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In celebration of women in developmental epigenetics

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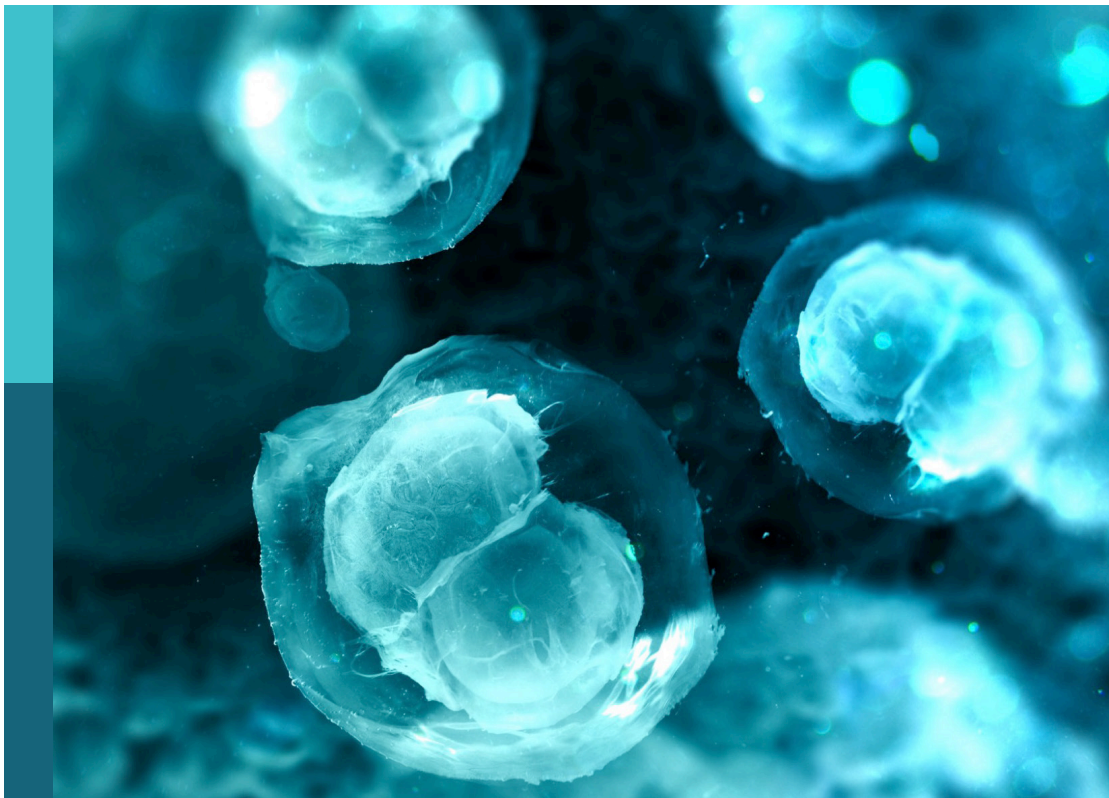

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In celebration of women in developmental epigenetics

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Editorial: In celebration of women in developmental epigenetics

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Editorial on the Research Topic
In celebration of women in developmental epigenetics

According to the United Nations, only 33% of researchers worldwide are women. This number drops dramatically as women move through the academic ranks. Each year, more women professors leave academia (6% assistant; 10% associate; 19% full, 2011–2020, United States), and fewer women are promoted (associate, 7%; full, 12%) compared to men (Spoon et al., 2023). Similarly, in Europe, retention rates in STEM remain low, with only 19% of women at the senior level (=full professor, 2021). These figures are reflected in manuscript submissions, where only 4%–22% of corresponding authors are women (Nature Editorial, 2024; Brück, 2023; Cell Editorial Team, 2022). Further compounding the gender disparity in publishing, women at all levels (graduate students to faculty) are less likely to be credited with authorship than men (Ross et al., 2022). To counteract these trends, this Research Topic is dedicated to publishing manuscripts by women scientists as the first and/or corresponding authors.

Women scientists have made pioneering contributions to the field of epigenetics. Mary Lyon's discovery of *X*-chromosome inactivation provided fundamental insights into dosage compensation mechanisms in mammals. Nobel laureate Barbara McClintock's work, on transposons and epigenetic silencing challenged traditional genetic paradigms and emphasized dynamic gene regulation. Susan Clark's development of bisulfite mutagenesis techniques was the bedrock for the precision mapping of global DNA methylation. Sarah Elgin's pioneering research on heterochromatin structure and function in *Drosophila* was key to understanding position effect variegation. These women, and many others, stand as role models for women scientists. Here, we highlight the contributions of these articles as a celebration of women in developmental epigenetics.

Genomic imprinting

Genomic imprinting is an epigenetic process that is dependent on the sex of the parent in which one parental allele is silenced, while the other parental copy is expressed (Barlow and Bartolomei, 2014). Genomic imprinting and its intersection with development have

long been championed by women researchers, such as Denise Barlow, Marisa Bartolomei, Shirley Tilghman, and Anne Ferguson-Smith, who identified the first imprinted genes (Barlow et al., 1991; Bartolomei et al., 1991; Ferguson-Smith et al., 1991). In this Research Topic, Weinberg-Shukron et al. reviewed the developmental regulation of the *Dlk1-Dio3* imprinted domain. The authors conclude with a discussion on "how to build an imprinted domain." Fang and colleagues also reviewed mechanisms of imprint regulation, assessing evidence for host defense mechanisms and endogenous retroviral elements in the establishment and maintenance of canonical and non-canonical imprints. Regmi et al. discovered that the *Dnmt1* P allele mutation reduced methylation levels throughout the mouse genome, except at gametic differentially methylated regions (DMRs). This protection did not extend to the corresponding secondary DMRs, suggesting that the maintenance mechanisms at gDMRs are different from those at non-imprinted sequences and secondary DMRs.

Epigenetic programming

Given the reliance of epigenetic modifications on metabolites (e.g., methyl groups), investigators have turned to analyses of the one-carbon cycle and nutrients to decipher their role in embryonic/fetal epigenetic programming and inheritance (Clare et al., 2019). Emma Whitelaw's pioneering work on the molecular regulation of the mouse Agouti locus exemplifies the interplay between nutrient sensitivity, environmental factors, transposable elements, and epigenetic regulation during development (Morgan et al., 1999). She coined the term metastable epialleles (MEs) to describe loci where DNA methylation status, and thus phenotype, varies between individuals (Rakyan et al., 2002). In this Research Topic, Sainty et al. reviewed the current knowledge on the early life environment, including maternal micronutrient availability, and disease risk later in life, with a specific focus on DNA methylation at MEs. Additionally, the authors describe the uniqueness of assessing DNA methylation in the placenta as a target tissue for studying MEs in mixed environmental exposures. Senner and co-authors investigated genome-wide DNA methylation in the placentas of mice with fetal growth restriction, using a hypomorphic mutation at the methionine synthase reductase gene, which encodes a key enzyme in one-carbon metabolism. Although regions with altered DNA methylation were identified in homozygous mutant placentas, including young endogenous retroviral elements with ectopic expression, a direct link between the methylome of mutant spermatozoa and that of mutant placentas was not found. Thus, the authors discounted DNA methylation as a mechanism for direct or multigenerational epigenetic inheritance of aberrant fetal growth. Ducreux and colleagues investigated the impact of commercial media and methionine supplementation on the embryonic transcriptome as a proxy for preimplantation epigenetic programming in human ART-produced embryos. Embryos cultured in Fericult (no amino acids) until day 2 had altered gene expression compared to those cultured in a Global medium, including downregulation of SETDB1, a lysine methyltransferase (H3K9me3). Further culture in Global until day 5 (Fericult-Global vs. Global-Global) minimized these transcriptional changes.

Epigenetic modifications are not limited to chromatin modifications. Non-coding RNAs (ncRNAs) also play a significant role in epigenetic regulation, including microRNAs, SINEUPs (natural antisense long ncRNAs that increase translation of partially overlapping mRNAs), telomerase RNAs, and promoter-associated long ncRNAs (Mattick and Makunin, 2006; Esteller, 2011). These ncRNAs interact with RNA-binding proteins to regulate gene expression, chromatin structure, and telomere length (Statello et al., 2020). In this Research Topic, Tokunaga and Imamura discussed the potential of analyzing ncRNAs to provide a new therapeutic approach to microcephaly, which is often associated with developmental disorders. Additionally, Le Breton et al. summarized the current understanding of the role of transposable elements (TEs) in the aging brain and in neurological conditions. They encouraged the investigation of aberrant TE activities and resulting products as potential biomarkers for neurological disorders or biological age.

Developmental exposures

Given the malleability of epigenetic modifications to cellular signals, it is not surprising that they also respond to environmental exposures (Ryznar et al., 2021; Mo et al., 2022; Tando and Matsui, 2023). This is especially true during prenatal and perinatal development, with epigenetic perturbation contributing to long-term adverse health outcomes. Using *C. elegans* as a model system, Susan Gasser examined chromatin organization and Histone 3 lysine 9 methylation in relation to perinuclear anchoring to the nuclear scaffold, as well as changes in chromatin state, phenotypic plasticity, and developmental fate in response to environmental factors, such as overcrowding pheromones (Meister et al., 2011; Gonzalez-Sandoval et al., 2015). Lawless and colleagues reviewed the impact of prenatal cadmium exposure on epigenetic alterations in the placenta, fetus, child/offspring, and adult, including in germ cells, potentially contributing to adverse multigenerational effects. Petroff and co-authors examined the effects of environmental toxicants on hydroxymethylation. Exposure to the plasticizer di (2-ethylhexyl) phthalate over a period from preconception to perinatal weaning in mice resulted in aberrant hydroxymethylation in male (blood and cortex) and female (blood) adults. Similar exposure to lead (Pb) altered hydroxymethylation only in the cortex of adult males. These findings emphasize the susceptibility of the developing male cortex to environmental toxicants.

Conclusion

This Research Topic serves as a flagship for all current and future women scientists and leads by example in making progress toward gender parity in publishing.

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Conflict of interest

The authors declare that the research and writing of this Editorial was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The inter- and multi- generational epigenetic alterations induced by maternal cadmium exposure

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Exposure to cadmium during pregnancy, from environmental or lifestyle factors, has been shown to have detrimental fetal and placental developmental effects, along with negatively impacting maternal health during gestation. Additionally, prenatal cadmium exposure places the offspring at risk for developing diseases in infancy, adolescence, and adulthood. Although given much attention, the underlying mechanisms of cadmium-induced teratogenicity and disease development remain largely unknown. Epigenetic changes in DNA, RNA and protein modifications have been observed during cadmium exposure, which implies a scientific premise as a conceivable mode of cadmium toxicity for developmental origins of health and disease (DOHaD). This review aims to examine the literature and provide a comprehensive overview of epigenetic alterations induced by prenatal cadmium exposure, within the developing fetus and placenta, and the continued effects observed in childhood and across generations.

KEYWORDS

cadmium, epigenetics, placenta, fetal development, maternal nutrition

1 Introduction

Cadmium is a naturally occurring metallic element used in many industrial processes, such as electroplating, galvanizing, producing batteries and solar panels, and zinc and iron smelting (Johri et al., 2010; Ramos-Ruiz et al., 2017). It is distributed widely in the environment, due to the rise of global industrialization, and is highly concentrated in cigarette smoke (Järup et al., 1998; Satarug et al., 2017). Individuals can become exposed to cadmium through the consumption of crops grown in contaminated soil and polluted seafood, as well as occupation or habitation in dense industrial areas and residences near toxic waste dump sites (Aoshima, 1987; Pizzol et al., 2014). Overconsumption or inhalation of this metal has been shown to induce genotoxicity, ROS production, and apoptosis, further leading to the development of cardiovascular disease, renal dysfunction, and carcinogenesis (Satoh et al., 2002; Waalkes, 2003; He et al., 2006). Moreover, cadmium exposure can disrupt essential metal ion concentrations, further leading to metabolic disruption, insulin resistance, and obesity (Jackson et al., 2022).

Cadmium has also been identified as an endocrine disruptor, making it particularly dangerous during prenatal exposure. Endocrine-disrupting chemicals can stimulate or inhibit hormone production, alter hormone transport throughout the body, and interfere with normal reproductive function (Vaiserman, 2014). Prenatal cadmium exposure has been shown to interfere with progesterone, testosterone, and leptin synthesis, which alters offspring's thyroid function and the development of their reproductive systems (Iijima et al., 2007; Ishitobi et al., 2007; Stasenko et al., 2010; Samuel et al., 2011; Banzato et al., 2012).

Furthermore, prenatal cadmium exposure has been linked to spontaneous abortions and premature delivery (Yang et al., 2006). Fetal growth restriction is also a common manifestation among offspring exposed to gestational cadmium, evidenced by decreased birth weight and height, and a reduced head circumference (Zhang et al., 2018). Additionally, cadmium exposure during pregnancy leads to a disturbed translocation of placental metal ions, such as zinc, and reduces the maintenance of fetal nutrition and viability, which negatively impacts fetal growth and development (Mikolić et al., 2015). Moreover, prenatal cadmium exposure has been associated with the development of offspring diseases as they reach adulthood. A mouse model revealed that prenatal cadmium exposure induced hyperglycemia as the offspring mice reached puberty and impaired glucose tolerance in adulthood (Yi et al., 2021). Increased maternal blood and hair levels of cadmium were also associated with an increased incidence of congenital heart defects, which significantly predisposes the offspring to poor cardiac outcomes and the development of cardiovascular disease in adulthood (Jin et al., 2016; Ou et al., 2017; Bokma et al., 2018; Bauer et al., 2019).

Recently, investigating the developmental origins of health and disease (DOHaD) has been given much attention. This theory, known as the Barker hypothesis, states that adverse prenatal and early life factors, such as poor nutrition or lifestyle influences, significantly impact fetal and childhood growth, and predispose the offspring to metabolic syndrome, subsequently leading to the onset of adolescent and adult diseases (Barker et al., 2002; Edwards, 2017). Mechanistically, epigenetic alterations highlight these adverse disease outcomes. Due to environmental or maternal lifestyle factors, these modifications have been shown to significantly induce offspring metabolic syndrome, obesity, heart disease, and hypertension (Ryznar et al., 2021). An accumulation of data has demonstrated that prenatal cadmium exposure induces many epigenetic alterations, such as DNA methylation and post-translational histone modifications, as well as influences on differential micro-RNA expression within the developing offspring (Vilahur et al., 2015). This review aims to summarize recent research findings regarding the effects of gestational cadmium exposure on epigenetic variations within both the placenta and fetus, as well as to examine how these epigenetic alterations influence the clinical outcomes of the next generations in both childhood and adult stages.

2 Cadmium-associated epigenetics alterations during pregnancy

Epigenetics is defined as the dynamic changes to DNA to affect gene expression apart from alterations to the underlying sequence (Berger, 2007). Examples of epigenetic alterations include DNA methylation, posttranslational modifications of histone proteins, and small non-coding RNA molecules that can interfere with gene expression (Vilahur et al., 2015). Modification of epigenetic patterns has been found to result from environmental and lifestyle stimulants, such as exposure to ecological contaminants, poor nutrition, obesity, and smoking. Furthermore, prenatal exposure to such environmental factors can impose an adverse uterine environment, predisposing the developing offspring to aberrant epigenetic alterations and increasing disease risk.

2.1 Cord blood

As previously mentioned, cadmium exposure has been shown to induce many epigenetic modifications during prenatal development. Maternal blood cadmium concentrations were found to be associated with genomic DNA hypomethylation of the gene, ATP9A, and variable methylations of the gene cg24904393, within the umbilical cord blood of human infants (Park et al., 2022). ATP9A is important for phospholipid transporting ATPase activity. Decreased expression of this gene has been shown to increase extracellular vesicle release and apoptosis, as well as interrupt the recycling process of critical transport proteins, such as GLUT-1 (Park et al., 2022). Additionally, the gene, cg24904393, encodes the plasminogen protein, which is vital in blood coagulation and fibrinolysis (Park et al., 2022). Dysregulation of this gene can increase the risk of thrombosis, which proves to be detrimental to the developing offspring (Park et al., 2022). Moreover, within the cord blood of cadmium-exposed infants, hundreds of cadmium-associated differentially methylated regions (DMRs) were identified. These DMRs were most commonly located within the maternal imprinting control regions. The top three functional categories associated with the cadmium-induced methylation modifications include BMI regulation, atrial fibrillation, and hypertension (Cowley et al., 2018).

2.2 Placental development

The placenta is an organ that exists during pregnancy to modulate nutrient, oxygen, and waste exchange from the mother to the fetus. Maternal spiral artery remodeling is an essential event in placental development that allows for sufficient blood to be delivered from the maternal circulation to the placenta (Woods et al., 2018). Concurrently, extensive villous branching and vascularization ensure to establish a highly specialized network of maternal blood and fetal capillaries within the placental layer adjacent to the fetus (Rossant and Cross, 2001). Proper development of this organ is vital to support the growth of the fetus, as obstructions in its formation result in impaired nutrient transport, leading to fetal growth restriction and maternal complications of pregnancy, such as pre-eclampsia (Wier et al., 1990; Wang et al., 2016a; McKinney et al., 2016; Zhang et al., 2016; Hung and Chen, 2018; Rana et al., 2019; Tenório et al., 2019).

Prenatal cadmium exposure has been shown to induce many different epigenetic alterations within the placenta, which significantly impact its function. In a human study of mother-infant pairs, placental cadmium levels were significantly associated with sex-specific DNA methylation changes within this organ. Within the female offspring, differential methylation was observed near transcriptional start sites for cell damage response genes, whereas methylation changes were observed in genes involved in placental development, cell differentiation, and angiogenesis in the males (Mohanty et al., 2015). Additionally, increased maternal cadmium levels were significantly associated

with decreased DNA methylation within the promoter region of *PCDHAC1* in the human placenta, which is a gene belonging to the protocadherin gene family and is very important for fetal growth (Everson et al., 2016). There was also a strong association found between decreased DNA methylation of placental *PCDHAC1* and an increased odds of small for gestational age (SGA) offspring and decreased head circumference upon prenatal cadmium exposure in this study (Everson et al., 2016). In mice, prenatal cadmium exposure was found to decrease the expression of placental GLUT-3, due to site-specific DNA methylation, consequently leading to fetal growth restriction (Xu et al., 2016). Moreover, the placenta is an organ rich in the expression of imprinted genes, which are genes defined by their preferential expression from one of the two parental alleles and are regulated by epigenetic marks responding to environmental stimuli (Xu et al., 2017; Cowley et al., 2018). Consistently, the maternal imprinted gene, *Cdkn1c*, was significantly upregulated, while the paternally imprinted gene, *Peg10*, was significantly downregulated in the cadmium-exposed mouse placenta, and these changes were associated with restricted offspring growth. This was found to be a consequence of a decreased methylation level in the promoter region of *Cdkn1c* and an increased methylation level within the promoter region of *Peg10* (Xu et al., 2017). These genes are important for nutrient transport and placentation, as differential expression of *Cdkn1c* and *Peg10* were associated with discrepancies in cell proliferation and survival, and fetal viability and labyrinth malformation, respectively (Koppes et al., 2015; Xu et al., 2017). Recently, the expression of *Cdkn1c* was found to be upregulated in the cadmium-exposed mouse placenta of both male and female offspring and was associated with decreased fetal growth (Simmers et al., 2022). The female offspring displayed no significant differences between the ratio of *Cdkn1c* transcripts derived from the maternally and paternally inherited alleles, while the male offspring had a significant increase in the expression percentage of *Cdkn1c* from maternally inherited alleles, indicating no loss of imprinting (Simmers et al., 2022). On the contrary, this increased expression of *Cdkn1c* in the placentas was not found to be a result of the differential DNA methylation, as discussed previously, since there were no changes in the methylation profile within the promoter region upon cadmium treatment (Simmers et al., 2022). Instead, the increased expression of *Cdkn1c* in this study was found to be a consequence of altered placenta morphology (Simmers et al., 2022). The differences in these epigenetic alterations could potentially be attributed to inconsistent study designs, as cadmium provided 5 weeks prior to pregnancy, during mating, and throughout pregnancy did not induce methylation differences, while decreased DNA methylation of *Cdkn1c* was observed when cadmium was provided at E7.5 (Xu et al., 2017; Simmers et al., 2022).

MicroRNAs (miRNAs) are epigenetic modifiers that are important for gene expression. Within the placenta, miRNAs are involved in regulating trophoblast differentiation, migration, invasion, and vasculogenesis (Mouillet et al., 2015; Hayder et al., 2018). However, exposure to environmental factors, such as cadmium, can induce alterations in the expression of these placental miRNAs, leading to changes in the regulation of genes involved in proper placental development and, subsequently, maternal complications of pregnancy, like preeclampsia, fetal growth restriction, and preterm birth (Kotlabova et al., 2011). Prenatal cadmium exposure has been found to significantly increase the expression of miR-509-3p and miR-193b-5p within the human placenta, which may affect both placental function and nervous system development (Tehrani et al., 2022). miR-509-3p is negatively associated with cell migration and invasion, two events that are vital for proper spiral artery remodeling in placental development (Su et al., 2015; Pan et al., 2016; Ahir et al., 2017). Consistently, increased miR-193b-5p expression is associated with cases of preeclampsia and fetal growth restriction due to hindered trophoblast migration and invasion (Zhou et al., 2016; Awamleh et al., 2019; Östling et al., 2019; Awamleh and Han, 2020). Furthermore, cadmium treatment of JEG-3 cells showed a significant decrease in cell migration, due to increased expression of the TGF-β pathway family members. These findings were also associated with altered miR-26a expression, indicating that cadmium modulates placental miRNAs, contributing to increased activation of TGF-β signaling and abnormal trophoblast migration (Brooks and Fry, 2017).

2.3 Fetal growth

Prenatal cadmium exposure has also been shown to induce many epigenetic alterations within the developing fetus. During early development, an increased occurrence of embryonic death, fragmentation, and developmental blockades upon cadmium treatment was observed in mice (Zhu et al., 2021). Interestingly, the surviving embryos experienced epigenetic changes at the 8-cell stage, including histone acetylation, evidenced by increased histone deacetylase 1, and genomic DNA methylation, manifested by *H19* hypomethylation (Zhu et al., 2021). *H19* is a gene involved in early embryonic development and implantation, along with increased ROS levels and DNA damage (Zhu et al., 2021). Additionally, differential DNA methylation was observed in dozens of genes relating to fetal gene expression, tissue morphology, cancer, lipid metabolism, and apoptosis within the cadmium-exposed infant (Sanders et al., 2014).

