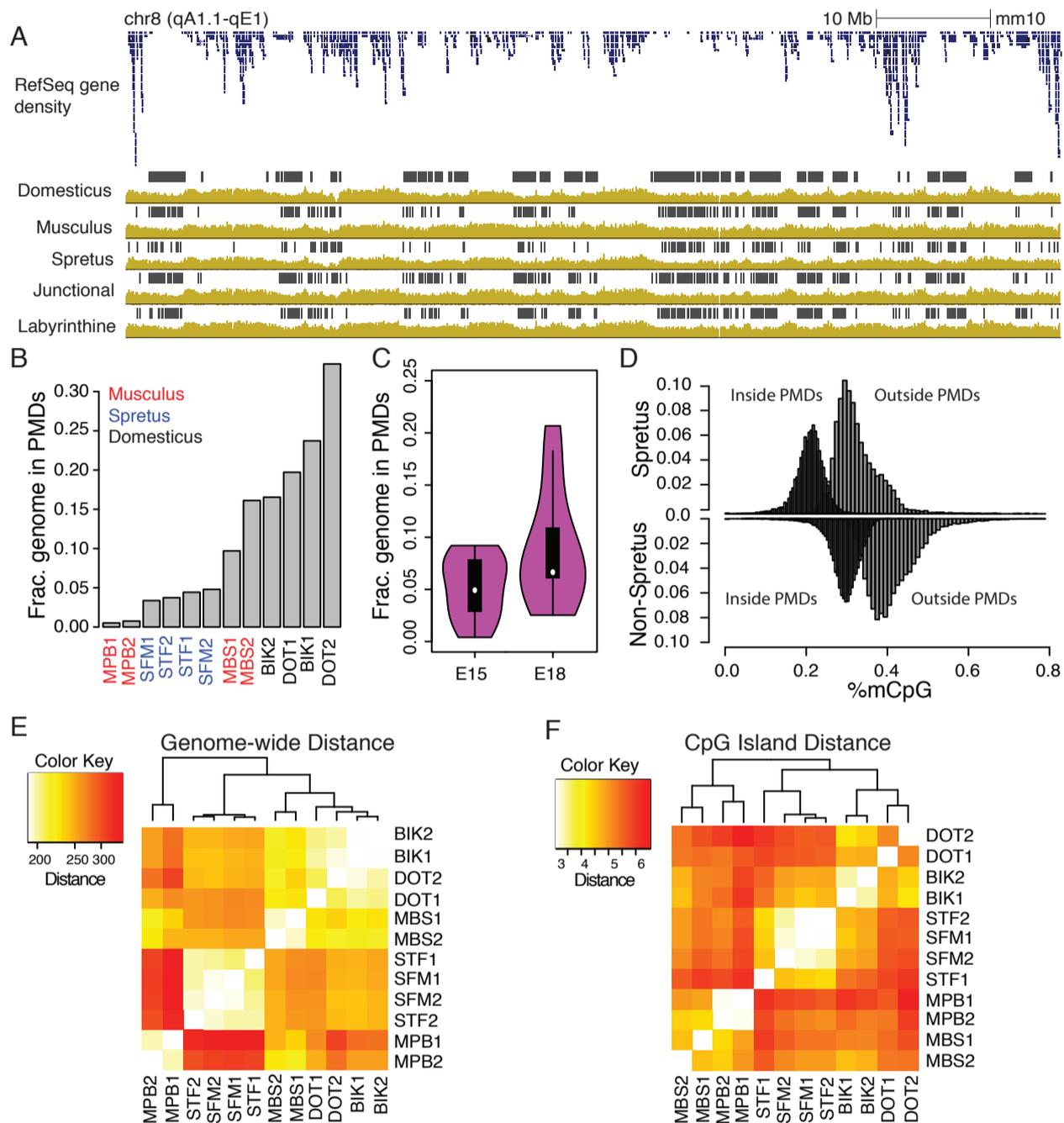
leading to large overall methylation differences between those and the other *M. musculus* samples (Figure 3E), CpG island methylation remained extremely close regardless of PMD presence. This suggests that CpG islands remain under direct regulation even inside PMDs (Figure 3F). Average methylation levels for CpG islands inside PMDs were slightly elevated in our mouse placenta samples, corroborating the finding in (Schroeder *et al.*, 2013). However, few CpG islands inside placental PMDs displayed methylation above 80% (Supplemental Figure 8B), while methylation of CpG islands inside cancer PMDs regularly exceeds 80% (Hansen *et al.*, 2011; Toyota *et al.*, 1999).