Although prenatal cadmium exposure has been shown to induce many different epigenetic alterations, there are inconsistencies among the modifications reported, leading some to suggest sex as a factor in cadmium-induced toxicities. In a human study, Kippler et al. (2013) reported that male offspring exhibited global hypermethylation, specifically of genes related to cell death pathways, within cord blood DNA, while the females showed global hypomethylation of genes associated with organ development, morphology, and bone mineralization (Kippler et al., 2013). These results provide compounding evidence to previous reports, in which fetal head and femur length in girls is decreased upon prenatal cadmium exposure and cadmium-associated osteoporosis and fractures are particularly observed in women (Engström et al., 2012; Kippler et al., 2012). Nevertheless, several of the individual CpG sites that were positively associated with cadmium were inversely correlated with birth weight, indicating that cadmium-induced fetal growth restriction potentially occurs through differential DNA methylation (Kippler et al., 2013). Additionally, cadmium-exposed female infants exhibited decreased birth weights, accompanied by hypomethylation of *PEG3*, which is a paternally expressed

imprinted gene that encodes a zing-finger protein that plays a role in p53-mediated apoptosis (Deng and Wu, 2000; Vidal et al., 2015). The cadmium-exposed male newborns were significantly lighter, but *PEG3* methylation was not affected (Vidal et al., 2015). Interestingly, female offspring of cadmium-exposed women with higher zinc levels showed increased methylation of *PEG3* when compared to those offspring of zinc-deficient mothers, indicating that adequate zinc intake may mitigate these cadmium-induced epigenetic alterations (Vidal et al., 2015). Furthermore, prenatal cadmium exposure in rats resulted in elevated expression of DNMT3A in the livers of the male offspring and a decreased expression within the female offspring. DNMT3A is involved in *de novo* CpG methylation (Castillo et al., 2012). This finding corresponded with hypermethylation of the glucocorticoid receptor within the male offspring and hypomethylation within the females (Castillo et al., 2012). This could induce altered glucocorticoid metabolism, which is significantly linked to an increased risk of cardiometabolic disorders in adulthood (Castillo et al., 2012).

3 Cadmium-associated epigenetic alterations during childhood

It has been speculated that many cadmium-associated epigenetic changes within the fetal stage of development contribute to adulthood diseases. A number of studies have employed an experimental design that includes follow-up data collection in the child and adulthood stages to investigate the status of the epigenetic alterations. As previously mentioned, prenatal cadmium exposure induces DNA methylation modifications within the developing fetus. Interestingly, Kippler et al. (2013) reported that similar methylation profiles about offspring growth observed in newborn cord blood were continually observed in children at 4.5 years old, along with the growth effects induced by prenatal cadmium exposure (Kippler et al., 2013). Furthermore, a higher maternal urinary cadmium concentration was significantly associated with a slower weight gain from age 3 months to 4 years, indicated by slower height and BMI trajectories (Chatzi et al., 2019). This effect was seemingly stronger in girls, specifically in those offspring exposed to cadmium during the first trimester, a period in which epigenetic remodeling is highly active (Chatzi et al., 2019). A recent study investigated if the epigenetic changes associated with prenatal cadmium exposure persist from birth into childhood. It reported several DNA methylation differences within the cord blood that appeared to still be associated with prenatal cadmium exposure at 9 years of age. Interestingly, these epigenetic changes were found to mainly be a consequence of gestational cadmium exposure rather than long-term childhood cadmium exposure (Gliga et al., 2022). However, this study was unable to significantly detect specific DNA methylation changes that persist from birth to prepubertal age due to small power and too few overlapping differentially methylated positions and regions to perform enrichment and pathway analyses, and it was unable to control for other environmental exposures and lifestyle factors (Gliga et al., 2022). Nevertheless, this data highlights the importance of determining gestational offenses as origins of disease rather than life-long exposure alone.

4 Cadmium-associated multigenerational epigenetic effects

During embryogenesis, the developing offspring experience two major cycles of epigenetic reprogramming. The first occurs during the preimplantation stage and the second during germ cell development (Li et al., 2019). During the preimplantation stage, the paternally inherited genome undergoes global DNA demethylation, followed by subsequent cell divisions to further induce the loss of epigenetic modifications (Sinclair et al., 2007). Interestingly, within the mouse, the maternally derived genome was found to retain its methylation patterns at this stage (Santos et al., 2005). As development continues into gastrulation and germ cell specification, both imprinted and non-imprinted genes are dynamically demethylated, ensuring that the inherited epigenetic alterations are deleted within the germline (Sinclair et al., 2007). This erasure of the gametic epigenetic patterns allows for the embryo to establish its own epigenetic profile indispensable for proper development (Huntriss, 2021). However, there are reports of genes in primordial germ cells escaping demethylation, thereby carring the epigenetic markers to F2 generation (Seisenberger et al., 2012; Hackett et al., 2013; Heard and Martienssen, 2014). Recently, transgenerational epigenetic inheritance was demonstrated, as two metabolic-related genes were specifically methylated and silenced in mouse embryonic stem cells, inducing abnormal metabolic phenotypes (Takahashi et al., 2023). Interestingly, these methylation and phenotypic changes were retained and transmitted across multiple generations, providing evidence contrary to the widely accepted notion that epigenetic patterns are erased during embryogeneis (Takahashi et al., 2023). Nevertheless, because the window of epigenetic reprogramming occurs very early in gestation, proceeding placentation and organogenesis, the embryo is vulnerable to epigenetic alterations caused by environmental stimuli, such as cadmium exposure, which can increase the risk of congenital defects and subsequent diseases in later life (Joubert et al., 2012; Manikkam et al., 2012; Vaiserman, 2014; Vilahur et al., 2015). The multigenerational effect of cadmium induced epigenetic alterations, though, remains controversial and poorly understood.

Cadmium has been shown to impact protein acetylation in testicular development. Briefly, lysine acetylation, specifically of histone H4, is an important step in the formation of sperm cells, as it is a prerequisite for histones to be replaced by transition nuclear proteins in spermatogenesis (Awe and Renkawitz-Pohl, 2010). Lysine succinylation is another post-translational modification that plays a regulatory role in cell differentiation and organ development (Xie et al., 2012). Cadmium exposure was found to impact GAPDH activity, and ATP and cAMP levels within germ cells in mice, which then further inhibited lysine acetylation and succinylation within the testes, resulting in reproductive injuries (Yang et al., 2018). Additionally, acetylation of histone H4K5 and H4K12 was also inhibited by cadmium treatment, further inducing spermatogenesis failure (Yang et al., 2018). These findings were also consistent with other reports of protein post-translational modifications impacting sperm function. Cadmium exposure to spermatozoa, *in vitro*, exhibited impacted GAPDH and AMPK activity and ATP production, further leading to inhibited sperm motility. This was found to be a result of increased tyrosine

phosphorylation (Wang et al., 2016b). Consistently, cadmium exposure in mice also induced dihydrolipoamide dehydrogenase tyrosine phosphorylation, which significantly impacted the activity of the TCA cycle and, subsequently, oxidative phosphorylation, leading to decreased ATP production and poor sperm motility within the developing testes (Li et al., 2016).

A rat model demonstrated that gestational cadmium exposure induced decreased levels of progesterone in offspring ovarian granulosa cells, due to significant decreases in the mRNA levels of the steroidogenic enzymes, StAR, and Cyp11a1, at post-natal day 56 (Liu et al., 2020). This gene expression decrease was found to be associated with an upregulation of the miRNAs, miR-27a-3p and miR10b-5p, within the ovarian granulosa cells (Liu et al., 2020). miR-27a-3p regulates estrogen and progesterone receptors' expression and mediates these hormones' metabolism (Liu et al., 2020). miR-10b-5p acts directly on StAR and mitigation of its expression can induce reproductive damage (Liu et al., 2020). Interestingly, decreased progesterone production and StAR expression were continually observed in the F2 rat offspring, indicating that prenatal cadmium-associated epigenetic alterations could have a transgenerational effect (Liu et al., 2020). Moreover, the apoptotic gene, Bcl2, was significantly altered in the ovarian granulosa cells of F1 and F2 rat offspring, accompanied by increased apoptotic cell bodies (Liu et al., 2021). This was accompanied by differential expression of miR-16-5p and miR92a-2-5p, which are regulators of Bcl2 expression (Liu et al., 2021). This indicated that prenatal cadmium exposure dysregulated the expression profile of these miRNAs within the ovarian granulosa cells, thereby leading to increased cell death through modulation of Bcl2, in both F1 and F2 offspring (Liu et al., 2021). Another recent study investigated the multigenerational effects of prenatal cadmium exposure on testicular function. The F1 and F2 male mice exhibited immature Sertoli and Leydig cells (Huang et al., 2020). Additionally, the F1 mice showed detachment of spermatogonia from the basement membrane, and the tubular diameter was impacted in both the F1 and F2 mice, indicating impacted spermatogenesis (Huang et al., 2020). Prenatal cadmium exposure also affected the secretion of male hormones, as a gonadotropin-releasing hormone, luteinizing hormone, progesterone, and testosterone were all significantly decreased in the F1 mice, while testosterone was significantly increased in the F2 mice (Huang et al., 2020). These results were found to be a result of differential expression of the steroidogenic enzymes, SF-1 and StAR, within both the F1 and F2 mice (Huang et al., 2020). These enzymes were regulated by the cadmium-induced expression of miR-328a-5 and miR-10b-5p, respectively (Huang et al., 2020). When the miRNA expression was increased, the enzyme expression was decreased, leading to consequential changes in both hormone production and testicular function in a multigenerational fashion (Huang et al., 2020).

5 Conclusion

Cadmium exposure, particularly during pregnancy, has been shown to induce many teratogenic effects and program the offspring to develop diseases later in life. Epigenetic alterations have been reported within cord blood, placenta, and fetal tissue upon cadmium exposure, leading to pregnancy complications, such as preeclampsia, and poor fetal growth. Sex-specific effects on epigenetic mechanisms are also observed, as the male offspring generally experience hypermethylation of genes, while the females show increased DNA hypomethylation, and many of the epigenetic alterations viewed in the fetal and neonatal stages persist into childhood. Interestingly, recent research has found that epigenetic alterations within germ cells pose multigenerational effects, in which adverse effects on the reproductive system were observed into the F2 offspring. However, there are limitations to the current knowledge. Comparing the studies, the experimental designs differ quite drastically, as cadmium is provided at different time points of pregnancy. Therefore, the results may differ between studies, and are not able to be accurately compared to draw conclusions. Additionally, most studies were focused on revealing genome-wide epigenetic changes, and the key genes that are adversely affected by epigenetic alterations are not clear. Further research on how the observed epigenetic changes influence gene expression, provoking disease development is warranted. More important, the selected epigenetic alterations and their associated genes should be validated in rigorously designed experiments. The fast developing gene editing technology has provided the possibility to introduce targeted epigenetic changes into animal models for verifying their roles in regulating gene expression and their multigenerational effects for disease development. Another limitation of currect research is that many of these studies observed epigenetic alterations in embryonic and fetal tissues, and discussed the potential for disease later in life, but these implications have yet to be investigated. Constructing experimental designs, in which the prenatal cadmium exposed offspring undergoes follow-up examination later in their life, either with or without continued cadmium treatment, is coveted for the advancement of the field. Moreover, information on the transgenerational effect of prenatal cadmium exposure on epigenetic alterations is lacking and warrants future research. Nevertheless, these results highlight cadmium's role in epigenetic programming within the prenatal period and its impact on offspring health and disease development later in life.

Author contributions

LL, LX, and KZ all contributed equally to the ideas, writing, and editing of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Developmental exposures to common environmental contaminants, DEHP and lead, alter adult brain and blood hydroxymethylation in mice

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Introduction: The developing epigenome changes rapidly, potentially making it more sensitive to toxicant exposures. DNA modifications, including methylation and hydroxymethylation, are important parts of the epigenome that may be affected by environmental exposures. However, most studies do not differentiate between these two DNA modifications, possibly masking significant effects.

Methods: To investigate the relationship between DNA hydroxymethylation and developmental exposure to common contaminants, a collaborative, NIEHS-sponsored consortium, TaRGET II, initiated longitudinal mouse studies of developmental exposure to human-relevant levels of the phthalate plasticizer di(2-ethylhexyl) phthalate (DEHP), and the metal lead (Pb). Exposures to 25 mg DEHP/kg of food (approximately 5 mg DEHP/kg body weight) or 32 ppm Pb-acetate in drinking water were administered to nulliparous adult female mice. Exposure began 2 weeks before breeding and continued throughout pregnancy and lactation, until offspring were 21 days old. At 5 months, perinatally exposed offspring blood and cortex tissue were collected, for a total of 25 male mice and 17 female mice (n = 5–7 per tissue and exposure). DNA was extracted and hydroxymethylation was measured using hydroxymethylated DNA immunoprecipitation sequencing (hMeDIP-seq). Differential peak and pathway analysis was conducted comparing across exposure groups, tissue types, and animal sex, using an FDR cutoff of 0.15.

Results: DEHP-exposed females had two genomic regions with lower hydroxymethylation in blood and no differences in cortex hydroxymethylation. For DEHP-exposed males, ten regions in blood (six higher and four lower) and 246 regions (242 higher and four lower) and four pathways in cortex were identified.

Pb-exposed females had no statistically significant differences in blood or cortex hydroxymethylation compared to controls. Pb-exposed males, however, had 385 regions (all higher) and six pathways altered in cortex, but no differential hydroxymethylation was identified in blood.

Discussion: Overall, perinatal exposure to human-relevant levels of two common toxicants showed differences in adult DNA hydroxymethylation that was specific to sex, exposure type, and tissue, but male cortex was most susceptible to hydroxymethylation differences by exposure. Future assessments should focus on understanding if these findings indicate potential biomarkers of exposure or are related to functional long-term health effects.

KEYWORDS

DNA methylation, DNA hydroxymethylation, lead (Pb), phthalate, DEHP (di-(2-ethylhexyl) phthalate), toxicoepigenetics, 5-hydroxymethylcytosine

1 Introduction

In early development, embryonic and fetal programming orchestrates finely tuned processes that help establish long-term health and wellbeing. During this time, adverse events, such as exposure to environmental contaminants (Heindel et al, 2015), may disrupt these processes, leading to a higher risk of health effects later in life. This hypothesis, called the Developmental Origins of Health and Disease (DOHaD) (Barker, 2007), has been widely studied in epidemiological and animal models. The biological processes behind the hypothesis, however, are poorly understood.

One process that may underly the DOHaD hypothesis is disruption to early epigenetic programming (Barouki et al, 2018). The epigenome can be defined as mitotically (and sometimes meiotically) heritable marks that help regulate gene expression without altering the genome itself (Murrell et al, 2005). These can include marks on nucleotides (e.g., DNA methylation, hydroxymethylation), histone modifications (e.g., acetylation, phosphorylation, ubiquitination), or noncoding RNA molecules, such as long noncoding RNA (lncRNA) (Greally, 2018). One of the most studied epigenetic marks is DNA methylation. DNA methylation is established early in life. In development, the ova and sperm methylome are erased immediately after fertilization and gradually rewritten throughout gestation (Monk et al, 1987). A secondary phase of fetal programming takes place during primordial germ cell development and migration (Seisenberger et al, 2012). The writing of DNA methylation occurs via a cyclical process: first methylation marks are added to the nucleotides (often cytosines upstream of guanines, CpGs, when methylated, 5-mC) by DNA methyltransferases (DNMTs). Methyl marks can then be oxidized to hydroxymethylation (5-hydroxymethylcytosine, or 5-hmC) by ten-eleven translocation (TET) dioxygenases (Tahiliani et al, 2009), which can be further modified to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). Both 5-fC and 5-caC can be removed and replaced with a naked cytosine via base excision repair (Moore et al, 2013).

DNA methylation and hydroxymethylation are stable and present throughout tissues in the body (Globisch et al, 2010; Li and Liu, 2011; Wu et al, 2011; Nestor et al, 2012) and likely have opposing regulatory effects on gene expression (Wu and Zhang, 2017). Traditionally, DNA methylation has been thought to have a gene "silencing" effect, but the exact effects are dependent on where the modifications are in the genome (Jones, 2012). Hydroxymethylation plays an essential role in normal development (Yan et al, 2023), helping regulate development in both the heart (Greco et al, 2016) and brain (Stoyanova et al, 2021). Especially in promotor regions, it may reverse the effects of methylation (Mellén et al, 2017). Throughout the genome, it can also act as a recruiter and signal for other epigenetic factors (Takai et al, 2014). While methylation and hydroxymethylation are inherently linked, the developmental programming of these marks do have some independence (Amouroux et al, 2016; Lopez et al, 2017; Yan et al, 2023). However, most environmental exposure studies evaluating the methylome and hydroxymethylome do not use methods that differentiate between these marks; instead, studies typically report on combined "total methylation" (Booth et al, 2012). Because adverse events during these processes could disrupt programming and alter the methylome and hydroxymethylome independently, it is essential to understand the unique response of each of these epigenetic marks.

Some environmental contaminants, such as the group of plasticizers known as phthalates, have developmental effects that have been recently identified. Exposure to phthalates is nearly universal (Woodruff et al, 2011; Zota et al, 2014) and has been linked with endocrine disrupting effects and an increased risk of metabolism and neurodevelopmental disorders and diseases (Braun, 2017). Other common contaminants, such as the metal lead (Pb), have been widely known as developmental toxicants for decades. Early life exposure to Pb can occur via drinking water, contaminated soil, or dust, which can subsequently disrupt brain development, slow growth, and impact the immune system (Bellinger et al, 2017). In animal models, other contaminants, like the plastic additive bisphenol A (BPA) may cause changes in hydroxymethylation (Kochmanski et al, 2018). Developmental exposure to both phthalates (Svoboda et al, 2020; Hsu et al, 2021; Liu et al, 2021) and Pb (Dou et al, 2019b; Wang et al, 2020; Hong et al, 2021; Svoboda et al, 2021) have been linked to differences in the epigenome-wide total methylation, but little is known about effects in the hydroxymethylome.

To understand differences in the hydroxymethylome after developmental exposure to both the phthalate, di(2-ethylhexyl) phthalate (DEHP), and Pb, a longitudinal mouse model was used. This study was conducted as a part of the National Institute of Environmental Health Sciences (NIEHS) Toxicant Exposures and Responses by Genomic and Epigenomic

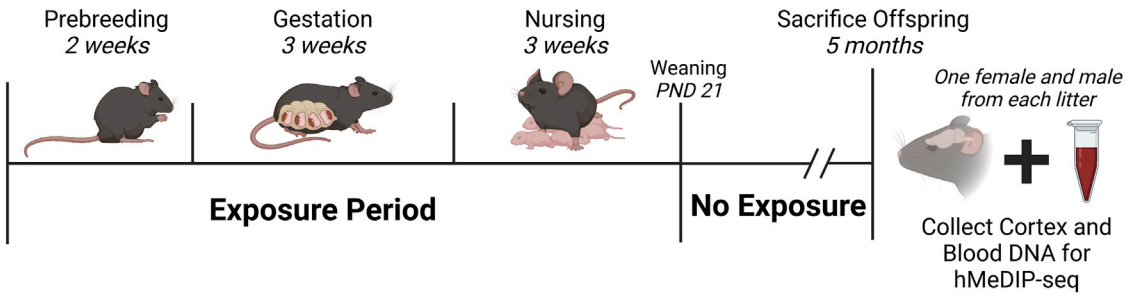

FIGURE 1
Study Design and Timeline. Nulliparous *a/a* mice were exposed to either 25 mg DEHP/kg food chow, or 32 ppm Pb-acetate in drinking water, or assigned to a control group. Exposures began 2 weeks before breeding, continued throughout pregnancy/gestation and lactation, and ceased at weaning (PND 21). At 5 months, one female and one male from each litter were sacrificed, and cortex and blood were collected for DNA hydroxymethylation analysis. Final sample sizes were: DEHP-females, n = 5; DEHP-males, n = 7 for cortex, n = 6 for blood; Pb-females, n = 6; Pb-males, n = 6; control-females, n = 6; control-males, n = 6. Created in Biorender. Abbreviations: DEHP, di(2-ethylhexyl) phthalate; hMeDIP-seq, hydroxymethylated DNA immunoprecipitation sequencing; Pb, lead; PND, postnatal day.

Regulators of Transcription II (TaRGET II) Consortium, which aims to determine how the environment affects disease susceptibility across the life course through changes to the epigenome. We hypothesized that perinatal DEHP and Pb exposure would result in tissue- and sex-specific changes in DNA hydroxymethylation in adulthood.

2 Materials and methods

2.1 Study Design

Wild-type non-agouti *a/a* mice were obtained from a 230+ generation colony of agouti viable yellow (A^{vy}) mice, which are genetically invariant and 93% identical to C57BL/6J mice (Dou et al, 2019a). Virgin *a/a* females (6–8 weeks old) were randomly assigned to one of three exposure groups: 5 mg DEHP/kg chow/day, 32 ppm Pb-acetate drinking water, or control (DEHP- and Pb-free). Exposure began 2 weeks prior to mating with virgin *a/a* males (7–9 weeks old) and continued until offspring weaning at postnatal day 21 (PND 21). Animals were maintained on phytoestrogen-free modified AIN-93 chow (Envigo Td.95092, 7% corn oil diet, Harlan Teklad) and housed in polycarbonate-free cages. All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with experimental procedures outlined by the NIEHS TaRGET II Consortium and the highest animal welfare standards (Wang et al, 2018).

DEHP and Pb exposures were conducted *ad libitum*. DEHP was dissolved in corn oil and used to create a 7% corn oil for chow. Assuming pregnant and nursing female mice weigh roughly 25 g and eat, on average, 5 g of chow per day, the resulting exposure level of 5 mg DEHP/kg bodyweight per day reflects human relevant exposures (Chang et al, 2017). Pb-acetate drinking water was prepared with distilled drinking water, with a concentration of 32 ppm to model human-relevant perinatal exposure. In previous work, we identified that this dose generates maternal blood levels (BLLs) ranging from 16 to 60 μg/dL (mean: 32.1 μg/dL) (Faulk et al, 2013). Individual animal exposures were not measured in the present study. At PND 21, all offspring were weaned and moved to either DEHP-free control chow or Pb-free control water and maintained until 5 months of age (Figure 1).

2.2 Tissue collection and DNA extraction

Immediately following euthanasia with CO_2 asphyxiation, blood was collected via cardiac puncture. Cortex tissue was dissected and immediately flash frozen in liquid nitrogen and stored at −80°C. The final sample size for this study included n ≥ 5 males and n ≥ 5 females in each exposure group (DEHP-exposed, Pb-exposed, and control) with 1 male and 1 female per litter per group. The final sample size for this study was n = 71, once tissues (i.e., cortex and blood) were collected. The AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Cat. #80224) was used to extract DNA from blood and cortex tissue. Extracted DNA was stored at −80°C until further processing.

2.3 hMeDIP-seq

Sample quality was assessed using the Agilent TapeStation genomic DNA kit (Agilent) and concentrations were measured using Qubit broad range dsDNA (Invitrogen). Ligation adapter arms were synthesized by IDT and hybridized in the University of Michigan Epigenomics Core Facility. Unless specified otherwise, the enzymes used for library preparation and dual-indexing primers were purchased from New England Biolabs.

For each sample, a total of 750 ng of genomic DNA was sheared by adaptive focused acoustics, using the Covaris S220 (Covaris). Sheared DNA was blunt-ended and phosphorylated. A single A-nucleotide was then added to the 3′ end of the fragments in preparation for ligation of adapter duplex with a T overhang. The ligated fragments were cleaned using Qiagen's MinElute PCR purification columns. DNA standards for hMeDIP-seq (Diagenode, 5-hmC, 5-mC, & cytosine DNA standard pack for hMeDIP, cat # AF-107–0040) were added to each sample before denaturation and resuspension in ice-cold immunoprecipitation buffer (10 mM Sodium Phosphate pH 7.0, 140 mM NaCl, 0.05% Triton X-100). A 10% volume was input before 2 μg of a 5-hmC-specific antibody

(Active Motif, Cat # 39791) was added for immunoprecipitation overnight at 4°C with rotation. Dynabeads Protein-G (Invitrogen) were added to the immunoprecipitation to perform the pull-down of 5-hmC-enriched fragments. The 5-hmC-enriched DNA fragments were then released from the antibody by digestion with Proteinase K (Ambion).

After cleanup with AMPure XP beads (Beckman Coulter), the percent input in the 5-hmC enriched fragments was evaluated by qPCR, using primers specific for the spike-ins. Samples with good percent input were then PCR amplified for the final library production, cleaned using AMPure XP beads, and quantified using the Qubit assay and TapeStation High Sensitivity D1000 kit. The libraries were pooled and then sequenced on a NovaSeq6000 instrument at the University of Michigan Advanced Genomics Core Facility.

2.4 Data processing and analysis

Reads were assessed for quality (FastQC v0.11.8), had adapter sequence trimmed (TrimGalore v0.4.5), and aligned to mm10 with Bowtie2 (v2.3.4.1) (Langmead and Salzberg, 2012) using default parameters (excepting -X 2000). Duplicate reads were marked with Picard (v2.20.2) and filtered out with samtools (v1.2) (Li et al, 2009). Alignments that overlapped ENCODE blacklisted regions were removed with bedtools (v2.28.0) (Quinlan and Hall, 2010) and the resulting reads were used for peak calling with macs2 (v2.1.2) (Zhang et al, 2008). Additional ChIP QC measures were determined with phantompeakqualtools (Landt et al, 2012) and DeepTools (v3.3.0) (Ramirez et al, 2016).

Analyses were conducted in R (v > 4.1) using Bioconductor packages (R Core Team, 2021). Sex, tissues, and exposures were analyzed separately. Using DiffBind (Stark and Brown, 2011; Ross-Innes et al, 2012), consensus peaks (overlapping in at least 66% of samples in each comparison group) were exacted. Individual consensus peaks were counted using 100 and 500 bp windows and normalized based on library size. Both 100 and 500 bp counts were analyzed using DESeq2 options, and regions with a significantly different count between exposed and control groups were identified, using a false-discovery rate (FDR) cutoff of 0.15. To minimize false positives, only regions with FDR<0.15 in both the 100 and 500 bp analyses were considered as true positives.

Regions were annotated to the mm10 genome using annotatr (Calvacante and Sartor, 2017). Random regions were generated and annotated to compare relative frequencies of annotations. For comparisons with more than 100 mapped genes on the differential regions, gene sets were assessed for gene ontology using ChIP-Enrich (Welch et al, 2014).

3 Results

3.1 Differential hydroxymethylation: DEHP

For females (n = 5 exposed, n = 6 control), there were two differentially hydroxymethylated regions (DhMRs) in 5-month blood comparing DEHP-exposed and control groups (Figure 2A; Table 1; Supplementary Table S1; Supplementary Figure S1A). In both regions, an intron region on the *Cit* gene and an open sea region on chromosome 19 were less hydroxymethylated in the DEHP group compared to the controls. These two regions or genes did not overlap with regions or genes from other comparisons (e.g., female DEHP cortices, male tissues, or any comparison from Pb exposures). There were no regions with differential hydroxymethylation in female cortex (n = 5 exposed, n = 6 control).

For male blood (n = 6 exposed, n = 6 control), there were ten genomic regions in 5-month blood that had differential hydroxymethylation, with six regions higher in exposed compared to control and four regions lower in exposed (Figure 2A; Table 1; Supplementary Table S2; Supplementary Figure S1B). Nearly 70% of these DhMRs mapped to introns of genes (Figure 2B); lower hydroxymethylated regions included introns in *Fhod3*, *Fbxl12*, *Nos1*, and *Tmem266*. Higher hydroxymethylation in the DEHP-exposed male blood included regions in introns in *Tph2*, *Bace1*, and *Phactr1* and two unnamed, open sea regions on chromosomes 3 and 5. Only one annotated DhMR gene overlapped with any other comparison (e.g., male DEHP cortex, female DEHP blood, or any comparison from Pb exposures)—*Phactr1* was also identified in male cortices exposed to Pb (Figure 2C).

Adult male cortices developmentally exposed to DEHP (n = 7 exposed, n = 6 control) had 246 differentially hydroxymethylated regions, of which, only four had less hydroxymethylation in exposed (Figure 2A; Table 1; Supplementary Table S3; Supplementary Figure S1C). These regions mapped to 100 genes, with about 45% annotating to each gene introns and the open sea (Figure 2B). There were several regions that mapped to lncRNA, including *Macrod2os1*, *Mir99ahg*, *Gm26820*, *Gm15581*, *RP23-418H8.3*, *9130204K15Rik*, *4930511M06Rik/1700066O22Rik*, and *D930019O06Rik*. Other regions mapped to the promoters on *Kif2b*, *Slc12a7*, *Creg2*, and *Kctd19*. Of all 100 genes, there were four overlapping with the male Pb cortex genes, including *Camkmt*, *Galnt2*, *Ccdc192*, and *Asic2*. In mapped genes, gene ontology suggested that four pathways had differential hydroxymethylation levels in the male DEHP exposed cortices (Table 2). One pathway was a molecular function (solute and proton antiporter activity), and three pathways were biological processes in androgen receptor signaling and bone and biomineral regulation.

3.2 Differential hydroxymethylation: Pb

For females (n = 6 exposed, n = 6 control for both blood and cortex), there were no differences of hydroxymethylation in either blood or cortices when comparing exposed to control groups across both blood and cortex.

For males (n = 6 exposed, n = 6 control for both blood and cortex), there were no differences in blood hydroxymethylation, but there were 385 regions that had universally higher hydroxymethylation in Pb-exposed cortex compared to controls (Figure 2A; Table 1; Supplementary Table S4; Supplementary Figure S1D). These DhMRs mapped to 325 unique genes, with annotations primarily in introns (~18%) and open sea regions (~60%) (Figure 2B). There were several DhMRs in lncRNA regions, including *Rian*, *Trerf1*, *Pvt1*, *Gm3294*, *Gm13575*, *Gm38190*, *Gm27247*, *Gm12278*, *Gm29295*, *Gm26883*, *Gm26904*, *Gm17435*, *Gm26691*, *Gm16183*, *RP23-304A10.2*, *RP23-363M4.1*, *2610203C22Rik*, *E130304I02Rik*, *2810407A14Rik*,

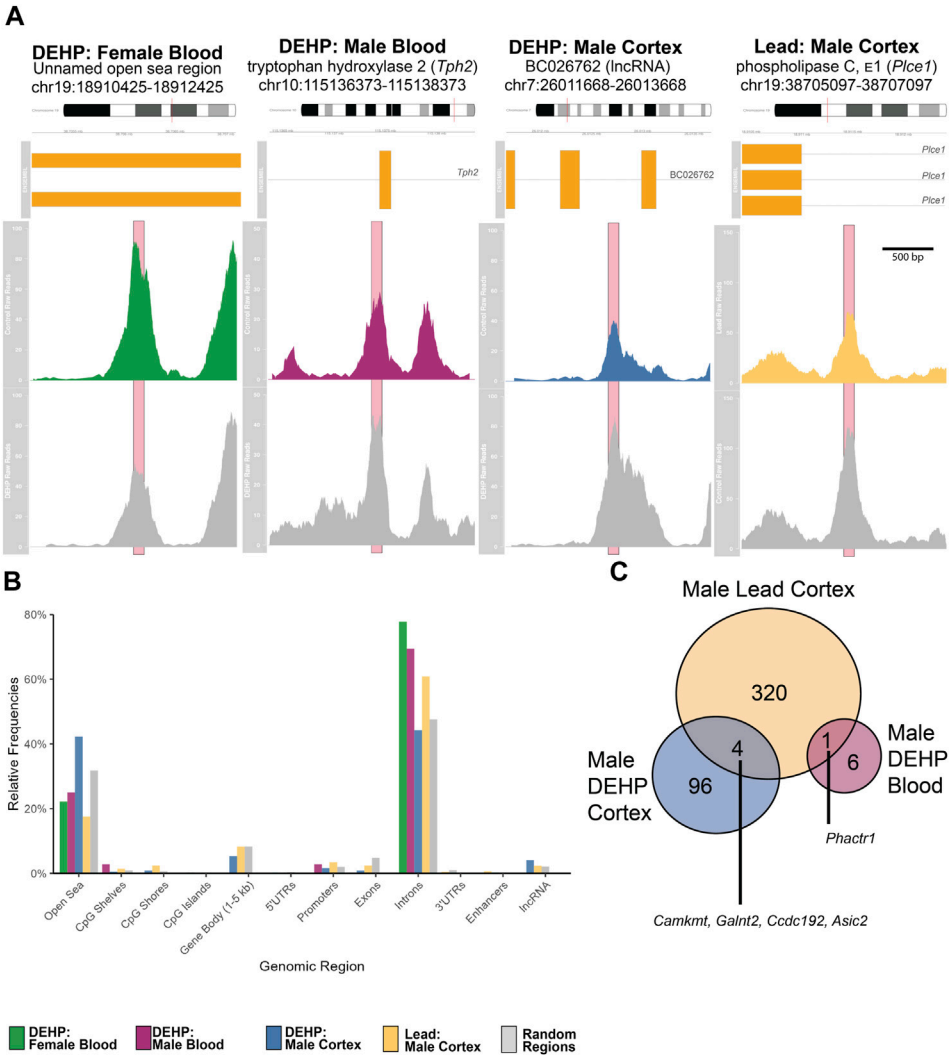

FIGURE 2
(A) Averaged Raw Reads from Top Hit in Each Comparison. Top colored panel in each panel represents the exposed group, bottom gray panel represents control. Significant peaks are marked with pink boxes. Gene region details show chromosomal and genomic location, as well as gene variants and exons (in yellow boxes). Each plot depicts the differentially hydroxymethylated region ± 1,000 bp. Detailed peak data is found in Supplementary Table. **(B)** Frequencies of Annotated Regions in Significant Peaks. Each bar shows the percentage of annotated peaks in that category using the annotatr package and mm10 genome in R. For each category, the left-most bar represents DEHP female blood peaks (green), second from left represents the DEHP male blood peaks (purple), the middle represents the DEHP male cortex peaks (blue), the second from the right represents the Pb male cortex peaks (yellow), and the right-most bar represents the randomly generated regions for comparisons (gray). **(C)** Venn Diagram of Annotated Differentially Hydroxymethylated Genes in Males. Unique genes that were overlapping in DEHP blood, DEHP cortex, and Pb (lead) cortex. No specific regions within those genes overlapped. There were no overlaps with female differentially hydroxymethylated regions. Abbreviations: CpG–cytosine-guanine site; DEHP–di(2-ethylhexyl) phthalate; lncRNA–long noncoding RNA; Pb–lead; UTR–untranslated region.

2610037D02Rik, and *F630040K05Rik*. Regulatory regions were also identified in promoters for calcium related genes (*Cacnb4*, *Camk2g*, and *Cabp1*), genes that interact with DNA or epigenetic processes (*Mxi1*, *Mnt*, *Mthfd2*, *Ldb1*, *Mfrp*, *Hnrnpk*, *mir7074*, and *Yeats2*), an imprinted gene (*Rian*), an oncogene related to AP1 transcription factor complex (*Jund*), and several other genes related to various cell functions (*Gpr156*, *Ttll6*, *Paip1*, *Marcksl1*, *Kcnq2*, *Septin8*, *Dxd18*, *Dhx37*, *Tmco1*, and *4833412C05Rik*). There were also 12 known enhancer regions on chromosomes 1, 4, 7, 9, 11, 15, 17, and 18 annotated to differentially hydroxymethylated regions. Gene ontology of the 325 genes suggested that pathways related to neuronal and neural function were the primary pathways that showed differential hydroxymethylation (Table 2).

Comparing log-fold differences in hydroxymethylation between regions in the Pb-male cortex and analogous regions in the DEHP-male cortex, sites were poorly correlated (Supplementary Figure S2).

4 Discussion

While the epigenome is comprised of many different modifications and molecules, there has been a strong focus on DNA methylation in studies on the effects of exposures to common chemicals. However, these studies typically use methods that do not differentiate between types of DNA modifications, even

TABLE 1 Number of differentially hydroxymethylated regions by exposure (FDR<0.15).

Sex	Tissue	Higher in exposed	Lower in exposed	Total
DEHP				
Females	Blood	-	2	2
	Cortex	-	-	0
Males	Blood	6	4	10
	Cortex	242	4	246
Pb (Lead)				
Females	Blood	-	-	0
	Cortex	-	-	0
Males	Blood	-	-	0
	Cortex	385	-	385

though methylation and hydroxymethylation act in biologically opposite ways. In this study, we found that the genome-wide mouse hydroxymethylome is affected by developmental exposure to both DEHP and Pb, with differences in hydroxymethylation observed in adulthood.

In the brain, hydroxymethylation accounts for 33%–50% of DNA modifications, a much higher proportion than in other tissues (Cui et al, 2020). This high occurrence may indicate its importance in normal brain function, such as memory formation (Kremer et al, 2018). Brain hydroxymethylation also plays a role in response to injury (Morris-Blanco et al, 2019; Madrid et al, 2021; Moyon et al, 2021) and oxidative stress (Delatte et al, 2015), likely in a region-specific manner (Doherty et al, 2016). Its role in injury and oxidative stress responses may also be why hydroxymethylation across the body appears to be more sensitive to environmental assaults than other epigenetic marks (Chatterjee et al, 2020).

Exposure to DEHP or other phthalates has been associated with differences in bulk measures of hydroxymethylation in both human urine (Pan et al, 2016) and rat testes (Abdel-Maksoud et al, 2015). Presently, we found that perinatal exposure to DEHP was associated with later-in-life differences in region-specific blood hydroxymethylation in males and females and brain hydroxymethylation in males. There were much fewer regional differences in blood compared to brain, which could be related to the brain's high levels of hydroxymethylation compared to other tissues. Perinatal DEHP exposure may also alter hydroxymethylation in a tissue specific manner, like with total DNA methylation in our same model (Wang et al, 2020; Liu et al, 2021; Svoboda et al, 2021). In the male brain, there were several regions in gene promoters responsible for microtubule control/cell division (*Kif2b*), cell transport (*Slc12a7*), neural-specific endoplasmic reticulum and Golgi functions (*Creg2*), and potassium channel function (*Kctd19*). These differences were also linked to gene ontology pathway enrichment in general cellular processes and in pathways related to androgen signaling and bone development. In humans, disruptions in androgen signaling have been one of the primary health effects of concern after developmental DEHP exposure. High exposures have been associated with decreased anogenital distances in males (Swan et al, 2005; Li and Ko, 2012) and long-term changes in growth and metabolism (Tsai et al, 2016; Tsai et al, 2018). More recently there have been concerns about bone growth and development in both animal models (Bielanowicz et al, 2016; Chiu et al, 2018) and humans (Heilmann et al, 2022).

Pb-exposed females showed no differences in hydroxymethylation; only Pb-exposed male brains had increases in hydroxymethylation in the brain. Hydroxymethylation differences after Pb exposure have been explored in various

TABLE 2 Gene ontology results from annotated differentially hydroxymethylated regions (FDR<0.1).

Ontology name	GO ID	Type	Genes in GO Set (N)	Genes in Data (n)	*p*-value	FDR
DEHP (Male Cortex)						
solute:proton antiporter activity	GO:0015299	MF	15	2	1.20E-05	0.011
androgen receptor signaling pathway	GO:0030521	BP	45	4	1.06E-05	0.054
regulation of bone mineralization	GO:0030500	BP	71	6	3.35E-05	0.085
regulation of biomineral tissue development	GO:0070167	BP	79	6	5.18E-05	0.087
Pb (Male Cortex)						
postsynapse	GO:0098794	CC	460	26	1.26E-04	0.044
postsynaptic density	GO:0014069	CC	231	16	2.30E-04	0.044
postsynaptic specialization	GO:0099572	CC	233	16	2.71E-04	0.044
asymmetric synapse	GO:0032279	CC	234	16	3.24E-04	0.044
neuron to neuron synapse	GO:0098984	CC	236	16	3.66E-04	0.044
nuclear periphery	GO:0034399	CC	110	7	8.75E-04	0.088

Gene ontology (GO) results using differential hydroxymethylated regions from the male cortex and ChIP-Enrich in R. All pathways were upregulated in exposed groups. Abbreviations: DEHP, di(2-ethylhexyl) phthalate; BP, biological processes; CC, cellular component; MF, molecular function; Pb, lead.

human tissues, including childhood blood (Rygiel et al, 2021), cord blood (Sen et al, 2015; Okamoto et al, 2022), toenail and placenta (Tung et al, 2022), and sperm (Zhang et al, 2021). Epigenome-wide differences were only assessed in toenails/placenta using the EPIC array (Tung et al, 2022) and in sperm using hMeDip-seq (Zhang et al, 2021). In toenails and placentas, most of the differentially hydroxymethylated sites were higher with Pb exposure (Tung et al, 2022), whereas sperm showed mostly lower hydroxymethylated regions (Zhang et al, 2021). Presently, all male cortex regions had higher hydroxymethylation with Pb exposure. All three epigenome-wide studies (Tung et al, 2022; Zhang et al, 2021, and the present study) identified differential hydroxymethylation in calcium genes or pathways. Because Pb is a bioanalogue of calcium, these similarities are expected. These three studies also consistently reported differences in pathways related to nervous system development and synapse function, even in non-neural tissues. This link should be further explored, as small, sparse, and local differences in hydroxymethylation have been associated with gene expression (Marion-Poll et al, 2022), potentially representing a "fine tuning" mechanism of gene regulation that is linked with transcription factor recruitment (Lercher et al, 2014). Because the brain is the primary target organ of Pb toxicity, collective results may be revealing differences in hydroxymethylation patterns that underlie the link between developmental Pb exposure and later life neurotoxic effects.

In Pb-exposed males, an imprinted gene (*Rian*) and several lncRNA genes were identified as differentially hydroxymethylated. Genomic imprinting is an epigenetically regulated process in which a gene is expressed from one allele in a parent of origin-specific manner. This phenomenon occurs in nearly 1% of the protein coding genes and includes maternally-expressed genes that are paternally imprinted, or vice versa (Barlow and Bartolomei, 2014; Kanduri, 2016). Imprinted genes are typically clustered to form imprinted domains, which also include the genetic code for at least 1-2 lncRNA. Imprinted domains often also contain gametic differentially methylated regions, one of which controls the entire domain to serve as an imprinting control region. The *Rian* lncRNA is part of a large imprinted domain on chromosome 12 (in mice). The human ortholog, *MEG8*, regulates vascular smooth muscle cell proliferation, migration, and apoptosis via miRNA interactions with the peroxisome proliferator activator receptor alpha (PPARα) (Zhang et al, 2019). Differential hydroxymethylation of imprinted genes was also identified in mice after developmental exposure to BPA (Kochmanski et al, 2018), which may confer broader patterns of disruption in imprinted genes after developmental exposure to common environmental contaminants.

While most identified regions were unique between exposures, there were four genes that overlapped between the DEHP and Pb in the male cortex, including *Camkmt*, *Galnt2*, *Ccdc192*, and *Asic2*. *Camkmt* encodes for a methyltransferase that assists in calcium dependent signaling, *Galnt2* encodes for a glycotransferase linked with metabolism functions, *Ccdc192* encodes for a long-noncoding RNA, and *Asic2* encodes for a protein in an ion channel, with high prevalence in the central nervous system. In the same cohort of mice from the present study, differential total methylation was identified in the *Galnt2* gene in heart tissue after DEHP and Pb exposure (Svoboda et al, 2021). Differential total methylation of the human ortholog of *Galnt2* was also identified in a human cohort exposed to Pb (Svoboda et al, 2021), representing either a potential biomarker of exposure or, due to the *Galnt2*'s large size, general epigenetic differences that broadly occur after environmental exposures. There was also one gene that overlapped in brain/blood across exposures–*Phactr1*, a gene that encodes for a phosphatase that regulates the actin cell structure. These overlapping genes may represent common areas of the hydroxymethylome that are particularly sensitive to environmental toxicants. Alternatively, as hydroxymethylation generally confers genome instability (Supek et al, 2014), these could be chance overlaps due to tertiary genome structure or other factors.

Hydroxymethylation is different between sexes and across ages, typically increasing throughout development (Cisternas et al, 2020). In our study, there were large differences between sexes, which may be a due to normal sex differences or due to sex differences in responses to environmental exposures. Additional studies with larger sample sizes and other model strains/species should be conducted, and the inclusion of physiological and behavioral endpoints should be emphasized, especially given the growing body of evidence on sex-specific responses to toxicants (Gade et al, 2021). Our smaller sample size could also result in false negatives. Validation studies with larger sample sizes should aim to include multiple developmental timepoints and tissues to understand hydroxymethylation differences in varying ages and across the body. Future validation studies could also consider PCR or nanopore techniques to confirm hMeDIP-seq results. Because we didn't measure individual animal dietary intake of either DEHP or Pb, future studies should also measure toxicant exposures in offspring to better estimate dose-response relationships. Additionally in the brain, it is important to consider the impacts of non-cytosine hydroxymethylation (Ma et al, 2017), the parallel differences in methylation, and the cell-type specific patterns in epigenetic modifications which were not presently assessed.