### Discussion

The placenta is a rapidly evolving organ that plays a temporary but essential role in mammalian development. In this study, we explored the role of the placental epigenome on its function and evolution. We characterized the placental epigenome as globally deregulated, possessing within-tissue methylation variance substantially higher than in other differentiated tissues. This noisy and globally hypomethylated state, relative to other tissues, remains a fundamentally distinct and poorly understood feature of placental cells. Recent studies have shown that this low methylation likely originated very early and persists through the trophoblast lineage (Branco *et al.*, 2016). In addition, the globally low rate of methylation appears to be a common

feature across distant species of mammal (Branco *et al.*, 2016; Schroeder *et al.*, 2015). We showed that global variation in placental methylation is conserved in differentiated placental layers and across later timepoints. The source of this variability could be rooted in the placenta’s transient nature, allowing its epigenome to erode during development without much harm to the overall success of the embryo. Another possibility is that the placenta’s adaptive response to its environment (Fowden and Moore, 2012; Sferruzzi-Perri and Camm, 2016) manifests in global methylation changes in subpopulations of cells not yet detectable without applying single-cell methods.

Despite globally heterogeneous methylation patterns, we observed similar levels of methylation across species at regulatory regions, including gene promoters and CpG islands both inside and outside of promoters (as annotated in the UCSC table browser, see Methods). While methylation in placental promoters showed within-tissue consistency, the actual distribution was placenta-specific, showing a shift in the “high” methylation range that remained correlated with repressive effects on gene expression. This opens up an interesting question: if the background methylation level in differentiated somatic cells is higher than needed for its role in gene expression, why does it remain at consistently high levels with such small variation between somatic cell types?



**FIG. 3.** Partially methylated domains exist in mouse placenta and are spatially conserved across species. (A) Methylation levels (yellow barplots) and identified PMD locations (grey boxes) in a selected genomic interval; genes shown in blue. (B) Genomic fraction covered by PMDs in whole placenta samples. (C) Genomic fraction covered by PMDs at E15 and E18 in intra-specific samples. (D) Distributions of CpG methylation levels inside and outside PMDs in *M. spretus* and non-*M. spretus* samples. (E) Pairwise distances between genome-wide methylation profiles (average level in 1kb bins). (F) Pairwise distance by species in CpG islands.

We produced the first methylomes of the junctional and labyrinthine zones and observed substantial *de novo* methylation between E15 and E18. Our results suggest that while Dnmt3a/3b may not be necessary in the early development of

the trophoblast (Branco *et al.*, 2016), it could play a role in later maturation of the placental layers. Using male and female placental methylomes, we indirectly explored methylation on the inactivated paternal X chromosome in females. We identified

globally lower methylation levels in females, but elevated CpG island methylation on the X chromosome, which contributes to X inactivation (Csankovszki *et al.*, 2001).

The impact of the globally lowered methylation state on retrotransposon activity in the placenta also remains poorly understood. Our analysis revealed that retrotransposon families were more likely to show species-specific methylation than expected, with differentially methylated CpGs especially enriched in members of the RLTR10 and RLTR20 subfamilies. This pattern stands in stark contrast to non-placental tissues, where retrotransposons are usually methylated (Bestor, 2000). This elevated tolerance to retro-element hypomethylation and expression may be required for placenta-specific phenomena, such as previously identified exaptation events of specific retrotransposons by the placenta to evade the maternal immune system (Feschotte and Gilbert, 2012; Mi *et al.*, 2000) or co-option of certain retrotransposon subfamilies as placenta-specific enhancer elements (Chuong *et al.*, 2013). In contrast to the study by Chuong *et al.* (2013), which identified placenta-specific enhancer elements at mouse-specific retrotransposons not present in the rat, we identified differential methylation in retrotransposons that are present in all species studied. This could represent more recent adaptations of the placental regulatory program, although further study is needed.