Overall, this study demonstrates that sex-specific hydroxymethylation is different in adulthood in response to developmental environmental exposures, in not just the brain (specifically cortex), but also blood. The differences, however, were limited and should be validated with future work. Results were observed using a method that is able to detect regional differences across >95% of the genome, mostly in areas with lower cytosine-guanine base density (Beck et al, 2022). Because environmental epigenetic studies have traditionally only used methods that capture the collective DNA modifications (e.g., methylation + hydroxymethylation) in high density cytosine-guanine areas of the genome, many environmental effects in the hydroxymethylome may be masked. Future research needs to distinguish DNA methylation and hydroxymethylation functions and responses to environmental exposures, with the aim of revealing potential biomarkers or interventions in the exposure-disease pathway. The hydroxymethylome has had some promising studies in these areas already (Kim et al, 2018; Morris-Blanco et al, 2019; Morris-Blanco et al, 2021). Going forward, its role and responses to the environment should be a major research focus, sharing the spotlight with the diverse modifications and molecules of the epigenome.

Data availability statement

The data presented in the study are deposited in the NCBI Geo repository, accession number GSE229717.

Ethics statement

The animal study was reviewed and approved by University of Michigan Institutional Animal Care and Use Committee (IACUC).

Author contributions

LS, DD, MS, JG, and JC constructed the study, supervised experiments and data collection. JC, KN, and CR helped create the research design. KN, BP, TJ, CR, and JC collected samples. CL prepared the libraries and ran the hMeDIP-seq. RCG processed the data. MS, KW, and RP helped design the statistical tests. RP analyzed data. RP, RC, RM, CL, and BP drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1198148/full#supplementary-material

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Emerging concepts involving inhibitory and activating RNA functionalization towards the understanding of microcephaly phenotypes and brain diseases in humans

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Microcephaly is characterized as a small head circumference, and is often accompanied by developmental disorders. Several candidate risk genes for this disease have been described, and mutations in non-coding regions are occasionally found in patients with microcephaly. Various non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), SINEUPs, telomerase RNA component (TERC), and promoter-associated lncRNAs (pancRNAs) are now being characterized. These ncRNAs regulate gene expression, enzyme activity, telomere length, and chromatin structure through RNA binding proteins (RBPs)-RNA interaction. Elucidating the potential roles of ncRNA-protein coordination in microcephaly pathogenesis might contribute to its prevention or recovery. Here, we introduce several syndromes whose clinical features include microcephaly. In particular, we focus on syndromes for which ncRNAs or genes that interact with ncRNAs may play roles. We discuss the possibility that the huge ncRNA field will provide possible new therapeutic approaches for microcephaly and also reveal clues about the factors enabling the evolutionary acquisition of the human-specific "large brain."

KEYWORDS

microcephaly, non-coding RNA, RNA-binding protein, human medicine, epigenetic, brain diversity

1 Introduction

Abnormal brain growth leads to aberrant brain size and developmental disorders. Microcephaly is defined as a head circumference < −2 standard deviations (SD) in humans (Whelan, 2010). Genetic mutations have been identified in half of such patients. Patients with severe microcephaly (<-3 SD) are more likely to be also have other developmental diseases such as epilepsy, cerebral palsy, autism, and intellectual disabilities simultaneously (Pirozzi et al., 2018). Numerous studies have revealed a variety of risk genes for microcephaly. For example, the *assembly factor for spindle microtubules (ASPM)* gene, which encodes a centrosomal protein, is one of the most frequent candidate genes for this symptom (Nicholas et al., 2009). Dysfunction of other centrosomal proteins such as WDR62, CEP135, CENPE, and MCHP1 also causes microcephaly, which indicates the importance of centrosomes for brain volume expansion in

infants (Pirozzi et al., 2018). On the other hand, non-genetic factors (e.g., Zika virus infection, excessive maternal alcohol drinking, drug overdose, and malnutrition) can also be causes for such diseases (Whelan, 2010). In addition, epigenetic factors are known to be involved in abnormal brain growth phenotypes. For example, Rett syndrome, an epigenetic disease, was first described in 1966 (Rett, 1966). The syndrome appears in approximately 1 in 10,000 female births. Patients grow and develop normally until 6-8 months of age, and then gradually lose speech and hand skills and appear to have stereotypic hand movements. The head circumference growth decelerates and patients are diagnosed with microcephaly (Weng et al., 2011). This disorder is caused by mutation in *X-linked methyl-CpG-binding protein 2 (MeCP2)*, whose protein product binds to methyl-CpG sites (Amir et al., 1999), affecting both genic and intergenic regions in the genome to modulate RNA transcription. The occurrence of the complex disease phenotypes is further supported by recent studies showing that many central nervous system (CNS) disorders are also associated with mutations in non-coding regions in the human genome (Simon-Sanchez and Singleton, 2008). Single nucleotide polymorphisms (SNPs) located within non-coding regions have occasionally been found in infants with microcephaly (Xia et al., 2017). A recent study indicated that ASPM is modulated by circular RNA and microRNA (miRNA), both of which are types of non-coding RNAs (ncRNAs) (Han et al., 2021). Therefore, better understanding of the involvement of non-coding regions in the pathogenesis of microcephaly through ncRNA transcription is needed. NcRNAs play important roles in genome transcription, RNA translation, RNA degradation, and protein scaffolding (Yan et al., 2021). For example, several miRNAs related to Feingold syndrome function in RNA interference in which the precursors of these miRNAs are transcribed by RNA polymerase II, and then the miRNA is incorporated into the miRNA-induced silencing complex called miRISC to degrade the target mRNA (de Pontual et al., 2011), as described later. In addition to miRNA, long ncRNA (lncRNA) with size greater than 200 nt (Novikova et al., 2013) also seems to function for regulating brain size by forming a complex structure with chromatinic DNA to regulate gene expression (Chi et al., 2019). Here, we will introduce diseases with microcephaly candidate genes including those for RNA-binding proteins (RBP) and with intergenic mutations that affect the generation of ncRNAs and discuss how ncRNAs are involved in establishing the nature of human-specific "large-brain" and how RNA-involving epigenetic mechanisms can be therapeutic targets (Figure 1). In fact, there are several brain diseases that affect brain size but are not annotated as microcephaly. Since little information on ncRNAs contributing to microcephaly is available, we will also refer to ncRNAs known to be physically and/or functionally connected to brain-size-affecting diseases (e.g., autism spectrum disorder: ASD) other than known microcephaly-related diseases.

2 Cytosolic function of ncRNAs in brain diseases

2.1 Microcephaly-related inhibitory ncRNAs in the cytoplasm

Feingold syndrome is an autosomal dominant syndrome including microcephaly, short stature, and short mesophalanx of the fifth finger (brachymesophalangy). Several ncRNAs are involved in the pathogenesis of this syndrome. In many cases, the deletion of either *MYCN* (type 1) or *MIR17HG* (type 2) seems to cause this type of disease (de Pontual et al., 2011). *MIR17HG* generates six miRNAs, namely, miR-17, 18a, 19a, 20a, 19b-1, and 92a-1 (Mendell, 2008), which have been reported to be involved in proliferation of various tumors (Tan et al., 2022). MYCN protein seems to regulate the expression of these miRNAs by binding to the *MIR17HG* promoter region to upregulate miRNA expression (de Pontual et al., 2011). MiR-17-92 cluster is described as a human oncogene in several cancers (Hayashita et al., 2005) (Mu et al., 2009). The deletion of the cluster promotes apoptosis because the miRNAs target *BIM* (Ventura et al., 2008), which initiates the intrinsic apoptotic pathway (Sionov et al., 2015). Mice models for Feingold syndrome type 2 exhibit brachymesophalangy, small body (short stature), and microcephaly. The homozygous deletion of *MIR17HG* frequently leads to perinatal lethality (de Pontual et al., 2011). *MIR17HG* targets *TGF-β receptor type 2 (TGFBR2)* (Ma et al., 2016) (Mirzamohammadi et al., 2018), and cases deficient for *MIR17HG* are associated with excessive TGF-β signaling, which is supported by the fact that treatment with a TGF-β receptor inhibitor, LY364947, prevented the skeletal defect and microcephaly in the Feingold syndrome type 2 mouse model. GW788388, another TGF-β receptor inhibitor, and 1D11, a neutralizing antibody against TGF-β ligands, also caused similar effects (Mirzamohammadi et al., 2018).

2.2 Gene-activating ncRNAs functioning in the cytoplasm

In recent years, patients with *de novo* mutation of *RAB11B* have been described. The symptoms include absent speech, epilepsy, hypotonia, and microcephaly (Lamers et al., 2017) (Jauss et al., 2022), in spite of the fact that, in mouse, *Rab11b* deficiency exhibits no phenotypes (Nassari et al., 2020). This suggests that human *RAB11B* has acquired human-specific functions. RAB11B is a small GTPase belonging to the Rab family. Rab forms and transfers vesicles, and fuses them with the cellular membrane (Stenmark and Olkkonen, 2001). *RAB11B* is expressed in the brain, heart, and testis (Lai et al., 1994). Mislocalization of abnormal RAB11B due to mutations at its GTP/GDP binding pocket causes disorganized brain structures and functions (Lamers et al., 2017). Interestingly, *RAB11B-AS1* is transcribed from the bidirectional *RAB11B* promoter to modulate *RAB11B* functions. *RAB11B-AS1* is expressed in humans including in the brain, and functions as a "SINEUP" RNA for *RAB11B*, that can promote *RAB11B* translation (Zarantonello et al., 2021). SINEUP is a category of lncRNAs that promote translation of partly overlapping mRNAs (Zucchelli et al., 2015). This mechanism involves Polypyrimidine tract-binding protein (PTBP1), which is also known to function in alternative splicing for *Filamin A (FLNA)* (Zhang et al., 2016), a causative gene for microcephaly in mice, and the deregulation of this alterative splicing leads to periventricular heterotopia (PH) in human (Lian et al., 2012). In addition, PTBP1 can function together with heterogeneous nuclear ribonucleoprotein K (HNRNPK) to bind to the SINEUP RNAs to target mRNAs. These two RBPs help to recruit ribosomal subunits for enhancing the translation of the target mRNAs (Toki et al., 2020). It is noteworthy that *RAB11B* and

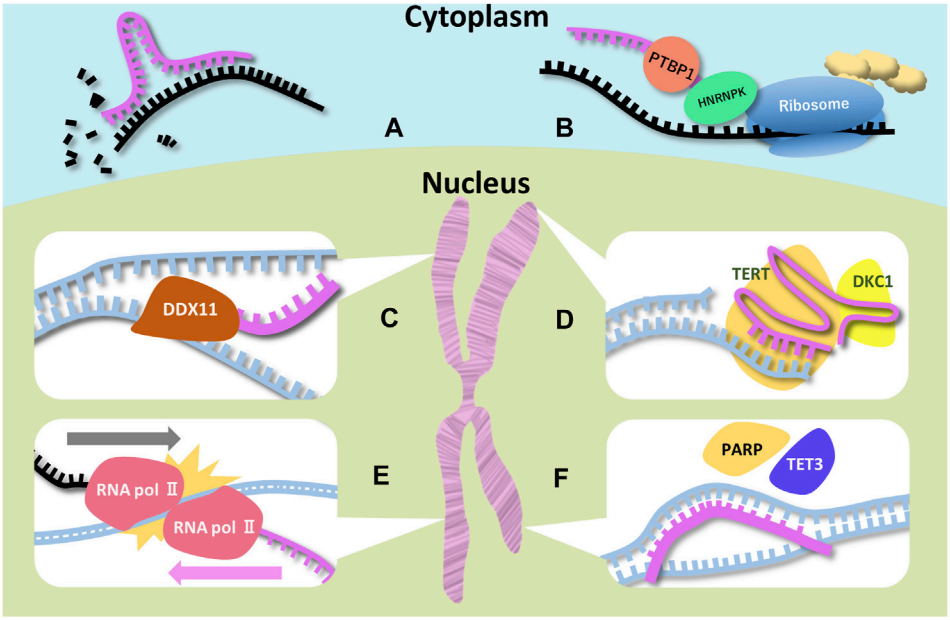

FIGURE 1
Schematic representations of function of ncRNAs and RBPs associated with microcephaly Six examples for ncRNAs (miRNA, SINEUP, DDX11-AS1 (CONCR), TERC, UBE3A-ATS, and pancRNA) are illustrated. Magenta, black, and blue strands indicate ncRNA, mRNA, and DNA respectively. **(A)** Degradation of the target mRNA is a miRNA function. MiRNAs have the complementary sequence of the target mRNAs. **(B)** Two RBPs (PTBP1 and HNRNPK) bind to SINEUP and recruit the ribosome. SINEUP upregulates translation through RBPs. **(C)** DDX-AS1 bind to DDX11 directly to promote its enzymatic activity. In addition, the lncRNA traps miRNA targeting DDX11 mRNA. **(D)** TERC is the template for telomere elongation. DKC1 is essential for the TERC stability. **(E)** RNA polymerases colliding is thought to lead to stopping elongation of UBE3A mRNA. **(F)** pancRNA recruits transcription and histone acetylation factors by changing DNA structure.

RAB11B-AS1 are downregulated by *CDH8* (Zarantonello et al., 2021), and therefore, known *CDH8* variants may modulate the cellular level of *RAB11B*, leading to macrocephaly and intellectual disability (Bernier et al., 2014). Although the molecular function of *RAB11B-AS1* in brain contexts is still obscure, it is possible that evolutionary acquisition of *RAB11B-AS1* was actively involved in enlarging the human brain because some studies have shown an association with cancer via oncogene effects such as cell proliferation (Li et al., 2020), migration and invasion (Niu et al., 2020).

3 Epigenetic function of ncRNAs in brain disease

3.1 Chromatinic ncRNAs acting on intergenic regions

Warsaw breakage syndrome is a recessive hereditary disease caused by mutation in *DEAD/H-box helicase 11* (*DDX11*, also known as *hChlR1*) (van der Lelij et al., 2010). The clinical features include microcephaly, hearing loss, and facial dysmorphia. DDX11 regulates chromatin structure (Pisani et al., 2018). *DDX11* also controls chromosome separation and sister chromatid condensation in mitosis. *DDX11* is hypothesized to prevent abnormal DNA structure in the replication folk (Leman et al., 2010). In line with this idea, defective sister chromatid cohesion is frequently observed in Warsaw breakage syndrome patients' cells (Pisani, 2019). Separation of the centromere and sister chromatid pairs observed in mitomycin C-induced chromosomal breakage is a remarkable feature of *DDX11* mutations (van der Lelij et al., 2010). The mutations in conserved helicase motifs result in unwinding forked duplex DNA substrates. DDX11 destabilization occurs due to misfolding of the protein (Santos et al., 2021). In mouse models, *Ddx11* is indispensable for mouse embryonic and placental development, and *Ddx11* knock out causes embryonic lethality (Inoue et al., 2007). In zebrafish models, embryonic lethality was increased and craniofacial and vertebral abnormalities were observed. In addition, *ddx11* dysfunction generated heterochromatic structures ectopically. This gene also affects histone epigenetic modifications (Sun et al., 2015). Interestingly, a lncRNA, *DDX11 antisense RNA 1* (DDX11-AS1, also known as *CONCR*) is transcribed bidirectionally from the *DDX11* promoter region. Although the molecular function of *DDX11-AS1* in microcephaly contexts is still obscure, deletion of *DDX11-AS1* causes a defect in sister chromatid condensation in mitosis like Warsaw breakage syndrome. Unlike SINEUP, the DDX11 protein level is not affected by the ncRNA knockdown. Levels of histone H3K9 acetylation at the *DDX11* promoter region and *DDX11* mRNA are also unchanged. Surprisingly, however, the ncRNA can bind DDX11 protein directly, and thus activates hydrolysis of ATP. *DDX11-AS1* maintains proper chromatin structure through promoting the enzymatic activity of DDX11 (Marchese et al., 2016). Another report indicated that *DDX11-AS1* also function to regulate *DDX11* through sponging miR-873-5p, which can target *DDX11* (Zhang et al., 2020).

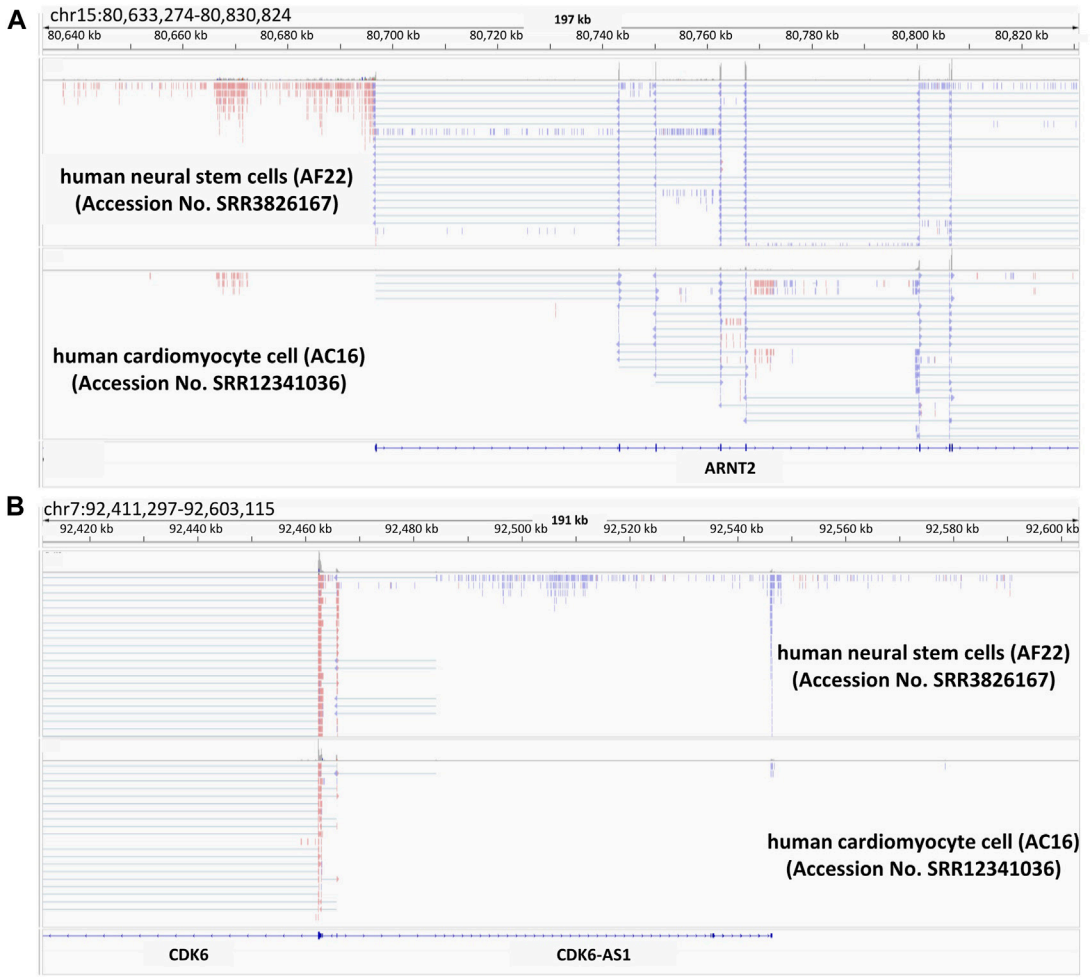

FIGURE 2
Presence of divergent lncRNAs in two examples of microcephaly-related genes Snapshots of the Integrative Genomics Viewer. Publicly available RNA-seq data of human-iPS cell-derived neural stem cell (AF22) and human hybrid cardiomyocyte (AC16) are shown. The data is from (Brattas et al., 2017; Lopacinski et al., 2021). ARNT2 **(A)** and CDK6 **(B)** are microcephaly related genes. In this figure, the colors indicate the differential strand usage.

3.2 ncRNA that maintains telomere length

Mutation of *dyskerin pseudouridine synthase 1 (DKC1)* frequently result in Hoyeraal-Hreidarsson syndrome, a microcephaly disease (Dehmel et al., 2016). DKC1 has TRUB (tRNA pseudouridine synthase B-like) and PUA (pseudouridine synthase and archaeosine transglycosylase) domains. The TRUB domain constitutes the catalytic core of DKC1, whereas the PUA domain seems to function as a RNA binding motif (Garus and Chantal, 2021). *DKC1* plays an important role in pseudouridylation of rRNA and telomere extension. The telomerase complex is composed of telomerase reverse transcriptase (TERT), telomerase RNA component (TERC), and other protein factors including DKC1 (Czekay and Kothe, 2021). Apoptosis and chromosomal aberrations increase and proliferative potential decreases in *Terc*$^{-/-}$ mouse cells (Wong et al., 2003). Both Hoyeraal-Hreidarsson syndrome patients and mouse models for DKC1 dysfunction show reduced rRNA processing and telomerase activities (Mochizuki et al., 2004). In fact, loss of telomere length cause dyskeratosis congenita characterized by bone marrow failure, hyperpigmentation, nail dystrophy and leukoplakia (AlSabbagh, 2020). In particular, *DKC1* is involved in the Xp28 and X-linked recessive dyskeratosis congenita, known as a profound type of dyskeratosis congenita, including growth retardation and microcephaly (Dehmel et al., 2016). In most cases of Hoyeraal-Hreidarsson syndrome, the variant A353V located in the PUA domain of DKC1 is observed (Knight et al., 1999). The same mutation attenuates the binding of DKC1 to the TERC, leading to TERC destabilization (Czekay and Kothe, 2021). Accordingly, some patients with mild dyskeratosis congenita also have telomere shortening (Vulliamy et al., 2001) (Yamaguchi et al., 2003). Because bone marrow failure also accompanies dyskeratosis congenita, the patients are frequently treated with hematopoietic cell transplantation (HCT) or androgen therapy (Savage and Niewisch, 2009). Considering the potential of ncRNAs as future therapeutic agents for curing such diseases, their physical association with EXOSC10, a component of the RNA exosome complex which eliminates TERC, may be notable, because its knockdown

TABLE 1 NcRNA-involving phenotypes in mouse model.

Name	Phenotype	Ref
BC1	Learning and memory impaired	Chung et al. (2017)
Neat1	Determination of behavioral responses under conditions of stress	Kukharsky et al. (2020)
AtLAS	Regulation of social hierarchy	Ma et al. (2020)
Linc-Brn1b	Generation of upper layer II-IV neurons in the neocortex	Sauvageau et al. (2013)
Pnky (lnc-pou3f2)	Neuronal differentiation	Ramos et al. (2015)
Malat1	Synapse formation and/or maintenance	Bernard et al. (2010)
GM12371	Regulation of expression of synaptic gene	Raveendra et al. (2018)
Bdnf-AS	Maintenance of stemness in neural stem cells	Modarresi et al. (2012)
Evf2	Formation of GABA-dependent neuronal circuitry	Bond et al. (2009)
Dali	Regulation of neural differentiation genes	Chalei et al. (2014)
Zfas1	Upregulating in status epilepticus mice model	Hu et al. (2020)
Dlx6-as1	Upregulating in Parkinson's disease (PD) mice model	Liu et al. (2022)

TABLE 2 NcRNAs related to human brain diseases.

ncRNA	Disease	Ref
BC200	Alzheimer's disease (AD)	Sosinska et al. (2015)
NEAT1	Amyotrophic lateral sclerosis (ALS), Epilepsy, SCZ (female), AD, PD	Safari et al. (2019); An et al. (2020)
BDNF-AS	ASD	Wang et al. (2015)
MSNP1AS	ASD	Kerin et al. (2012)
DGCR5	SCZ	Meng et al. (2018)
RP5-998N21.4	SCZ	Guo et al. (2022)
DODA-AS1 (G30)	SCZ, bipolar disorder	Detera-Wadleigh and McMahon (2006)
Cyrano (OIP5-AS1)	SCZ (female)	Safari et al. (2019)
FAS-AS1	SCZ (male)	Safari et al. (2019)
Gomafu (MIAT/RNCR2)	Multiple sclerosis (MS), SCZ	Barry et al. (2014); Fenoglio et al. (2018)
TUNA	Huntington's Disease (HD)	Lin et al. (2014)
RMST	PD	Chen et al. (2022)
PTCHD1-AS	ASD	Ross et al. (2020)
MEG3	ASD	Taheri et al. (2021)

restored telomerase activity in *DKC1* knockdown cells (Shukla et al., 2016).

3.3 The convergent regulation of gene expression by ncRNA

Angelman syndrome, another microcephaly disease, was first described in 1965. It was characterized by unusual arm position and jerky movement (Kishino et al., 1997). Major characteristics include severe intellectual disability, lack of speech, sleep disruption, and microcephaly (Levin et al., 2022). Mouse models for Angelman syndrome frequently exhibit motor dysfunction and deficits in learning and memory. Abnormal electroencephalogram (EEG) is also observed (Miura et al., 2002). Mutation in the *E6-AP ubiquitin-protein ligase gene (UBE3A)* was found in chromosome 15 of many patients (Kishino et al., 1997) (Matsuura et al., 1997). Normally, *UBE3A* is expressed only from the maternal allele in the brain, while the paternal allele is silenced by genome imprinting. Some patients have a UBE3A mutation in the maternal allele, and others have paternal uniparental disomy (PUD) and/or imprinting defects (ID) (Saitoh et al., 2005) (Bai et al., 2014). The deletion patients have

more profound effects than PUD and ID patients (Lossie et al., 2001). A *UBE3A* antisense transcript, *UBE3A-ATS*, suppresses *UBE3A* on the paternal chromosome (Meng et al., 2012). In the paternal chromosome, *UBE3A* and *UBE3A-ATS* are transcribed at the same time. However, in contrast to SINEUP, *UBE3A-ATS* prevents *UBE3A* transcription at the expressing paternal allele. It has been thought that 2 opposing RNA polymerases collide and stop the elongation of *UBE3A* mRNA (Wang et al., 2021) (Mabb et al., 2011). Although the molecular function of *UBE3A-ATS* in microcephaly contexts is still obscure, disrupting *UBE3A-ATS* transcription is noted as a potential therapy to increase *UBE3A* expression in the gene therapy field. For example, a clinical trial using antisense oligonucleotides is ongoing (Schmid et al., 2021). In a mouse model, such treatment can recover paternal *UBE3A* expression. Early treatment in mouse models (at postnatal day 1) is more effective compared with treatment of the adult (at 2 to 4 months of age). Partial improvement of motor deficiency and anxiety is observed only in young models. However, the behavioral phenotypes are hardly recovered. Nonetheless, both early and adult treatments ameliorate the memory impairment in fear conditioning tests (Milazzo et al., 2021) (Meng et al., 2015). Creation of indels located between the *Ube3a* 3' UTR and *Snord115 (Small Nucleolar RNA, C/D Box 115)* by CRISPR/Cas9 rescued the behavioral phenotype (Schmid et al., 2021). Cas9 targeting the *Snord115* cluster also prevent the motor deficiency (Wolter et al., 2020). Injection of the adeno-associated virus (AAV) expressing Zinc finger-based artificial transcription factors (ATFs), that repress *Ube3a-ats* to induce *Ube3a* expression (Bailus et al., 2016), also recovers the behavioral phenotype in mouse models (O'Geen et al., 2023). It is noteworthy that these targetings simultaneously truncate *UBE3A-ATS*, supporting the idea that allele-specific artificial removal of *UBE3A-ATS* is essential for ongoing therapies. How the lncRNA represses *UBE3A* expression is still uncertain, and elucidation of the mechanism will enable more effective therapy for Angelman syndrome.

3.4 ncRNAs leading to divergent transcription

We have illuminated various points of ncRNA actions in the above sections. Independently from convergent lncRNAs, we have found a different class composed of thousands of lncRNAs resulting from divergent transcription that originates from protein-coding gene promoters (Uesaka et al., 2014). Later on, we will introduce the functional mode and the potentials of such divergent ncRNAs based on our previous and other studies. As shown in the upper panel of Figures 2A lncRNA seems to be transcribed in the reverse direction to the partner gene. Comparison between RNA-seq reads from human neural stem cells (Brattas et al., 2017) and those from human cardiomyocyte cells (Lopacinski et al., 2021), revealed that *aryl hydrocarbon receptor nuclear translocator 2 (ARNT2)* is more highly expressed in brain than in cardiomyocytes. A variant of the gene causes Webb-Dattani syndrome, of which the features include microcephaly (Webb et al., 2013). Likewise, *cyclin dependent kinase 6 (CDK6)* is also microcephaly candidate gene (Naveed et al., 2018) and lncRNA expression was synchronized with that of mRNA. It would be interesting to see the possible effects of divergent lncRNAs on the pathogenesis of microcephaly-related diseases. As noted above, we have discovered a new type of divergent lncRNAs, called promoter-associated lncRNAs (pancRNAs) that are transcribed in the reverse direction to a set of tissue-specific genes (Uesaka et al., 2017). Approximately half of mammalian promoters show CpG-rich sequences and lack of TATA elements. In these CpG island-type promoters without TATA elements, TATA-binding protein (TBP) is recruited together with CpG-rich sequence-specific transcription factors such as Sp1 (Wu and Sharp, 2013) in both strands, thereby driving bidirectional transcription (Mahpour et al., 2018). Although enormous numbers of genes, including housekeeping genes, have CpG-rich promoters, the characteristics of the promoters for pancRNA-partnered genes include the acquisition of a G- and/or C-skewed motif, while such a skew cannot be seen in housekeeping genes. In addition, the lack of a poly(A) site sequence in the body of the pancRNAs has enabled then to get longer (An et al., 2021). Promoter-proximal Ser2 phosphorylation further reinforces a longer RNAPII dwell time at the start site, which may be beneficial for recruiting U1 snRNP upstream of the gene, thereby suppressing the recognition of poly(A) sites and the coupled termination of divergent transcription (Almada et al., 2013). In line with the concordant expression of pancRNAs and the partnered genes, as shown in Figures 2A,B, pancRNA production is associated with DNA demethylation, H3K4 trimethylation (Hamazaki et al., 2015), and H3K27 acetylation (Uesaka et al., 2017). In terms of the biological functions of pancRNAs, these are dependent on the roles of the downstream genes. For example, in rat PC12 cells, *pancNusap1* functions in *nucleolar and spindle associated protein 1 (Nusap1)* activation through histone acetylation, accelerating the cell cycle since Nusap1 plays a role in spindle microtubule organization (Yamamoto et al., 2016). Another example is mouse *pancIl17d*, which enhances demethylation of the *interleukin 17days (Il17d)* promoter by recruiting ten-eleven translocation 3 (Tet3) and poly ADP-ribose polymerase (Parp). Silencing *pancIl17d* is embryonic lethal, probably because Il17d functions to support proliferation/differentiation of pluripotent stem cells, which has been evidenced by the fact that supplying Il17d protein rescues embryonic survival (Hamazaki et al., 2015). pancRNAs occasionally form a triple helix structure with the DNA duplex of promoters and/or enhancers, and interact with some regulatory proteins, such as histone modifiers and transcription factors, to regulate gene transcription in cis. A second mechanism is based on transcriptional activation via formation of a DNA-RNA hybrid (R-loop). In mammalian cells, the asymmetrical distribution of cytosine and guanine, one of the characteristics of CpG islands for tissue-specific genes as discussed above, makes it easy to form R-loops (Chen et al., 2017). Therefore, targeting these structures triggered by pancRNA expression might be a strategy to mitigate microcephaly-related diseases in the future.

4 RBPs as potential targets for brain diseases

Although the information on ncRNAs in microcephaly is still limited, we can learn more about ncRNAs in relation to brain diseases. In addition to the examples of functional ncRNAs noted above, several other ncRNAs that specify social interactions and

behavior have been identified by using mouse models (Table 1). For example, brain cytoplasmic 1 (BC1), which has a motif for dendritic localization (Robeck et al., 2016), regulates neuronal activity-dependent translation in neurons (Eom et al., 2014). Memory and learning dysfunction were observed in some mouse knockout models of BC1 ncRNAs (Chung et al., 2017). The lncRNA nuclear paraspeckle assembly transcript 1 (Neat1) sponges various miRNAs (Azizidoost et al., 2022). The knockout model of Neat1 lost interest in a social interaction (Kukharsky et al., 2020). The ncRNA of synapsin2 (syn2) is decreased in mice with dominant behavior. The ncRNA modulates the social rank thorough binding syn2b pre-mRNA directly and protecting against its destabilization (Ma et al., 2020). In ASD and schizophrenia (SCZ), differentially expressed lncRNAs were detected (Ziats and Rennert, 2013) (Chen et al., 2016) (Table 2). One can hypothesize that most of the lncRNAs function together with RBPs. Although we still do not know of RBPs specifically functioning in the context of microcephaly, in some cases of brain diseases, detailed relationships between RBPs and ncRNAs have been revealed. Cyrano (OIP5-AS1), which is a schizophrenia candidate gene in females (Safari et al., 2019), sponges HuR (human antigen R) and inhibits the protein (Kim et al., 2016). Gomafu (RNCR2/MIAT) binds to the RNA-binding protein Celf3 and splicing factor SF1. The complex is speculated to control splicing and transcription (Ishizuka et al., 2014). TUNA (Tcl1 Upstream Neuron-Associated lincRNA) forms an RNA-RBP complex with three RBPs, PTBP1, HNRNPK, and nucleolin (NCL), and the complex binds to the *sox2* promoter (Lin et al., 2014). Rhabdomyosarcoma 2-associated transcript (RMST) and SOX2 interaction plays an important role in neural stem cell fate specification (Ng et al., 2013). A recent study has shown that lncRNAs determine Sox2's genomic localization (Hamilton et al., 2023). In another example, the interaction of the transcription factor POU3F3 and DNMT1-associated long intergenic (Dali) was described (Chalei et al., 2014). We believe that accumulating evidence further opens up the possibility of lncRNAs as therapeutic targets to artificially regulate their association with various RBPs.

5 Conclusion

In this review, we described five ncRNAs that regulate microcephaly-related genes. Although little information is available on ncRNAs responsible for microcephaly, multiple factors are known to provoke microcephaly. For example, 30% of case of ASD are accompanied by the features of diminishing brain size (Fombonne et al., 1999). Table 2 shows the ncRNAs known to be related to brain diseases. In fact, there are many ncRNAs related to ASD. Interestingly, most of the ncRNAs in this list are categorized as lncRNA species. Therefore, it would be interesting to confirm whether the lncRNA class rather than the small RNA class brain function tends to affect the determination of brain size by analyzing the lncRNAs listed in Table 2. Along with understanding of the human genome, tailor-made medicine is a center of attention these days. Acquisition of the sequences of individual genomes become easier and less expensive, revealing mutations that occur not only in coding genes but also in intergenic regions. In particular, accessible and affordable sequence reading enables us to find new intergenic mutations that could have been missed previously because of mild disease symptoms and poor sequencing technology. The resultant studies on intergenic regions allow us to highlight the potentials of ncRNAs for understanding human pathology in clinical research. Since the intergenic regions are poorly conserved among the enormous variety of organisms, and the large size and complicated functions of the brain are human-unique features, it is intriguing possibility that the intergenic regions contribute a big controlling center for determining such interesting traits. Considering the human-specific features of the brain structure and function, it seems likely that model animals such as mouse, zebra fish, and fruit fly would be of limited use for searching for human-specific ncRNAs. Leveraging human brain organoids, genome-wide association studies (GWASs), and massive annotation of human-specific ncRNA functions are essential for pioneering this vast ncRNA field. This field will lead us to new treatments for brain disease and understanding what makes us human.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Assessing the influence of distinct culture media on human pre-implantation development using single-embryo transcriptomics

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The use of assisted reproductive technologies is consistently rising across the world. However, making an informed choice on which embryo culture medium should be preferred to ensure satisfactory pregnancy rates and the health of future children critically lacks scientific background. In particular, embryos within their first days of development are highly sensitive to their micro-environment, and it is unknown how their transcriptome adapts to different embryo culture compositions. Here, we determined the impact of culture media composition on gene expression in human pre-implantation embryos. By employing single-embryo RNA-sequencing after 2 or 5 days of the post-fertilization culture in different commercially available media (Ferticult, Global, and SSM), we revealed medium-specific differences in gene expression changes. Embryos cultured pre-compaction until day 2 in Ferticult or Global media notably displayed 266 differentially expressed genes, which were related to essential developmental pathways. Herein, 19 of them could have a key role in early development, based on their previously described dynamic expression changes across development. When embryos were cultured after day 2 in the same media considered more suitable because of its amino acid enrichment, 18 differentially expressed genes thought to be involved in the transition from early to later embryonic stages were identified. Overall, the differences were reduced at the blastocyst stage, highlighting the ability of embryos conceived in a suboptimal *in vitro* culture medium to mitigate the transcriptomic profile acquired under different pre-compaction environments.

KEYWORDS

assisted reproductive technologies, culture media, embryo, RNA-seq, transcriptome

1 Introduction

Assisted reproductive technologies (ARTs) have allowed the birth of millions of children worldwide. In Europe, for instance, over two million children were born following ARTs (Wyns et al., 2021), and numbers continuously rise, proving that tackling infertility is a huge challenge for decades to come (de Geyter et al., 2020). However, significant variability in

ART practice and effectiveness exists between countries and even at the regional scale (Munné et al., 2017; Chambers et al., 2021). In particular, embryo culture is at the core of ARTs, but making an informed decision on which culture medium to use is still a subtle task (Lane and Gardner, 2007). Embryo culture media are not expected to perfectly mirror *in vivo* environment conditions (Vajta et al., 2010), but they should, nonetheless, provide the required biological content to sustain satisfactory embryo development compared to natural conceptions. A myriad of embryo culture media is nowadays commercially available. However, owing to trade confidentiality, their exact composition is unknown, which obscures the scientific decisions for choosing one culture medium over another for embryologists (Biggers and Summers, 2008). Although the competitive commercial race to optimize embryo culture media greatly contributed to increased pregnancy rates in ARTs, the scientific basis behind their formulation is unclear, which is a matter of concern for ART-related biovigilance (Sunde et al., 2016). Very few studies have followed up the health of children born in relation to different embryo culture media used in ART cycles, but they tend to indicate that certain media may be suboptimal, with a potential long-term health impact (Kleijkers et al., 2014; Zandstra et al., 2015; Bouillon et al., 2016).

The early embryo closely interacts with its environment, particularly during the cleavage stage (Zander et al., 2006; Bolnick et al., 2017). After fertilization, the embryo transits through the oviduct until reaching the uterus, where it may be implanted. This journey throughout the maternal track exposes the embryo to multiple molecules, including growth factors, hormones, and metabolites, which promote complex reactions (Paria and Dey, 1990; Kölle et al., 2020; Saint-Dizier et al., 2020). This period also coincides with critical epigenetic reprogramming, which influences gene expression (Morgan et al., 2005; Messerschmidt et al., 2014). Substantial evidence has linked adverse environmental maternal exposures and transcriptome changes in human embryonic stem cells and newborns' cord blood (Winckelmans et al., 2017; Guo et al., 2019). This likely reflects the adaptation of the embryo to external stressors, displaying remarkable plasticity at the molecular and cellular levels (Bateson et al., 2004; Ramos-Ibeas et al., 2019).

Compared to natural conception, *in vitro* conditions inherent to ARTs can be a source of additional stress (Roseboom, 2018). Indeed, the *in vitro* environment could adversely affect the postnatal phenotype of the offspring born via *in vitro* fertilization (IVF: with or without sperm microinjection) (Fernández-Gonzalez et al., 2004; Watkins and Fleming, 2009; Gardner and Kelley, 2017). The many processes involved in ARTs and, in particular, the osmotic stress, substrate imbalance, volatile organics, and contaminant pollution linked to *in vitro* culture can trigger embryonic stress response mechanisms, but this has been barely assessed to date (Leese, 2002; Puscheck et al., 2015; Cagnone and Sirard, 2016). As Thompson et al. (2007) highlighted, "there is no adaptation by an embryo to its environment that has no consequence." In particular, differences in embryo culture medium composition may lead to differences in adaptive responses to stress.

The embryo is mostly transcriptionally silent until day 3 (four- to eight-cell stage), when embryonic genome activation (EGA) mainly occurs and relies on maternally provided mRNAs for early embryo development (Braude et al., 1988; Vassena et al., 2011; Leng et al., 2019), although transcription initiation has been reported in human embryos at the one-cell stage, acting as a proxy for early epigenetic programming (Asami et al., 2022). During this period, the capacity of the embryo to maintain metabolism and cellular homeostasis may, thus, be limited (Edwards et al., 1998; Lane and Gardner, 2001). Accordingly, short exposure to ammonium before compaction was shown to compromise the ability to further develop compared with the same exposure after compaction in mice (Zander et al., 2006). After EGA, dynamic changes in gene expression accompany embryonic lineage specification, and anomalies in these sequential expression changes can lead to developmental arrest (Sha et al., 2020). These examples highlight that the early embryo is sensitive to its micro-environment and that many parameters in IVF centers should be tightly controlled, especially embryo culture.

Evidence from animal models showed that *in vitro* culture can affect embryonic gene expression and epigenetic marks compared with *in vivo* conditions (Mann et al., 2004; Rinaudo and Schultz, 2004; Fauque et al., 2007; Wright et al., 2011). Most importantly, these molecular effects can be worsened depending on the culture medium (Rinaudo and Schultz, 2004; Schwarzer et al., 2012; Feuer et al., 2017), with a reported sensitivity of imprinted gene expression (Market-Velker et al., 2010; Ramos-Ibeas et al., 2019). Comparatively, studies in humans have mainly focused on the clinical efficiency of various culture media (live birth rate, implantation rate, clinical pregnancy rate, birthweight, placental weight, and pre-term birth rate) (Dumoulin et al., 2010; Eskild et al., 2013; Youssef et al., 2015; Kleijkers et al., 2016). Only two studies compared the transcriptomic profile of blastocysts cultured in two different media: using microarrays, they reported misregulation of genes involved in cell cycle, apoptosis, protein degradation, and metabolism, which have the capacity to impair embryo development (Kleijkers et al., 2015; Mantikou et al., 2016). This justifies pursuing efforts to identify the biological origin of embryo culture effects.

In this study, we investigated, for the first time, the impact of different culture media used in IVF centers (Ferticult, Global, and SSM) on the transcriptome of a unique collection of day-2 and -5 human embryos using single-embryo RNA sequencing. Analyzing day-2 embryos (four-cell stage) will provide insight into effects of culture media on maternal-provided transcripts and early embryonic gene activation (epigenetic programming). The importance of analyzing day-5 embryos (blastocyst stage) is that it will increase our understanding of effects on molecular processes after compaction. Embryo culture media rich in amino acids, such as Global, are nowadays preferentially used among IVF centers over media depleted in amino acids, such as Ferticult. The SSM medium evaluated in this study is no longer used due to under-performance in terms of pre-implantation and pregnancy rates (Bouillon et al., 2016). In addition, we tested whether supplementation with methionine, an essential amino acid for embryo development, could modulate the embryonic transcriptome.

We found evidence for medium-specific transcriptomic differences at day 2 (embryos at the four-cell stage), affecting major genes involved in embryonic development. In a second experiment, embryos cultured in two different media until day 2 were cultured until the blastocyst stage (day 5) in the same media considered more suitable because of its amino acid

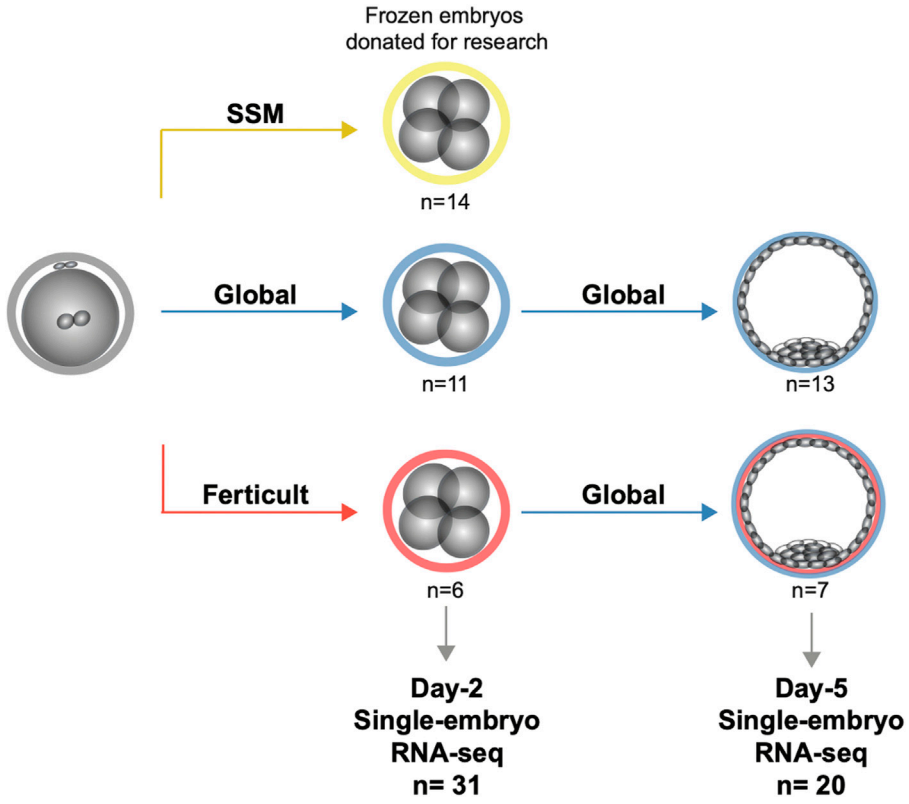

FIGURE 1
Study design for the transcriptomic analysis of human embryos cultured until days 2 and 5.

enrichment, and differences tended to reduce, reflecting the possible adaptation of the embryonic transcriptome to the culture medium. Using an expression pseudotime approach based on previously described datasets, altered expression of some genes thought to be involved in the transition from early to later embryonic stages was still identified in these blastocysts depending on their culture media pre-compaction. In addition, supplementing the embryo culture medium with methionine nearly four times the concentration found in culture media did not modify gene expression.