Though selection to maintain genome integrity in extra-embryonic tissues will certainly be lower than in the embryo or its germline, too much retro-element expression still represents a potential danger to genome integrity. Retro-element hypomethylation may be possible due to the redundant nature of mechanisms for retrotransposon silencing (Aravin *et al.*, 2007; Reichmann *et al.*, 2013), allowing their sequences to act as an enhancers for nearby genes while limiting their transcriptional activity. In turn, differentially methylated subfamilies may help fuel the rapid diversification of placenta-specific regulatory networks. Although the mechanisms of retrotransposon-derived enhancers have been studied previously (McDonald *et al.*, 1997; Ruda *et al.*, 2004), further study is needed to explore the direct impact of differential retrotransposon methylation state between species on the transcription of nearby orthologous genes, and to understand what, if any, role retrotransposon-mediated transcription may play in the human placenta.

To function properly, the placenta must invade and integrate with maternal tissues, a process that shares some similarities to the invasive behaviors of some cancers (Novakovic and Saffery, 2013). Partially methylated domains exist in the mouse placenta, are absent in our smallest, developmentally young embryos, and widen between E15 and E18, suggesting that they arise as a function of developmental time. These PMDs

share conserved locations across species, layer, and are found in the same gene-poor regions as in cancers. PMDs correlate with late replicating domains in human (Berman *et al.*, 2012), and therefore may arise in both cancer and placental cells as a consequence of rapid cell division outpacing the maintenance of methylation in these regions. CpG island hypermethylation is a hallmark of cancer methylomes and is enriched within cancer PMDs. Mouse placenta PMDs show no CpG island hypermethylation of the type reported in the PMDs of cancer methylomes (Berman *et al.*, 2012). Further studies are required to determine if PMDs have any significance in placental function. However, this shared feature of placental and cancer methylomes is striking and any model to explain PMDs will be more appealing if its explanatory power extends to both cancer and placenta epigenomes.

### Materials and Methods

#### Inter-species whole placenta tissue collection

All animal husbandry, experimental procedures, and personnel were approved by the University of Southern California’s Institutional Animal Care and Use Committee, protocol #11394. Mice were housed under a 14:10 hour light cycle with food and water *ad libitum*. To investigate species differences in placental methylation, crosses between wild derived inbred strains were established, developed and distributed by François Bonhomme and colleagues (U. Montpellier). For *M. domesticus*, we made reciprocal crosses

between strains BIK (originally isolated from Kefar Galim, Israel) and DOT (Tahiti); for *M. musculus*, MPB (Bialowieza, Poland) and MBS (Sokolovo, Bulgaria); and for *M. spretus*, STF (Fondouk Djedid, Tunisia) and SFM (Montpellier, France). For all crosses, a single stud and dam were housed together for four and a half days, and then split. 10 days later, females were euthanized, uteri were collected and the number of viable conceptuses counted, leading to gestational ages between E11 and E16. The embryos and placenta were dissected and weighed. Two placentae were sampled from a single litter in each cross direction and snap frozen in liquid nitrogen. Placenta are named according to their maternal strain. We attempted to sample only female placenta, through two replicated attempts to PCR-amplify two Y- and one X-linked region (Kunieda *et al.*, 1992), but for logistical reasons we had to include two male placentae.

#### Intra-species junctional and labyrinthine zone tissue collection

All experiments were carried out under the UK Home Office Animals (Scientific Procedures) Act 1986. C57Bl/6 females were housed under dark:light 12:12 conditions with free access to water and the standard diet used in the University of Cambridge Animal Facility. At 8-10 weeks, females were mated with C57Bl/6 males and the day a copulatory plug was found was denoted as embryonic day 1 of pregnancy (term=20.5 days). On embryonic days 15 or 18 of pregnancy

(days correspond to the periods of rapid placental and fetal growth respectively), mouse dams were schedule 1 killed by cervical dislocation. Uteri were collected and the number of viable conceptuses counted. Embryos and placentas were dissected and weighed. Each placenta from the litter was rapidly separated into the functionally distinct zones, the labyrinthine transport and junctional endocrine zones (Sferruzzi-Perri *et al.*, 2009), in ice cold sterile PBS before rapid snap freezing in liquid nitrogen. Fetal tails were kept for sexing using standard genotyping methods including using primers to detect the SRY gene (5'-CCCAGCATGCAAAATACAGA-3' and 5'-TCAACAGGCTGCCAATAAAA-3'), an internal control gene (5'-AGTGGCTAACGCTGAGTGGT-3' and 5'-GTGCCTGTCGGAGGAGAAC-3') and with agarose gel electrophoresis. From each litter, the male and female placenta with its weight closest to the litter mean was used for further analysis.

#### DNA Extraction and Methylation Assay

Using a Qiagen DNA extraction kit, DNA was extracted and purified for all inter- and intra-species samples. DNA was fragmented to 100-300bp fragments by sonication and end repaired before ligation of methylated sequencing adapters. Bisulfite treatment was performed using the Zymo EZ DNA Methylation Gold kit. Following bisulfite treatment, DNA fragments were de-salted and size selected to produce a 200-300bp short-insert

library, subjected to PCR, and size selected again before 100bp paired-end reads were sequenced using an Illumina Hiseq4000.

#### Data Analysis

Reads were mapped to the mm10 reference genome using WALT (Chen *et al.*, 2016). Calculation of methylation levels, bisulfite conversion rate, and identification of PMDs was performed as described in (Song *et al.*, 2013), and all values are available in Supplemental Tables 1 and 2. Weighted methylation levels as defined by Schultz *et al.* (2012) were used to calculate average methylation levels in genomic regions. All browser plots were created using the UCSC genome browser tool (Kent *et al.*, 2002). Promoters were defined as +/-1kb from the mm10 RefSeq TSS based on the observation that hypomethylation frequently occurs there on the kilobase scale (Molaro *et al.*, 2011). CpG islands were identified as described in Gardiner-Garden and Frommer (1987). Retrotransposon copies were annotated by RepeatMasker (Smit *et al.*, 1996) and analysis was performed only on the LINE, SINE, and LTR classes (removing all non-retrotransposons from the annotation). Pearson correlation and Euclidean distance at single CpG resolution in whole placenta samples were calculated from the 2,801,446 CpG sites with 5x or greater sequencing depth across all samples. Pairwise comparisons between whole placental samples included only within-species comparisons. Intraspecies single CpG site

correlations and distances were computed from 8,180 CpG sites with 3x or greater sequencing depth across all samples. Boxplots for brain, intestine, and blood were produced with a random set of CpG sites downsampled to the number covered in placental samples. Pearson correlation, Euclidean distance, and distributions were produced using only promoters, CpG islands, and retrotransposons with at least 5 CpG observations to reduce the discretizing effect of low-coverage observations.

ANOVA between global methylation levels with species as the factor was performed in R as a one-way analysis of variance. Differentially methylated CpGs between species were called using (Dolzhenko and Smith, 2014) with species-specific methylation signatures identified using the other two species as background samples. Multiple testing correction of combined DM CpG p-values was done as described in (Benjamini and Hochberg, 1995) using an alpha level of 0.05. Observed over expected (O/E) ratios used for enrichment and depletion analysis of subfamilies were calculated as follows:

$$\frac{O}{E} = \frac{\text{DM CpGs in subfamily}}{\text{DM CpGs total}} \bigg/ \frac{\text{CpGs in subfamily}}{\text{CpGs genome-wide}} \quad (1)$$

Public RNA-seq reads were mapped using STAR (Dobin *et al.*, 2013). BAM files were converted to read counts using HT-seq (Anders *et al.*, 2014) and differentially expressed genes were identified using edgeR (Robinson *et al.*,

2010). We placed an upper bound on the counts per million (CPM) of differentially expressed genes analyzed to focus on those genes that were nearly silenced in one cell type relative to the other.