2 Materials and methods

2.1 Ethics statement

This research was authorized by the National Biomedicine Agency (legal decision published in the Journal Officiel under the reference JORF N°0233- 6 October 2016 and extended under the reference JORF n°0303- 31 December 2019).

2.2 Embryo selection- experimental design

We used embryos donated for research by couples and cryopreserved at the Reproductive Lab of Dijon Hospital during a relatively short period (maximum 2 years long). Embryos were included if they originated from couples ≤42 years of age, in conventional IVF or intracytoplasmic sperm injection (ICSI) attempt. Embryos from attempts with surgical spermatozoa (testicular or epididymal sperm) or performed in a viral context (HIV or viral hepatitis) were excluded. Clinical information and the number of embryos per couple are available in Supplementary Table S1. Freezing and thawing were performed with strict procedures as previously described (Bechoua et al., 2009) and detailed in Supplementary Methods. In brief, all embryos used in this study were cryopreserved individually 2 days post-fertilization by a slow-cooling protocol. Then, embryos were *in vitro* cultured in different culture media (Global medium, LifeGlobal; Ferticult IVF medium, FertiPro; SSM, and Irvine Scientific). However, to limit environmental variability, experiments were performed in parallel from different groups, using the same consumables and equipment. We analyzed only embryos cultured in these three different culture media but with identical morphological criteria, i.e., at the four-cell stage with less than 15% of anucleate fragments and regular cleavage (Figure 1). In another experiment, we selected day-2 cryopreserved embryos with identical morphological criteria as described previously from embryo cohorts cultured up to day 2 either in Ferticult or Global, which were then cultured up to day 5 in Global. Finally, for methionine supplementation, we included day-2 cryopreserved embryos from cohorts from the same patients (sibling embryos) with at least four embryos with identical embryo morphological criteria (four-cell stage). Precisely, after

thawing, these sibling embryos were randomly cultured in the Global medium without or with methionine supplementation (200 μM; concentration nearly thrice that in the Global medium), and we analyzed by single-embryo RNA-seq sibling embryos that reached the blastocyst stage in both groups (without or with methionine supplementation).

Embryos were thawed according to the strict protocol routinely used in human IVF-clinic to maintain their integrity as much as possible (Bechoua et al., 2009). Immediately after thawing, embryos were transferred into pre-equilibrated embryoslides (Unisense Fertilitech, Vitrolife) with 25 μL of the culture medium and covered with 1.2 mL of oil (Nidoil, Nidacon). They were cultured up to the blastocyst stage at 37°C and tri-gas atmosphere (6%CO2, 5%O2, and 89% N2). According to the classification of Gardner and Schoolcraft (1999), only blastocysts with at least a B2 blastocoel cavity without lysis were analyzed. At the time of sequencing, embryos were between the B2 and B4 blastocyst stages (Supplementary Table S1). We also paid attention to using the same batches of culture media in all experiments.

2.3 Single-embryo RNA sequencing

A previously described scRNA-seq method was applied to single embryos (Pérez-Palacios et al., 2021). In brief, zona pellucida-free embryos (after using acidic Tyrode's solution) were individually placed in a lysis buffer containing 1.35 mM MgCl2 (4379878, Applied Biosystems), 4.5 mM DTT, 0.45% Nonidet P-40 (11332473001, Roche), 0.18 U/mL SUPERase-In (AM2694, Ambion), and 0.36 U/mL RNase-inhibitor (AM2682, Ambion). Then, we performed a reverse transcription reaction (SuperScript III reverse transcriptase—18,080–044, Invitrogen, final concentration: 13.2 U/mL) and poly(A) tailing to the 3' end of the first-strand cDNA (by using terminal deoxynucleotidyl transferase—10,533–073, Invitrogen, final concentration: 0.75 U/mL). After the second-strand cDNA synthesis, 20 and 18 cycles (at day 2 and day 5, respectively) of PCR were performed to amplify the embryo cDNA using the TaKaRa ExTaq HS (TAKRR006B, Takara, final concentration: 0.05 U/mL) and IS PCR primer (IDT, final concentration: 1 mM). Following purification using a Zymoclean Gel DNA Recovery Kit (ZD4008, Takara), product size distribution and quantity were assessed on a Bioanalyzer using an Agilent 2,100 high-sensitivity DNA assay kit (5,067–4,626, Agilent Technologies).

The library preparation (KAPA Hyper Plus Library prep kit) and sequencing were performed by the ICGex – NGS platform (Institut Curie) on HiSeq 2,500 for day-2 embryos and on NovaSeq 6,000 Illumina sequencer for day-5 embryos for 100 bp paired-end sequencing.

2.4 Data pre-processing and quality control

We computed sequencing quality checks with FastQC v0.11.9 and trimming of adapters and low-quality sequences using TrimGalore! V0.6.6. Paired-end read alignment was performed onto human reference genome (hg38) with STAR v2.7.9a (Dobin et al., 2013) reporting randomly one position, allowing 6% of mismatches. Following previous recommendations (Teissandier et al., 2019), repeat annotation was downloaded from RepeatMasker and joined with basic gene annotation from Gencode v19. The merged file was used as an input for quantification with featureCounts v2.0.1. Genes with a minimum of count per million (cpm) > 1 in at least four samples were retained for further analysis. Principal component analyses were implemented with PCAtools v2.8.0 on log2(cpm+1) for all genes for single datasets and common genes for multiple datasets, excepting the 10% genes with the lowest variance.

2.5 Differential expression analysis

Differential expression analysis was performed using *edgeR*'s normalization (v3.38.1) combined with *voom* transformation from the *limma* package v3.52.1. *p*-values were computed using *limma* and adjusted with the Benjamini–Hochberg correction for multiple testing. Genes were declared as differentially expressed if FDR<0.1.

2.6 Gene Ontology and gene set enrichment analysis

We used Metascape v3.5 to calculate and visualize over-representation of gene ontologies in our list of differentially expressed genes (DEGs) (Zhou et al., 2019). Metascape applies hypergeometric tests and FDR corrections to identify ontology terms that comprise significantly more genes in a given gene list than what would be expected with a random gene list. For each gene list tested, we provided an appropriate background gene list corresponding to all expressed genes in all samples for a given experiment. We selected "Express Analysis" to capture relevant gene annotations from multiple sources (GO, KEGG, Reactome, canonical pathways, and CORUM). The *p*-value cutoff was kept at 0.01.

Gene set enrichment analysis (GSEA) was implemented with the *clusterProfiler* R package (v4.4.1) setting the adjusted *p*-value significance threshold at 0.05. Beforehand, imputed gene lists were pre-ranked by logFC.

2.7 Processing of public single-cell RNA-seq datasets in human embryos

We compared our data with three early embryos single-cell RNA-seq studies (Xue et al., 2013; Yan et al., 2013; Petropoulos et al., 2016). Reads alignment and quantification were executed as described previously onto raw reads downloaded from the European Nucleotide Archive (study accessions PRJNA153427, PRJNA189204, and PRJEB11202).

2.8 Trajectory inference and pseudotime computing

After pre-processing, read counts data from all 1,529 cells from the work of Petropoulos et al. were pre-clustered and normalized

with *scran* and *scater* packages (v1.24.0) after removing lowly expressed genes. Next, *Seurat* (v4.1.1) was used to scale the data and regress variables on total RNA. To identify genes whose expression dynamically changes across early embryonic development, in a continuous manner, independently of the embryo stage, we applied PHATE dimensionality reduction, a recently developed method that has been previously applied to human embryonic stem cells (Moon et al., 2019). We chose PHATE because of its ability to capture heterogeneity and reduce noise better than other dimensionality reduction methods (Moon et al., 2019). As the information geometry relies on diffusion dynamics, PHATE is especially suitable for early development (Moon et al., 2019). PHATE dimension reduction was applied with *phateR* v1.0.7 embedding three dimensions. We then inferred existing lineages and pseudotime using *slingshot* v2.4.0, a method adapted for branching lineage structures in low-dimensional data.

2.9 Differential expression along pseudotime

We used *tradeSeq* v1.10.0 (van den Berge et al., 2020) to fit a negative binomial generalized additive model (NB-GAM) for each gene. After examining diagnostic plots of the optimal number of knots (k) according to the Akaike Information Criterion (AIC), k was set to 6 as an optional parameter in the NB-GAM model. We selected DEGs along pseudotime with *associationTest*() function if p-value<0.05 and meanLogFC>2. This function relies on Wald tests to assess the null hypothesis that the expression of a gene is constant along pseudotime. DEGs with the culture medium were cross-checked with the list of DEGs along pseudotime according to Petropoulos et al.'s (2016) dataset. For further investigation, a dataset from Yan et al. (2013) was used to visualize these changes in a larger window, from the oocyte to late blastocyst stage.

3 Results

3.1 Study design and quality control analysis by comparison with previous studies

To analyze the impact of embryo culture media on the embryonic transcriptome, we performed single-embryo RNA-seq (Pérez-Palacios et al., 2021) on 51 frozen/thawed donated embryos after day 2 or day 5 of culture (Figure 1). Clinical characteristics of donors, embryo origin, and morphology are available in Supplementary Table S1, and information regarding survival after thawing can be retrieved from Supplementary Table S2. We compared three different media: Global (LifeGlobal, United States), SSM (Irvine Scientific, United States), and Ferticult (FertiPro, Belgium). Global and SSM have very similar components, except different forms of glutamine and the presence of taurine in SSM (Bouillon et al., 2016), and are intended to be used as one-step media up to day 5/6 of human embryo development (Supplementary Table S3). Ferticult differs from both in that it does not contain amino acids and is fitted to be used up to day 2/3. We processed 31 day-2 embryos (SSM: n = 14, Global: n = 11, and Ferticult: n = 6) and 20 day-5 embryos (Global: n = 13 without (n = 9) or with (n = 4) methionine supplementation and Ferticult: n = 7). At day 5, the number of samples was independent of the rate of embryos that survived the thawing process and reached at least the B2 stage (42.3% and 50.0% in Global and Ferticult groups, respectively). An average of 3.3 million reads per embryo at day 2 and 13.1 million reads at day 5 were generated, with an average mapping rate of 90.1% across all samples (Supplementary Table S4). We were able to detect the expression of 30% and 26% of all RefSeq genes and transposable elements at day 2 and day 5, respectively.

To assess the quality of our generated single-embryo RNA-seq datasets, we relied on previous high-quality studies that performed single-cell RNA-seq (scRNA-seq) in human early embryos. According to the criteria that an expressed gene should have a count per million (cpm) value greater than 1 in at least half of the samples in each embryo stage, we found consistent numbers of 12,056, 10,022, and 11,213 genes being expressed in four-cell stage embryos in the work of Yan et al. (2013), the work of Xue et al. (2013), and our own dataset, respectively (Supplementary Figure S1). In blastocysts, we identified 12,790 expressed genes in our data, compared with 8,204 genes in Yan et al.'s (2013) dataset in which blastocysts were collected a day later, at day 6.

Principal component analysis (PCA) and hierarchical clustering of global gene expression further confirmed the high similarity of our data with those two previous studies: our day-2 embryo samples clustered near four-cell samples, and our day-5 embryos samples clustered beyond morula and before late blastocyst stages (Supplementary Figures S2A,B).

3.2 Transcriptomic comparison of day-2 embryos cultured in different media

We first focused on transcriptome differences in short-term culture, at the four-cell stage (day-2), between embryos conceived in different media. The highest number of DEGs was found comparing Ferticult and Global media groups, with 266 DEGs (1.5% of all transcripts analyzed) showing adjusted p-value<0.1 (Figure 2A; Supplementary Table S5). In contrast, only one and five DEGs were found comparing SSM with either Ferticult or Global groups, respectively (Supplementary Table S5). However, the global transcriptomic analysis showed that SSM was transcriptionally closer to Global than to Ferticult (r = 0.95 versus r = 0.9, Spearman's correlation), which is consistent with their similar composition (Supplementary Figure S3A). Histograms of p-value distribution for all genes corroborate the observation of a strong effect of the culture medium on transcriptomic differences between Ferticult and Global and to a lesser extent between Ferticult and SSM, whereas p-values tended to be uniformly distributed between SSM and Global and far from statistical significance (Supplementary Figure S3B; Supplementary Table S5).

Among the 266 DEGs in the Ferticult-to-Global comparison, 145 were upregulated and 121 downregulated. Most of them (88.3%) displayed absolute log2(fold change, FC) > 2.5, which revealed substantial differences in the transcriptome of day-2 embryos depending on the culture medium (Supplementary Table S5; Supplementary Figures S4, S5A). Only eight DEGs are likely to be maternal transcripts because their expression is strictly declining in embryonic stages succeeding oocyte as assessed with Yan et al.'s

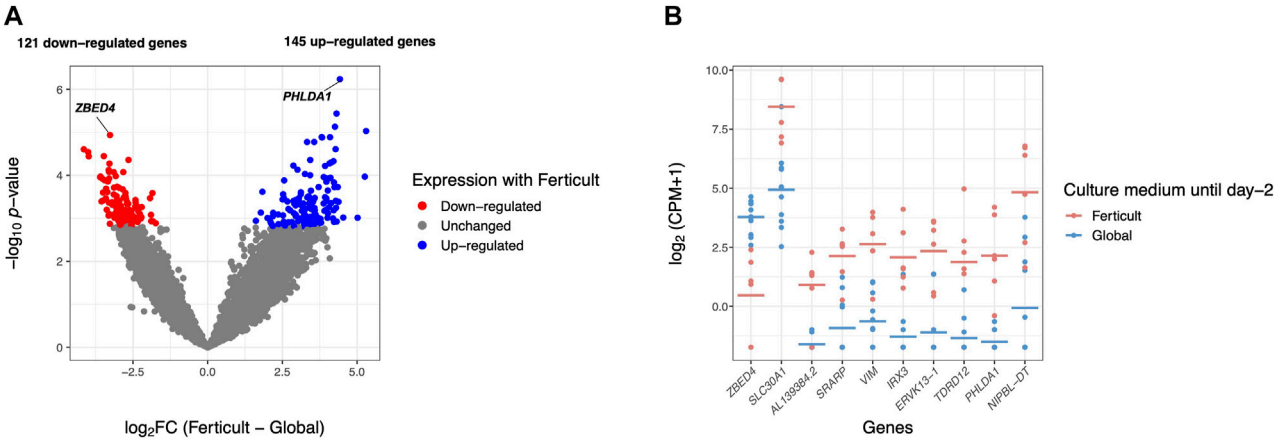

FIGURE 2
Differential gene expression analysis in day-2 embryos between Ferticult and Global media. **(A)** Volcano plot of gene expression between Ferticult and Global media at day 2. **(B)** Dot plot of the expression of top 10 Ferticult-to-Global DEGs, ordered by ascending log2FC (from left to right). Group mean is represented by the line. Dots represent individual embryos.

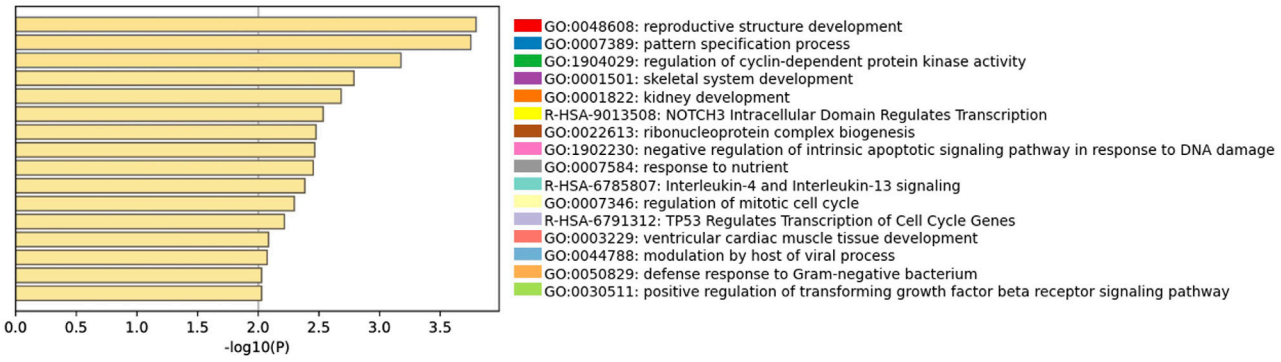

FIGURE 3
Gene Ontology analyses of Ferticult-to-Global DEGs at day 2. Bar plot of the most significant GO terms from clusters of significant pathways over-represented in day-2 DEGs, ordered by significance. Each term was selected by Metascape using a heuristic algorithm that selected the most informative term from clusters of proximal significant GO terms.

(2013) reference dataset (Supplementary Figure S5B). Top 10 most dysregulated genes including *ZBED4*, *SLC30A1*, AL139384.2, *SRARP*, *VIM*, *IRX3*, *ERVK13-1*, *TDRD12*, *PHLDA1*, and *NIPBL-DT* are shown in Figure 2B for each individual embryo according to the culture medium group. Six of them can be considered as mixed maternal/embryonic transcripts, while the four others appear to be transcribed from the embryonic genome. Gene Ontology (GO) analysis with Metascape revealed an over-representation of DEGs related to development (pattern specification process, reproductive structure development, skeletal system development, and kidney development), regulation (regulation of the mitotic cell cycle, regulation of cyclin-dependent protein kinase activity, negative regulation of the intrinsic apoptotic signaling pathway in response to DNA damage, and positive regulation of the transforming growth factor), ribonucleoprotein biogenesis complex, and response to nutrients (Figure 3; Supplementary Figure S6). Adjustments for maternal age or fertilization method were performed in all differential expression analyses, but the top DEGs remained the same (Supplementary Table S5). In parallel, we decided to perform a GSEA which provides a broader view of the overall biological processes that may be up- or downregulated with the use of either Ferticult or Global, which may not be detected by focusing on DEG interpretation. Top 10 significant ontologies indicate that genes involved in embryonic development and cell division are, respectively, likely to be differentially up- and downregulated with Ferticult (Supplementary Figure S7; Supplementary Table S6).

Focusing on major genes involved in chromatin-based processes such as DNA methylation, heterochromatin modulators, histone modifiers, and remodeling complexes, we found two histone modifiers to be significantly downregulated among the Ferticult-to-Global DEGs: the Aurora kinase A gene *AURKA* (FDR<0.1, log2FC = −1.84), which regulates many aspects of mitosis, and *SETDB1* (FDR<0.1, log2FC = −2.92), which catalyzes trimethylation

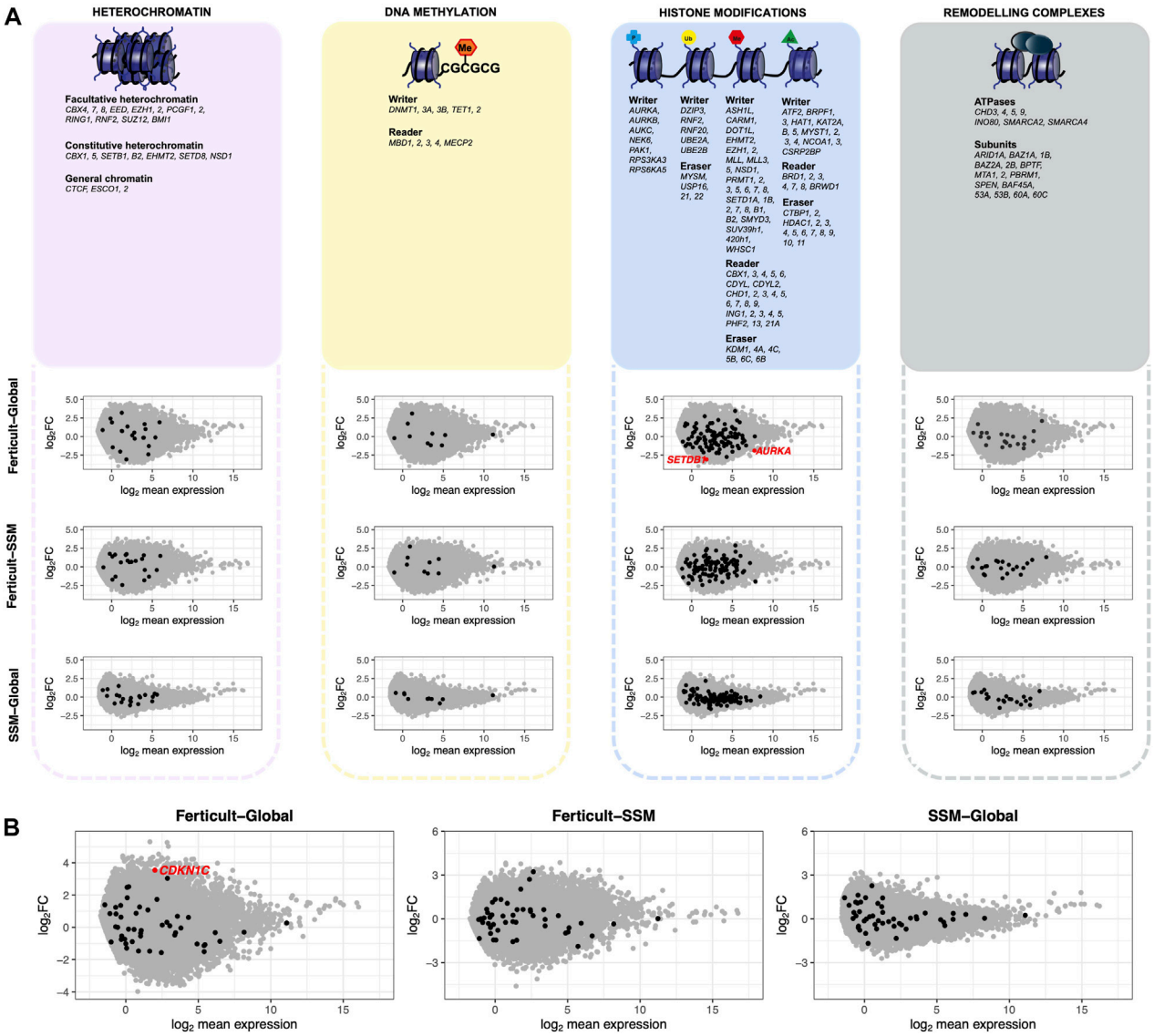

FIGURE 4
Expression differences of genes involved in chromatin-based mechanisms, imprinted genes, and transposable elements between Ferticult, SSM, and Global samples (day-2 embryos). **(A)** List of major genes identified as involved in chromatin-based processes with their scatter plot of differential expression analysis. Log2 mean expression was calculated by taking the average log2(cpm+1) expression in compared culture media groups. Each point represents a gene: misregulated genes from the aforementioned list (red dots), unchanged genes from the aforementioned list (black dots), and other expressed genes (gray dots). **(B)** Scatter plot of differential expression analysis focused on imprinted genes. Log2 mean expression was calculated by taking the average log2(cpm+1) expression in compared culture media groups. Each point represents a gene: misregulated imprinted genes (red dots), unchanged imprinted genes (black dots), and other expressed genes (gray dots).

of lysine 9 of histone H3 (H3K9me3) (Figure 4A). Additionally, focusing on imprinted genes, we only found the cyclin-dependent kinase inhibitor 1 (*CDKN1C*) gene among the 266 Ferticult-to-Global DEGs (Figure 4B). Finally, we also analyzed transposable elements and found three families to be differentially expressed between Ferticult and Global media (*CR1-12_1Mi*, *LTR6A*, and *LTR7C*). Although the top 20 expressed transposable element families were not differentially expressed in any comparison, their expression was higher in Ferticult, which resulted in fold change intensities higher in Ferticult comparisons (Supplementary Figures S8A,B).