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### Author Contributions

BED, ADS, and MDD designed the study. Bioinformatic analysis was performed by BED and ADS. Whole placental sample dissection and library preparation was performed by MDD. Placental layer dissection was performed by JLT and ANSP. The manuscript was written by BED, MDD and ADS with input from ANSP. All authors read and approved the final manuscript.

### References

- Anders, S., Pyl, P. T., and Huber, W. 2014. Htseq: a python framework to work with high-throughput sequencing data. *Bioinformatics*, page btu638.
- Aravin, A. A., Hannon, G. J., and Brennecke, J. 2007. The piwi-pirna pathway provides an adaptive defense in the transposon arms race. *science*, 318(5851): 761–764.
- Banister, C. E., Koestler, D. C., Maccani, M. A., Padbury, J. F., Houseman, E. A., and Marsit, C. J. 2011. Infant

- growth restriction is associated with distinct patterns of dna methylation in human placentas. *Epigenetics*, 6(7): 920–927.
- Benjamini, Y. and Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, pages 289–300.
- Berman, B. P., Weisenberger, D. J., Aman, J. F., Hinoue, T., Ramjan, Z., Liu, Y., Noushmehr, H., Lange, C. P., van Dijk, C. M., Tollenaar, R. A., *et al.* 2012. Regions of focal dna hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. *Nature genetics*, 44(1): 40–46.
- Bestor, T. H. 2000. The dna methyltransferases of mammals. *Human molecular genetics*, 9(16): 2395–2402.
- Bird, A. P. 1985. CpG-rich islands and the function of dna methylation. *Nature*, 321(6067): 209–213.
- Boyes, J. and Bird, A. 1991. Dna methylation inhibits transcription indirectly via a methyl-cpg binding protein. *Cell*, 64(6): 1123–1134.
- Branco, M. R., King, M., Perez-Garcia, V., Bogutz, A. B., Caley, M., Fineberg, E., Lefebvre, L., Cook, S. J., Dean, W., Hemberger, M., *et al.* 2016. Maternal dna methylation regulates early trophoblast development. *Developmental cell*, 36(2): 152–163.
- Chen, H., Smith, A. D., and Chen, T. 2016. Walt: fast and accurate read mapping for bisulfite sequencing. *Bioinformatics*, page btw490.
- Chuong, E. B., Tong, W., and Hoekstra, H. E. 2010. Maternal–fetal conflict: rapidly evolving proteins in the rodent placenta. *Molecular biology and evolution*, 27(6): 1221–1225.
- Chuong, E. B., Rumi, M. K., Soares, M. J., and Baker, J. C. 2013. Endogenous retroviruses function as species-specific enhancer elements in the placenta. *Nature genetics*, 45(3): 325–329.
- Coan, P., Ferguson-Smith, A., and Burton, G. 2005. Ultrastructural changes in the interhaemal membrane and junctional zone of the murine chorioallantoic placenta across gestation. *Journal of anatomy*, 207(6): 783–796.
- Crespi, B. and Nosal, P. 2013. Conflictual speciation: species formation via genomic conflict. *Trends in ecology & evolution*, 28(1): 48–57.
- Cross, J. C. 2000. Genetic insights into trophoblast differentiation and placental morphogenesis. In *Seminars in cell & developmental biology*, pages 105–113. Elsevier.
- Csankovszki, G., Nagy, A., and Jaenisch, R. 2001. Synergism of xist rna, dna methylation, and histone hypoacetylation in maintaining x chromosome inactivation. *The Journal of cell biology*, 153(4): 773–784.
- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T. R. 2013. Star: ultrafast universal rna-seq aligner. *Bioinformatics*, 29(1): 15–21.
- Dolzhenko, E. and Smith, A. D. 2014. Using beta-binomial regression for high-precision differential methylation analysis in multifactor whole-genome bisulfite sequencing experiments. *BMC bioinformatics*, 15(1): 215.
- Ehrlich, M., Gama-Sosa, M. A., Huang, L.-H., Midgett, R. M., Kuo, K. C., McCune, R. A., and Gehrke, C. 1982. Amount and distribution of 5-methylcytosine in human dna from different types of tissues or cells. *Nucleic acids research*, 10(8): 2709–2721.
- Feschotte, C. and Gilbert, C. 2012. Endogenous viruses: insights into viral evolution and impact on host biology. *Nature Reviews Genetics*, 13(4): 283–296.
- Fowden, A. and Moore, T. 2012. Maternal-fetal resource allocation: co-operation and conflict. *Placenta*, 33: e11–e15.
- Furukawa, S., Kuroda, Y., and Sugiyama, A. 2014. A comparison of the histological structure of the placenta in experimental animals. *Journal of toxicologic pathology*, 27(1): 11–18.