3.3 Monitoring 2-day culture medium-induced differences at the blastocyst stage

Given the transcriptomic differences of day-2 embryos resulting from the amino-acid-free Ferticult medium over Global medium, we wanted to further analyze whether amino acid deprivation during the first 2 days of development may have extended effects on the transcriptome of blastocyst embryos. For that purpose, a second batch of embryos cultured until day 2 in Global (n = 13) or Ferticult (n = 7) media was selected for their strict identical embryo morphology and subsequently cultured until the blastocyst stage,

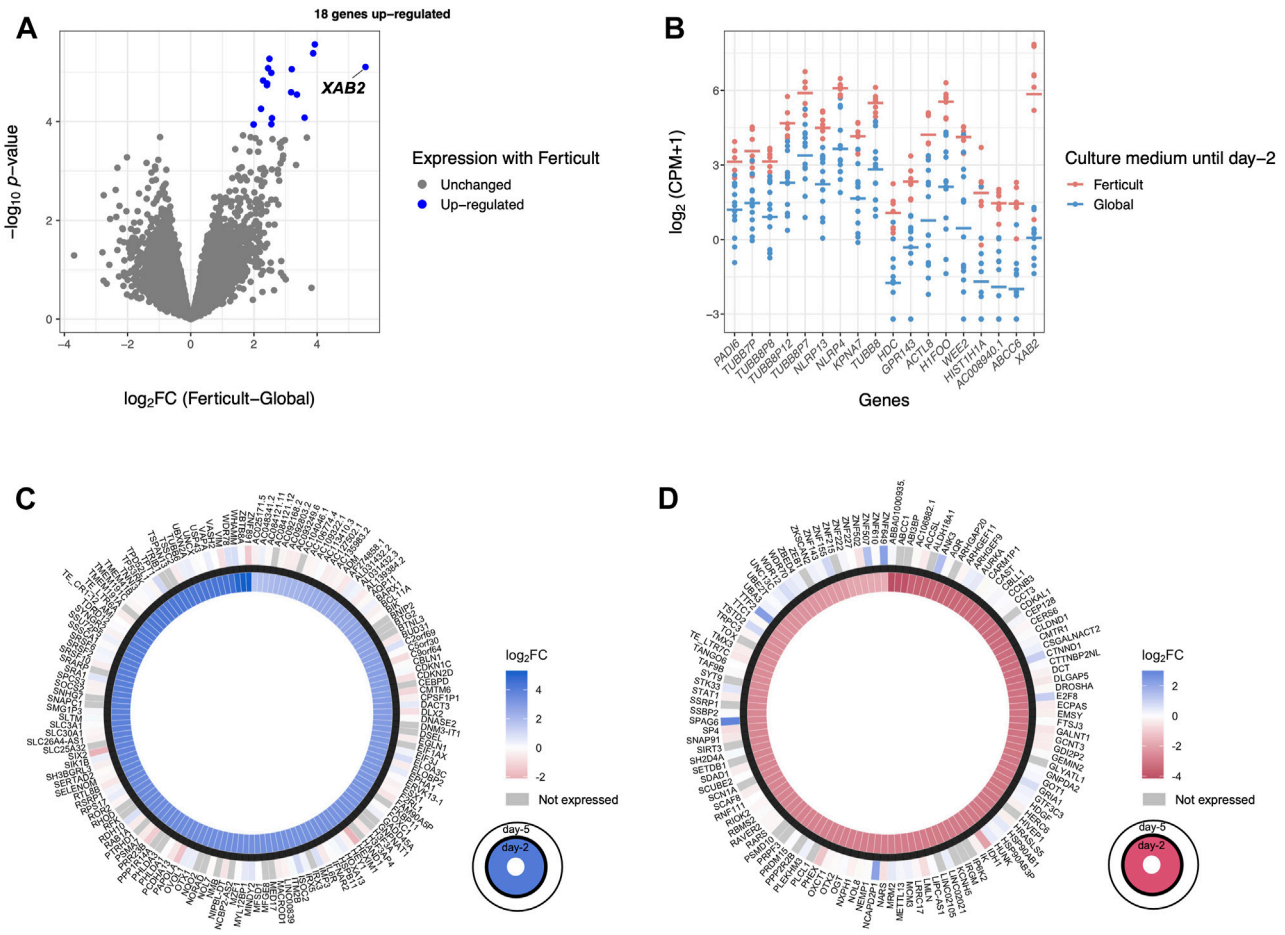

FIGURE 5
Differential expression analysis at day 5 of culture. **(A)** Volcano plot of differential gene expression between Ferticult and Global media. **(B)** Dot plot of the expression of all 18 Ferticult-to-Global DEGs, ordered by ascending log2FC (from left to right). Group mean is represented by the line. Dots represent individual embryos. **(C)** Circle plot of the expression of upregulated Ferticult-to-Global DEGs at day 2 and their expression at day 5. Plot displays the log2 fold change of the 147 DEGs upregulated with Ferticult at day 2 (interior layer) and the log2 fold change with Ferticult for the same genes at day 5 (exterior layer). Cells colored in gray correspond to genes that were not expressed at day 5. **(D)** Circle plot of the expression of downregulated Ferticult-to-Global DEGs at day 2 Global and their expression at day 5. Plot displays the log2 fold change of the 122 DEGs downregulated with Ferticult at day 2 (interior layer) and the log2 fold change with Ferticult for the same genes at day 5 (exterior layer). Cells colored in gray correspond to genes that were not expressed at day 5.

all in the Global medium (Figure 1A). Performing differential expression analysis after single-embryo RNA-seq, we found 18 DEGs in blastocysts that were previously cultured in the Ferticult medium until day 2 versus blastocysts cultured all along in Global: *ABCC6*, AC008940.1, *ACTL8*, *GPR143*, *H1FOO*, *HDC*, *HIST1H1A*, *KPNA7*, *NLRP4*, *NLRP13*, *PADI6*, *TUBB7P*, *TUBB8*, *TUBB8P7*, *TUBB8P8*, *TUBB8P12*, *WEE2*, and *XAB2* (Figures 5A,B; Supplementary Table S7). These genes were all upregulated with the Ferticult medium condition until day 2, with half showing a log2FC > 2.5 and the *XAB2* gene showing the highest overexpression score (>5.5) (Figure 5A). The GO analysis indicated a functional link with the meiotic cycle (DEGs associated with this pathway: *H1FOO*, *TUBB8*, and *WEE2*). Importantly, none of the previous expression differences observed at day 2 remained significant at day 5. Circular plots showed that fold changes of gene differences observed at day 2 were largely minimized by day 5 (Figures 5C,D). Conclusions were unchanged when adjusting for maternal age (Supplementary Table S7).

3.4 Effects of methionine supplementation

Methionine is an essential amino acid present in embryo culture media that serves as a precursor for protein synthesis and DNA methylation and, therefore, could modulate the transcriptome. We tested whether the addition of methionine at day 2 would impact the transcriptome of day-5 blastocysts cultured in Global by comparing nine samples cultured in Global and four samples cultured in Global supplemented with methionine after day 2, from sibling embryos (i.e., a pair of embryos of each condition were coming from the same couples) (Supplementary Figure S9). The differential expression analysis revealed no significant DEG.

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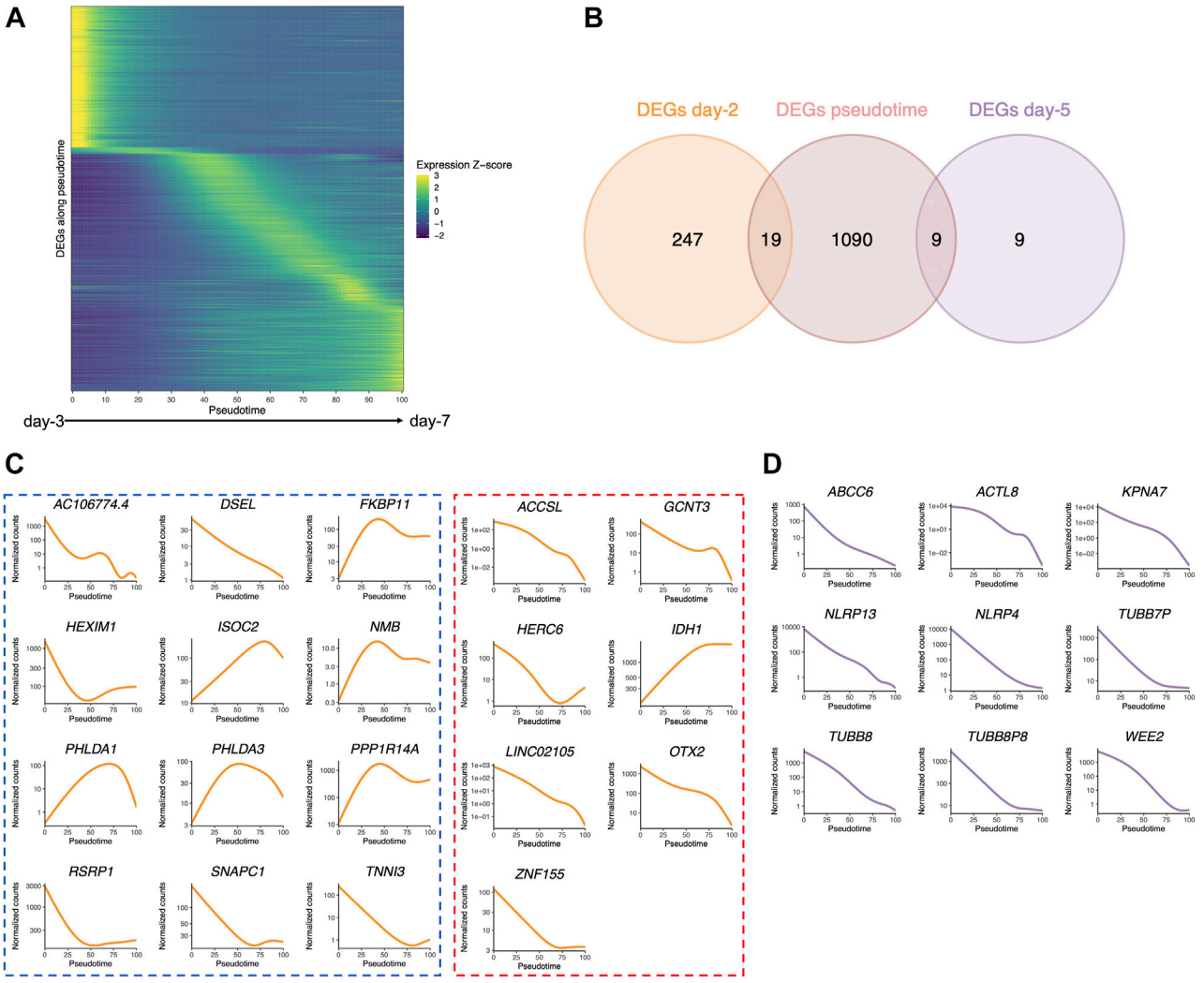

FIGURE 6
Pseudotime differential expression during human preimplantation development. Pseudotime is a metric that could be interpreted as a timing distance between one cell and its precursor cell and helps identify the ordering of cells along a lineage based on their gene expression profile. **(A)** Heatmap of the expression of the 1,110 genes that were found to be differentially expressed along pseudotime (from public datasets established from the eight-cell stage (Petropoulos et al., 2016), ordered by the timing of peak expression (arbitrary unit). Expression Z-score: Z-score of TMM-adjusted cpm. **(B)** Venn diagram of the number of DEGs along pseudotime that are differentially expressed between Ferticult and Global at days 2 and 5 of the embryonic culture. **(C)** Dynamics of the expression of the 19 day-2 Ferticult-to-Global DEGs that are differentially expressed along pseudotime according to Petropoulos et al.'s (2016) dataset. The curve corresponds to the NB-GAM fitted normalized counts (TMM-adjusted cpm). Left panel surrounded by a blue line corresponds to genes upregulated with Ferticult. Right panel surrounded by a red line corresponds to genes downregulated with Ferticult. **(D)** Expression dynamics of the nine day-5 Ferticult-to-Global DEGs that are differentially expressed along pseudotime according to Petropoulos et al.'s (2016) dataset. The curve corresponds to the NB-GAM fitted normalized counts (TMM-adjusted cpm).

3.5 Effects of the culture medium on the transcriptional trajectory of early embryos

Early embryos undergo profound transcriptional changes during the first stages of development. In an attempt to address whether the embryo culture medium may affect these sequential modifications of gene expression, we used a public dataset of 1,529 single-cell RNA-seq of human embryos from days 3 to 7 (cultured in either CCM (Vitrolife) or G-1 Plus (Vitrolife) media) (Petropoulos et al., 2016), which previously allowed delineating the transcription signature of each embryonic lineage and their dynamics during embryonic lineage segregation (Meistermann et al., 2021). Our objective was to identify genes whose expression dynamically changes across early embryonic development, in a continuous manner, independent of the embryo stage. For that purpose, we applied the PHATE dimensionality reduction method (Moon et al., 2019) and inferred existing lineages and pseudotime (van den Berge et al., 2020)—a metric that could be interpreted as a timing distance between 1 cell and its precursor cell. On the public scRNA-seq dataset (Petropoulos et al., 2016), we were able to identify eight clusters by using k-means clustering to group cells with high transcriptomic similarities. We also identified three distinct lineages (Supplementary Figure S10A) that shared the same

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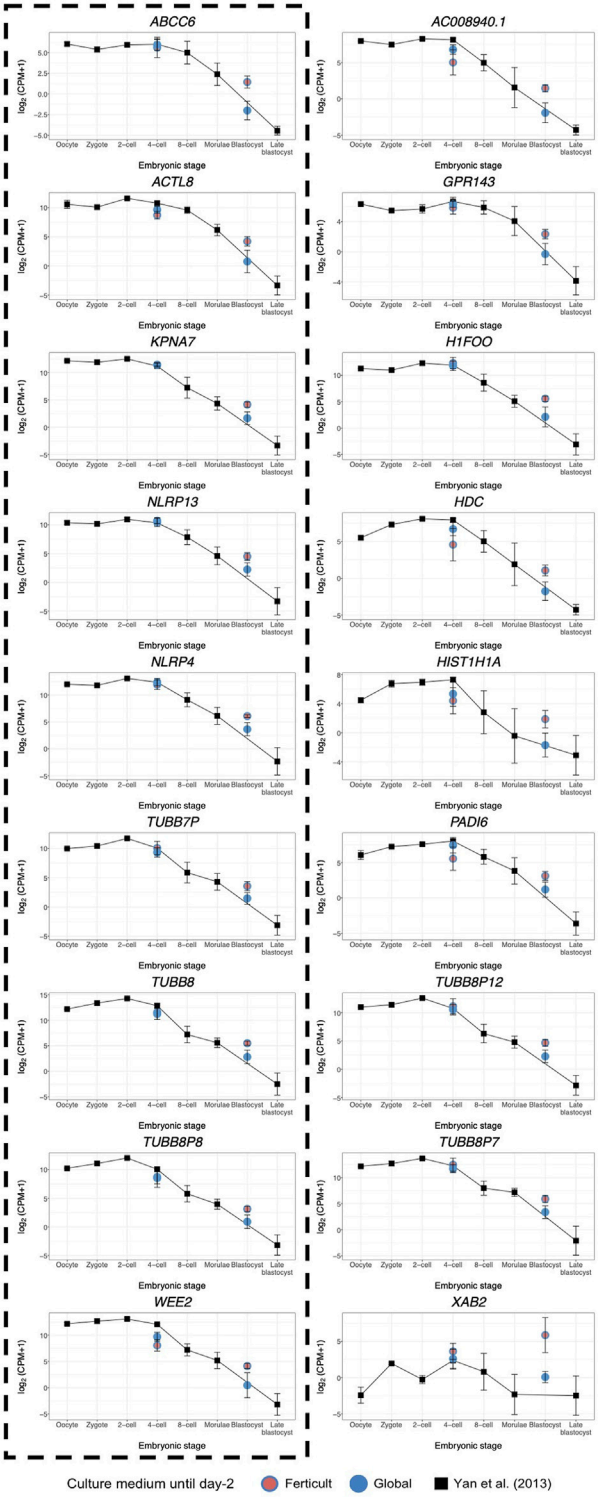

FIGURE 7
Expression dynamics of day-5 Ferticult-to-Global DEGs from the oocyte to the blastocyst stage as compared to the literature. We plotted the public scRNA-seq dataset from the work of Yan et al. (2013) obtained from the oocyte to the late blastocyst stage as the reference level of the expression of early embryonic genes. Each colored point represents the mean log2(cpm+1) value of all samples for each culture medium group. Black squares represent the mean log2(cpm+1) for all samples from the work of Yan et al. (2013) for each embryonic stage. Error bars represent the standard deviation between

(*Continued*)

FIGURE 7 (Continued)
samples from the same group. Day-2 embryos were represented at the four-cell stage. Day-5 embryos were represented at the blastocyst stage. DEGs surrounded by a dashed black line were also found to be differentially expressed along pseudotime according to Petropoulos et al.'s (2016) dataset.

structure when considering cells from clusters 1 to 5 but separated into clusters 6, 7, and 8. Using the cell classification adopted in previous studies (Petropoulos et al., 2016; Meistermann et al., 2021), the three lineages corroborated with the demarcation into epiblast (EPI), primitive endoderm (PrE), and trophectoderm (TE) cells (Supplementary Figures S10B,C). Each cell was then assigned a pseudotime to reflect its "transcriptomic age" along each of the three lineages (Supplementary Figure S10D). Along with this inferred pseudotime, we identified 1,110 genes with a dynamic expression pattern (Figure 6A).

Considering the question of the impact of the culture medium, we crossed these 1,110 dynamic genes with our list of DEGs identified at days 2 and 5 in the Ferticult-to-Global comparison. Remarkably, 19 out of 266 DEGs at day 2 and 9 out of 18 DEGs at day 5 showed dynamic expression changes across pre-implantation pseudotime, meaning that the choice of the culture medium has an impact on the expression of genes that are dynamically regulated during early development (Figure 6B). Temporal expression of those genes is shown in Figures 6C,D. While the 19 DEGs at day 2 that were also differentially expressed pseudotime showed a diverse profile of expression (Figure 6C), the 9 DEGs at day 5 that are also differentially expressed along pseudotime displayed a declining expression over embryo development in the reference pseudotime (Figure 6D). To confirm these results, we used an independent public scRNA-seq dataset from the work of Yan et al. (2013) obtained with a broader window, from the oocyte to late blastocyst (Figure 7). When considering our own datasets, embryos continuously cultured in Global up to day 5 also showed this declining trend of expression from days 2 to 5, with levels that were congruent with the reference dataset from the work of Yan et al. (2013) (Figure 7). However, day-5 embryos previously cultured in Ferticult until the four-cell stage showed over-expression for all DEGs, suggesting that these embryos retained abnormally high levels for their embryonic stage. On average, these blastocyst embryos showed expression levels that were closer to the morula stages.

4 Discussion

We provide here in-depth characterization of the transcriptomic effects exerted by different culture media on human embryos after 2 and 5 days of culture. In this study, media determined as a worse-case scenario, Ferticult and SSM, were compared to Global, as a better-case scenario, providing insight into the impact of culture media on the transcriptome of early ART-produced human embryos. It should be noted that for the best-case scenario, *in vivo*-derived embryos cannot ethically be obtained. It yields several insights into how culture medium composition can induce transcriptomic responses as an adaptation of the embryo

to its micro-environment, pre- and post-compaction. In line with the importance of our research questions, two of the media we tested are no longer used for human embryo culture (one was removed from the market).

First, we focused on embryos cultured until day 2, as this pre-compaction period is likely to be sensitive to environmental stressors. The most pronounced transcriptional divergence was between Ferticult and Global, with 266 DEGs. Among these DEGs, the majority were transcribed from the embryonic genome or consisted of mixed maternal/embryonic transcripts. It is in agreement with studies using animal models, where it has been shown that the culture environment influences the maternal-to-embryonic transition, which itself influences the maternal transcript clearance (Tesfaye et al., 2004; Zhang et al., 2022). A total of 19 of these DEGs could have a key role in early development, based not only on their dynamic expression changes across development but also on their association with GO terms related to essential developmental pathways, and were mostly upregulated in Ferticult. GSEA also depicted global differential regulation of important developmental pathways regarding the use of different culture media, notably cell division and pattern specification processes. Our results are congruent with two previous microarray studies that measured transcriptomic differences between embryos cultured in two culture media (G5 and HTF) related to cell cycle and metabolism-associated genes (Kleijkers et al., 2015; Mantikou et al., 2016). Despite SSM and Global having proximal components, the Ferticult–SSM comparison yielded only one DEG, but the overview of global patterns of expression still assumes the existence of transcriptomic differences between Ferticult and SSM media. The few composition disparities between SSM and Global would explain why the embryonic response to the SSM culture is not completely equal to that of Global.