- Gardiner-Garden, M. and Frommer, M. 1987. CpG islands in vertebrate genomes. *Journal of molecular biology*, 196(2): 261–282.
- Hansen, K. D., Timp, W., Bravo, H. C., Sabunciyan, S., Langmead, B., McDonald, O. G., Wen, B., Wu, H., Liu, Y., Diep, D., *et al.* 2011. Increased methylation variation in epigenetic domains across cancer types. *Nature genetics*, 43(8): 768–775.
- Harten, S. K., Oey, H., Bourke, L. M., Bharti, V., Isbel, L., Daxinger, L., Faou, P., Robertson, N., Matthews, J. M., and Whitelaw, E. 2015. The recently identified modifier of murine metastable epialleles, rearranged l-myc fusion, is involved in maintaining epigenetic marks at cpG island shores and enhancers. *BMC biology*, 13(1): 1.
- Hogg, K., Blair, J. D., McFadden, D. E., von Dadelszen, P., and Robinson, W. P. 2013. Early onset pre-eclampsia is associated with altered dna methylation of cortisol-signalling and steroidogenic genes in the placenta. *PLoS one*, 8(5): e62969.
- Hon, G. C., Rajagopal, N., Shen, Y., McCleary, D. F., Yue, F., Dang, M. D., and Ren, B. 2013. Epigenetic memory at embryonic enhancers identified in dna methylation maps from adult mouse tissues. *Nature genetics*, 45(10): 1198–1206.
- Hu, D. and Cross, J. C. 2010. Development and function of trophoblast giant cells in the rodent placenta. *International Journal of Developmental Biology*, 54(2): 341.
- Hughes, A. L., Green, J. A., Garbayo, J. M., and Roberts, R. M. 2000. Adaptive diversification within a large family of recently duplicated, placentally expressed genes. *Proceedings of the National Academy of Sciences*, 97(7): 3319–3323.
- Jones, P. A. 2012. Functions of dna methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, 13(7): 484–492.
- Kaaij, L. T., van de Wetering, M., Fang, F., Decato, B., Molaro, A., van de Werken, H. J., van Es, J. H., Schuijers, J., de Wit, E., de Laat, W., *et al.* 2013. Dna methylation dynamics during intestinal stem cell differentiation reveals enhancers driving gene expression in the villus. *Genome biology*, 14(5): 1.
- Karolchik, D., Hinrichs, A. S., Furey, T. S., Roskin, K. M., Sugnet, C. W., Haussler, D., and Kent, W. J. 2004. The ucsc table browser data retrieval tool. *Nucleic acids research*, 32(suppl 1): D493–D496.
- Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., and Haussler, D. 2002. The human genome browser at ucsc. *Genome research*, 12(6): 996–1006.
- Kieffer-Kwon, K.-R., Tang, Z., Mathe, E., Qian, J., Sung, M.-H., Li, G., Resch, W., Baek, S., Pruett, N., Grøntved, L., *et al.* 2013. Interactome maps of mouse gene regulatory domains reveal basic principles of transcriptional regulation. *Cell*, 155(7): 1507–1520.
- Kulkarni, A., Chavan-Gautam, P., Mehendale, S., Yadav, H., and Joshi, S. 2011. Global dna methylation patterns in placenta and its association with maternal hypertension in pre-eclampsia. *DNA and cell biology*, 30(2): 79–84.
- Kunieda, T., Xian, M., Kobayashi, E., Imamichi, T., Moriwaki, K., and Toyoda, Y. 1992. Sexing of mouse preimplantation embryos by detection of y chromosome-specific sequences using polymerase chain reaction. *Biology of reproduction*, 46(4): 692–697.
- Lambertini, L., Lee, T.-L., Chan, W.-Y., Lee, M.-J., Diplas, A., Wetmur, J., and Chen, J. 2011. Differential methylation of imprinted genes in growth-restricted placentas. *Reproductive sciences*, 18(11): 1111–1117.
- Li, Z., Dai, H., Martos, S. N., Xu, B., Gao, Y., Li, T., Zhu, G., Schones, D. E., and Wang, Z. 2015. Distinct roles of dnmt1-dependent and-independent methylation patterns in the genome of mouse embryonic stem cells. *Genome biology*, 16(1): 115.
- Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z., Ngo, Q.-M., *et al.* 2009. Human dna methylomes at base resolution show widespread epigenomic differences.

- nature*, 462(7271): 315–322.
- Lister, R., Mukamel, E. A., Nery, J. R., Urich, M., Puddifoot, C. A., Johnson, N. D., Lucero, J., Huang, Y., Dwork, A. J., Schultz, M. D., *et al.* 2013. Global epigenomic reconfiguration during mammalian brain development. *Science*, 341(6146).
- Lu, F., Liu, Y., Jiang, L., Yamaguchi, S., and Zhang, Y. 2014. Role of tet proteins in enhancer activity and telomere elongation. *Genes & development*, 28(19): 2103–2119.
- McDonald, J. F., Matyunina, L. V., Wilson, S., Jordan, I. K., Bowen, N. J., and Miller, W. J. 1997. Ltr retrotransposons and the evolution of eukaryotic enhancers. In *Evolution and Impact of Transposable Elements*, pages 3–13. Springer.
- Mi, S., Lee, X., Li, X.-P., 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(6771): 785–789.
- Molaro, A., Hodges, E., Fang, F., Song, Q., McCombie, W. R., Hannon, G. J., and Smith, A. D. 2011. Sperm methylation profiles reveal features of epigenetic inheritance and evolution in primates. *Cell*, 146(6): 1029–1041.
- Mould, A. W., Pang, Z., Pakusch, M., Tonks, I. D., Stark, M., Carrie, D., Mukhopadhyay, P., Seidel, A., Ellis, J. J., Deakin, J., *et al.* 2013. Smchd1 regulates a subset of autosomal genes subject to monoallelic expression in addition to being critical for x inactivation. *Epigenetics Chromatin*, 6(1): 19.
- Novakovic, B. and Saffery, R. 2013. Placental pseudo-malignancy from a dna methylation perspective: unanswered questions and future directions. *Front. Genet*, 4(285): 10–3389.
- Pai, A. A., Bell, J. T., Marioni, J. C., Pritchard, J. K., and Gilad, Y. 2011. A genome-wide study of dna methylation patterns and gene expression levels in multiple human and chimpanzee tissues. *PLoS Genet*, 7(2): e1001316.
- Pidoux, G., Gerbaud, P., Gnidehou, S., Grynberg, M., Geneau, G., Guibourdenche, J., Carette, D., Cronier, L., Evain-Brion, D., Malassiné, A., *et al.* 2010. Zol-1 is involved in trophoblastic cell differentiation in human placenta. *American Journal of Physiology-Cell Physiology*, 298(6): C1517–C1526.
- Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Manly, T., Sciaky-Gallili, N., and Cedar, H. 1984. Variations in dna methylation during mouse cell differentiation in vivo and in vitro. *Proceedings of the National Academy of Sciences*, 81(8): 2275–2279.
- Reichmann, J., Reddington, J. P., Best, D., Read, D., Öllinger, R., Meehan, R. R., and Adams, I. R. 2013. The genome-defence gene tex19. 1 suppresses line-1 retrotransposons in the placenta and prevents intra-uterine growth retardation in mice. *Human molecular genetics*, 22(9): 1791–1806.
- Roberts, J. and Cooper, D. 2001. Pathogenesis and genetics of pre-eclampsia. *The Lancet*, 357(9249): 53–56.
- Robinson, M. D., McCarthy, D. J., and Smyth, G. K. 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26(1): 139–140.
- Ruda, V., Akopov, S., Trubetskoy, D., Manuylov, N., Vetchinova, A., Zavalova, L., Nikolaev, L., and Sverdlov, E. 2004. Tissue specificity of enhancer and promoter activities of a herv-k (hml-2) ltr. *Virus research*, 104(1): 11–16.
- Sarver, B., Keeble, S., Cosart, T., Tucker, P., and Dean, M. D. in press. Phylogenomic insights into genome evolution and speciation in mice. *Genome Biology and Evolution*.
- Schroeder, D. I., Blair, J. D., Lott, P., Yu, H. O. K., Hong, D., Crary, F., Ashwood, P., Walker, C., Korf, I., Robinson, W. P., *et al.* 2013. The human placenta methylome. *Proceedings of the National Academy of Sciences*, 110(15): 6037–6042.

- Schroeder, D. I., Jayashankar, K., Douglas, K. C., Thirkill, T. L., York, D., Dickinson, P. J., Williams, L. E., Samollow, P. B., Ross, P. J., Bannasch, D. L., *et al.* 2015. Early developmental and evolutionary origins of gene body dna methylation patterns in mammalian placentas. *PLoS Genet*, 11(8): e1005442.
- Schultz, M. D., Schmitz, R. J., and Ecker, J. R. 2012. Leveling the playing field for analyses of single-base resolution dna methylomes. *Trends in genetics: TIG*, 28(12): 583.
- Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W., and Reik, W. 2012. The dynamics of genome-wide dna methylation reprogramming in mouse primordial germ cells. *Molecular cell*, 48(6): 849–862.
- Sferruzzi-Perri, A. N. and Camm, E. J. 2016. The programming power of the placenta. *Frontiers in physiology*, 7.
- Sferruzzi-Perri, A. N., Macpherson, A. M., Roberts, C. T., and Robertson, S. A. 2009. Csf2 null mutation alters placental gene expression and trophoblast glycogen cell and giant cell abundance in mice. *Biology of reproduction*, 81(1): 207–221.
- Sheaffer, K. L., Kim, R., Aoki, R., Elliott, E. N., Schug, J., Burger, L., Schübeler, D., and Kaestner, K. H. 2014. Dna methylation is required for the control of stem cell differentiation in the small intestine. *Genes & development*, 28(6): 652–664.
- Smit, A. F., Hubley, R., and Green, P. 1996. Repeatmasker. *Published on the web at <http://www.repeatmasker.org>.*
- Song, Q., Decato, B., Hong, E. E., Zhou, M., Fang, F., Qu, J., Garvin, T., Kessler, M., Zhou, J., and Smith, A. D. 2013. A reference methylome database and analysis pipeline to facilitate integrative and comparative epigenomics. *PloS one*, 8(12): e81148.
- Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B., and Issa, J.-P. J. 1999. CpG island methylator phenotype in colorectal cancer. *Proceedings of the National Academy of Sciences*, 96(15): 8681–8686.
- Tucker, P. K. 2006. Systematics of the genus mus. *The Mouse in Biomedical Research: History, Wild mice, and Genetics*, 1: 13–21.
- Walsh, C. P., Chaillet, J. R., and Bestor, T. H. 1998. Transcription of iap endogenous retroviruses is constrained by cytosine methylation. *Nature genetics*, 20(2): 116–117.
- Wold, S., Esbensen, K., and Geladi, P. 1987. Principal component analysis. *Chemometrics and intelligent laboratory systems*, 2(1-3): 37–52.
- Yearim, A., Gelfman, S., Shayevitch, R., Melcer, S., Glaich, O., Mallm, J.-P., Nissim-Rafinia, M., Cohen, A.-H. S., Rippe, K., Meshorer, E., *et al.* 2015. Hp1 is involved in regulating the global impact of dna methylation on alternative splicing. *Cell reports*, 10(7): 1122–1134.
- Yuen, R. K., Peñaherrera, M. S., von Dadelszen, P., McFadden, D. E., and Robinson, W. P. 2010. Dna methylation profiling of human placentas reveals promoter hypomethylation of multiple genes in early-onset preeclampsia. *European Journal of Human Genetics*, 18(9): 1006–1012.