Imprinted genes are candidates for high susceptibility to environmental conditions, and disruption of imprinted expression has been linked to developmental pathologies in humans (Maher and Reik, 2000). Accordingly, animal studies indicated that some embryo culture media were associated with hypomethylation of maternally expressed genes (such as *H19* and *SNRPN*), resulting in aberrant biallelic expression (Mann et al., 2004; Market-Velker et al., 2012). In our study, only one imprinted gene, *CDKN1C*, was upregulated after 2 days in culture in Ferticult compared to Global. *CDKN1C* is a key regulator of cell growth and proliferation, and aberrant expression is observed in syndromes with overgrowth, tumor predisposition, and congenital malformations, such as Beckwith–Wiedemann syndrome, notably in mouse embryos and fetuses (Andrews et al., 2007; Tunster et al., 2011). We also found that the expression of *SETDB1*, which is involved in histone methylation, was downregulated in Ferticult samples. These elements may reflect direct and indirect influences of the culture medium on the embryonic epigenome.

The link between culture medium composition and transcriptomic effects is still unclear, but transcriptional changes may reflect an adaptation to a sub-optimal environment. For those reasons, we further tested whether the differences observed at day 2 in Ferticult over Global were maintained later on, at the blastocyst stage, after being cultured in Global, which is considered more suitable because of its amino acid enrichment (Rinaudo and Schultz, 2004). Only 18 DEGs were retrieved, and importantly, none of the early differences observed at day 2 were conserved at this later stage. Notably, the differences in expression levels of *AURKA*, *SETDB1*, and the imprinted *CDKN1C* gene observed at day 2 no longer existed at day 5. The original transcriptional changes may not have persisted because, in post-compaction, the embryo acquires an increasing ability to mitigate the transcriptomic profile acquired under different pre-compaction environments and to correct transcriptional errors (Wale and Gardner, 2016). A second hypothesis is that the Global medium composition itself may have allowed the embryo to recover a favorable transcriptomic landscape. Finally, we cannot rule out that only viable embryos were able to develop until the blastocyst stage, and only embryos with functional abilities were, therefore, selected in our analysis.

Our analysis of genes that are differentially expressed along pseudotime brought evidence that the use of distinct culture media prior to compaction can alter the sequential gene expression changes linked to later embryo development. Genes activated or downregulated at the wrong time may impact development and cause lasting effects (Calle et al., 2012; Bertoldo et al., 2015). Notably, Ferticult was associated with the over-expression of some genes at day 5. It is, therefore, possible that 2-day culture in Ferticult induces a delay in clearance of some maternal RNAs. Accordingly, two of the 18 DEGs at day 5 were maternal effect genes (*PADI6* and *TUBB8*) (Mitchell, 2022), which may indeed reflect longer retention of maternal transcripts. *PADI6* is a member of the subcortical oocyte complex (Yu et al., 2014; Bebbere et al., 2016), while *TUBB8* is the major constituent of the oocyte meiotic spindle assembly in primates (Feng et al., 2016).

Nevertheless, our pseudotime analysis of genes differentially expressed over the course of development also identified genes that are thought to be involved in the transition from early to later embryonic stages, such as *ABCC6*, *ACTL8*, *KPNA7*, *NLRP4*, *NLRP13*, *TUBB7P*, *TUBB8P8*, and *WEE2*. *TUBB7P* and *TUBB8P8* encode beta-tubulins of major importance in cell division and morphology. Karyopherin subunit alpha 7 (*KPNA7*) is involved in nuclear protein transport (Tejomurtula et al., 2009), and *Kpna7*-deficient mice fail to develop to the blastocyst stage or show developmental delays (Hu et al., 2010). Whether *ABCC6*, *ACTL8*, *NLRP4*, *NLRP13*, and *WEE2* are involved in early embryogenesis remains unknown. Additionally, XPA-binding protein 2 (*XAB2*), whose expression does not appear to be stage specific, was particularly high in Ferticult (log2FC>5.5). *XAB2* plays a role in DNA repair (Hou et al., 2016) and is required for embryo viability (Yonemasu et al., 2005; Yanez et al., 2016). The activation of DNA repair mechanisms may be reflective of stress conditions experienced by pre-implantation embryos. Because embryos were cultured in the same medium after day 2 in our study and because the embryo is transcriptionally silent until EGA, we can hypothesize that the blastocyst transcriptome was influenced by alterations that occurred pre-compaction.

Finally, we investigated whether adding methionine to the culture medium, an essential amino acid whose concentration varies greatly between commercial media (Tarahomi et al., 2019), could affect embryo gene expression. Methionine is a precursor of S-adenosylmethionine, a key component in the one-carbon metabolism and methylation processes (Steegers-Theunissen et al., 2013). Methionine is necessary for proper embryo development, but in excessive concentration, it could negatively affect embryo abilities, as demonstrated in several animal models (Dunlevy et al., 2006; Rees et al., 2006; Kwong et al., 2010). Reassuringly, we did not identify any DEGs in sibling embryos cultured in Global until day 5, with or without methionine

supplementation (concentration nearly thrice that in the Global medium), suggesting that excessive methionine concentration from day 2 did not have a major influence on the blastocyst's transcriptome. It is possible that the original methionine concentration in the Global medium (50 μM as evaluated in Morbeck et al. (2014)) and the supplementation concentration (200 μM) assessed in this study are both within the physiological range. Consequently, the absence of significant differences after supplementation would be normal. Analyzing the early effects of methionine addition before EGA could be important.

Evidence that the embryo culture medium can impact gene expression has long been described in animals (Rinaudo and Schultz, 2004; Schwarzer et al., 2012; Feuer et al., 2017). Interestingly, in pig, adding reproductive fluids during *in vitro* culture allows producing blastocysts with closer chromatin and transcriptomic profiles compared to natural conditions (Canovas et al., 2017). It will be important to develop culture media closer to natural fluid even if we showed that the embryo is highly adaptable to different conditions. Additionally, if this study is reassuring, we might not forget that many other processes in the IVF laboratory environment constitute environmental stressors (temperature, pH, co-culture, light, oxygen tension, and manipulation). In our design, culture conditions other than culture media were identical for all samples, but a gamete or an embryo exposed to a stressful condition might be even more vulnerable to other stressors. In addition, identical freezing protocol was used, the slow freezing protocol, now optimized by vitrification. This freezing protocol might be a factor of the cumulative stress effect.

We cannot rule out that some differential expression observed at the blastocyst stage did stem from differences in embryo morphology between the groups, even if the blastocysts included were mostly B2. However, it is likely that if there are morphological differences, they may be substantially related to the use of the different culture media. In this study, we cannot exclude specific effects of couple characteristics (stimulation protocol, age, and infertility causes) on the embryonic transcriptome, but we showed that maternal age did not change the overall results.

For the first time in humans, we employed single-embryo RNA-seq on a unique collection of day-2 and -5 embryos to assess to what extent different culture conditions might affect the developing embryo transcriptome. Even though marked transcriptomic differences were observed between culture media at day 2, when embryos totally deprived in amino acids during their first days of development were returned to favorable culture conditions, these differences were reduced at the blastocyst stage. The few differences observed at day 5 may be attributed to a delay in molecular processes specific to the use of one medium. Altogether, our study emphasizes the abilities of the embryo to recover an expected transcriptomic landscape post-compaction. Consecutively, to rule out potential long-lasting epigenetic effects, it would be important to investigate whether the methylome also adapts to different media formulations. In addition, whether different embryo culture media used post-compaction could modulate the embryonic transcriptome, and notably, the expression of genes characteristic of lineage specification remains to be elucidated.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GEO accession number: GSE212811.

Ethics statement

The studies involving human participants were reviewed and approved by the National Biomedicine Agency. The patients/participants provided their written informed consent to participate in this study.

Author contributions

PF, JB, and BD took primary responsibility for conceptualization and investigation. PF and RP-P were responsible for the methodology. MG, JB, and PF were involved in resources, experiments, and visualization. BD and AT conducted the data curation and formal analysis. BD and PF were involved in original draft preparation. DB participated in review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1155634/full#supplementary-material

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One-carbon metabolism is required for epigenetic stability in the mouse placenta

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One-carbon metabolism, including the folate cycle, has a crucial role in fetal development though its molecular function is complex and unclear. The hypomorphic *Mtrr*^{gt} allele is known to disrupt one-carbon metabolism, and thus methyl group availability, leading to several developmental phenotypes (e.g., neural tube closure defects, fetal growth anomalies). Remarkably, previous studies showed that some of the phenotypes were transgenerationally inherited. Here, we explored the genome-wide epigenetic impact of one-carbon metabolism in placentas associated with fetal growth phenotypes and determined whether specific DNA methylation changes were inherited. Firstly, methylome analysis of *Mtrr*$^{gt/gt}$ homozygous placentas revealed genome-wide epigenetic instability. Several differentially methylated regions (DMRs) were identified including at the *Cxcl1* gene promoter and at the *En2* gene locus, which may have phenotypic implications. Importantly, we discovered hypomethylation and ectopic expression of a subset of ERV elements throughout the genome of *Mtrr*$^{gt/gt}$ placentas with broad implications for genomic stability. Next, we determined that known spermatozoan DMRs in *Mtrr*$^{gt/gt}$ males were reprogrammed in the placenta with little evidence of direct or transgenerational germline DMR inheritance. However, some spermatozoan DMRs were associated with placental gene misexpression despite normalisation of DNA methylation, suggesting the inheritance of an alternative epigenetic mechanism. Integration of published wildtype histone ChIP-seq datasets with *Mtrr*$^{gt/gt}$ spermatozoan methylome and placental transcriptome datasets point towards H3K4me3 deposition at key loci. These data suggest that histone modifications might play a role in epigenetic inheritance in this context. Overall, this study sheds light on the mechanistic complexities of one-carbon metabolism in development and epigenetic inheritance.

KEYWORDS

DNA methylation, folate, histone methylation, MTRR, sperm, transposable elements, trophoblast, epigenetic inheritance

1 Introduction

It is well established that the vitamin folate (also known as folic acid) is important for fetal development. A highly recognisable example is increased risk of neural tube closure defects (e.g., spina bifida) in babies that result from maternal dietary folate deficiency (Emery et al., 1969). In fact, folic acid supplementation during pregnancy and folate fortification programmes improves pregnancy outcomes (MRC Vitamin Study Research Group, 1991; Gelineau-van Waes et al., 2008). Beyond the neural tube, other developmental defects [e.g., fetal growth restriction (Furness et al., 2008; Chen et al., 2018), congenital heart defects (Christensen et al., 2015)] and pregnancy disorders (Mislanova et al., 2011; Wu et al., 2015) are associated with dietary deficiency and/or mutations in key enzymes involved in its metabolism. Although well studied, the molecular role of folate metabolism during development is complex and not well understood. One-carbon metabolism, which includes the folate and methionine cycles, is required by all cells for thymidine synthesis and for methyl groups involved in a broad range of methylation reactions (Lin et al., 2022). As a result, it is hypothesised that rapidly proliferating cells in a developing fetus and placenta requires one-carbon metabolism for DNA synthesis and general epigenetic regulation. The specific genomic targets of one-carbon metabolism that drive developmental phenotypes remain unclear.

To explore the specific molecular role of one-carbon metabolism during development, we study a mouse model with a hypomorphic mutation in the methionine synthase reductase gene (*Mtrr*^{gt}) (Padmanabhan et al., 2013). During one-carbon metabolism, folate metabolites are required to transmit methyl groups for the methylation of homocysteine by methionine synthase (MTR) to form methionine and tetrahydrofolate (Shane and Stokstad, 1985). Methionine acts as precursor for S-adenosylmethionine (SAM), which in turn serves as the sole methyl-donor for substrates involved in epigenetic regulation (e.g., DNA, histones, RNA) among other substrates (Ducker and Rabinowitz, 2017). Importantly, MTRR activates MTR through the reductive methylation of its vitamin B_{12} co-factor (Leclerc et al., 1998; Yamada et al., 2006; Elmore et al., 2007). The hypomorphic *Mtrr*^{gt} mutation reduces *Mtrr* transcript expression to a level that is sufficient to diminish MTR activity by 60% of controls (Elmore et al., 2007; Padmanabhan et al., 2013). Consequently, the progression of one-carbon metabolism is disrupted by the *Mtrr*^{gt} mutation as evidenced by plasma hyperhomocysteinemia (Elmore et al., 2007; Padmanabhan et al., 2013) and widespread changes in DNA methylation patterns (Padmanabhan et al., 2013; Bertozzi et al., 2021; Blake et al., 2021). Additionally, *Mtrr*$^{gt/gt}$ mice display several phenotypes similar to the clinical features of folate deficiency in humans (Krishnaswamy and Madhavan Nair, 2001) or human *MTRR* mutations (Schuh et al., 1984; Wilson et al., 1999) including macrocytic anemia (Padmanabhan et al., 2018) and neural tube closure defects (NTDs) (Padmanabhan et al., 2013; Wilkinson et al., 2021). Beyond this, other phenotypes have emerged in *Mtrr*$^{gt/gt}$ mice reflecting a broader influence of impaired one-carbon metabolism on development. These phenotypes include fetal growth defects (such as fetal growth restriction (FGR), fetal growth enhancement (FGE), or developmental delay) (Padmanabhan et al., 2013; Padmanabhan et al., 2017), complications during implantation (e.g., twinning, skewed implantation) (Padmanabhan et al., 2013; Wilkinson et al., 2021), haemorrhages, and/or congenital malformations (such as congenital heart defects and poor placentation) (Deng et al., 2008; Padmanabhan et al., 2013; Wilkinson et al., 2021). Therefore, the *Mtrr*^{gt} mouse line is ideal for exploring the molecular consequences of defective one-carbon metabolism during growth and development.

Remarkably, the *Mtrr*^{gt} mouse line is also a unique mammalian model of transgenerational epigenetic inheritance that occurs via the maternal grandparental lineage (Padmanabhan et al., 2013; Blake et al., 2021). Through highly controlled genetic pedigrees and embryo transfer experiments, we previously showed that an *Mtrr*$^{+/gt}$ genotype in male or female mice (i.e., the F0 generation) initiates multigenerational inheritance of developmental phenotypes in their wildtype (*Mtrr*$^{+/+}$) grandprogeny (i.e., the F2–F4 generations) (Padmanabhan et al., 2013; Padmanabhan et al., 2017). This effect occurs through their F1 wildtype daughters (Padmanabhan et al., 2013). In general, the mechanism of epigenetic inheritance is not well understood. In the context of the *Mtrr*^{gt} mouse line, we hypothesise that alterations in the epigenome of the F0 germline caused by abnormal one-carbon metabolism is inherited by the wildtype offspring of the next generation (and potentially beyond) to influence gene expression during development (Padmanabhan et al., 2013; Blake and Watson, 2016; Blake et al., 2021). Given the role of MTRR in one-carbon metabolism, and thus in cellular methylation, we initially focused on how the *Mtrr*^{gt} mutation alters DNA methylation patterns across generations. Through a targeted analysis, we previously determined that developmental phenotypes at E10.5 in *Mtrr*$^{gt/gt}$ conceptuses or F2 *Mtrr*$^{+/+}$ conceptuses derived by an F0 *Mtrr*$^{+/gt}$ maternal grandparent were associated with locus-specific changes in DNA methylation linked to gene misexpression (Padmanabhan et al., 2013; Blake et al., 2021). The effect was particularly striking in the placenta at key genes involved in the regulation of fetal growth and metabolism (Padmanabhan et al., 2013). It was also clear that epigenetic instability of DNA methylation occurs in mature spermatozoa from *Mtrr*$^{+/gt}$ and *Mtrr*$^{gt/gt}$ males as well as from F1 *Mtrr*$^{+/+}$ male progeny of F0 *Mtrr*$^{+/gt}$ males (Blake et al., 2021), which otherwise display normal spermatogenesis and spermatozoa function (Blake et al., 2019). However, the extent to which these altered germline methylation patterns are recapitulated in (or inherited by) the somatic cells of the progeny and grandprogeny is currently not well understood in the *Mtrr*^{gt} mouse line.

In this study, we use genome-wide approaches to investigate the global impact of one-carbon metabolism on the placental methylome in *Mtrr*$^{gt/gt}$ homozygous mice and in F2 *Mtrr*$^{+/+}$ mice derived from F0 *Mtrr*$^{+/gt}$ maternal grandfathers. In doing so, we probe the underlying impact on fetal growth and whether the differentially methylated regions (DMRs) are functionally important and/or inherited. We reveal that the *Mtrr*$^{gt/gt}$ placental methylome is unstable with implications for phenotype establishment, transposable element regulation, and genetic stability. We also determine that specific DMRs observed in spermatozoa of *Mtrr*$^{gt/gt}$ males are reprogrammed in *Mtrr*$^{gt/gt}$ placentas and as a result, we explore other epigenetic mechanisms for inheritance including histone methylation (H3K4me3). We integrate published ChIP-seq datasets from wildtype embryonic and trophoblast lineages with our *Mtrr*$^{gt/gt}$

spermatozoan methylome and placental transcriptome datasets. Overall, these analyses delve into the mechanistic complexities of one-carbon metabolism during development and epigenetic inheritance of phenotype.

2 Materials and methods

2.1 Ethics statement

This research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body.

2.2 Mouse model

Mtrr$^{Gt(XG334)Byg}$ (MGI:3526159) mouse line, referred to as the *Mtrr*^{gt} mouse line, was generated when a *β-geo* gene-trap (gt) vector was inserted into intron 9 of the *Mtrr* gene in 129P2Ola/Hsd embryonic stem cells (ESCs) (Elmore et al., 2007; Padmanabhan et al., 2013). *Mtrr*^{gt} ECSs were injected into C57Bl/6J blastocysts and upon germline transmission, the *Mtrr*^{gt} allele was backcrossed into the C57Bl/6J genetic background for at least eight generations (Padmanabhan et al., 2013). *Mtrr*$^{+/+}$ and *Mtrr*$^{+/gt}$ mice were generated from *Mtrr*$^{+/gt}$ intercrosses. *Mtrr*$^{gt/gt}$ mice were generated by *Mtrr*$^{gt/gt}$ intercrosses. Since the *Mtrr*^{gt} allele has a multigenerational effect (Padmanabhan et al., 2013; Bertozzi et al., 2021; Blake et al., 2021), C57Bl/6J mice from The Jackson Laboratories (www.jaxmice.jax.org) were used as controls and were bred in-house and maintained separately from the *Mtrr*^{gt} mouse line. The effects of the maternal grandpaternal *Mtrr*^{gt} allele were determined by the following pedigree: F0 *Mtrr*$^{+/gt}$ males were mated to C57Bl/6J females. The resulting F1 *Mtrr*$^{+/+}$ females were mated to C57Bl/6J males to generate F2 *Mtrr*$^{+/+}$ conceptuses. Genotyping for *Mtrr*$^{+}$ and *Mtrr*^{gt} alleles was performed using PCR on DNA extracted from ear tissue or yolk sac using a three-primer reaction resulting in a wildtype band at 252 bp and a mutant band at 383 bp (Padmanabhan et al., 2013). Primer sequences: primer *a* (5′-GAGATTGGGTCCCTCTTCCAC), primer *b* (5′-GCTGCGCTTCTGAATCCACAG), and primer *c* (5′-CG ACT TCCGGAGCGGATCTC) (Padmanabhan et al., 2013). All mice were housed in a temperature-and humidity-controlled environment with a 12 h light-dark cycle. All mice were fed a normal chow diet (Rodent No. 3 chow, Special Diet Services) *ad libitum* from weaning, which included (per kg of diet): 1.6 g choline, 2.73 mg folic acid, 26.8 μg vitamin B_{12}, 3.4 g methionine, 51.3 mg zinc.

2.3 Dissections and tissue collection

Noon of the day that the vaginal plug was detected was defined as embryonic (E) day 0.5. Mice were euthanized by cervical dislocation. Fetuses and placentas were dissected in cold 1x phosphate buffered saline (PBS) at E10.5 using a Zeiss SteReo Discovery V8 microscope, scored for phenotypes, and photographed. Fetuses and placentas were weighed and measured separately and snap frozen in liquid nitrogen (stored at −80°C). Both male and female placentas were assessed since no phenotypic sexual dimorphism was identified at E10.5 (Padmanabhan et al., 2017).

2.4 Phenotyping

Conceptuses were rigorously scored for gross phenotypes during dissection and allocated to the phenotypic categories that were previously defined, including phenotypically normal (PN), fetal growth enhancement (FGE), fetal growth restriction (FGR), developmental delay, severe abnormalities (e.g., congenital heart defects, neural tube closure defects, hemorrhages, skewed conceptus orientation, twinning, etc.), and resorption (Padmanabhan et al., 2013; Wilkinson et al., 2021). Notably, conceptuses with >1 phenotype were counted once and classified by the most severe phenotype observed. Only PN, FGR, and FGE conceptuses were assessed in this study. Phenotype parameters are defined below.

2.4.1 PN conceptuses

Fetuses and placentas met all developmental milestones appropriate for the developmental stage according to e-Mouse Atlas Project (https://www.emouseatlas.org/emap/home.html). PN fetuses at E10.5 contained 30–39 somite pairs and had crown-rump lengths that were within two standard deviations (sd) from the mean of C57Bl/6J fetuses at E10.5, putting them within the normal range for growth. All PN conceptuses lacked abnormalities identified via gross assessment.

2.4.2 FGR and FGE conceptuses

Conceptuses with FGR and FGE lacked abnormalities identified via gross dissection and met the staging criteria for E10.5 (i.e., 30–39 somite pairs). Yet, the fetuses displayed crown-rump lengths that were ≥2 sd below (for FGR) or above (for FGE) the mean crown-rump length for C57Bl/6J fetuses (Padmanabhan et al., 2013). Conceptus size was unaffected by litter size in all pedigrees and stages assessed (Padmanabhan et al., 2017).

2.5 Methylated DNA immunoprecipitation (meDIP) and next-generation sequencing

Whole placentas at E10.5 were homogenized using a MagNA Lyser Instrument (Roche) and incubated on an Eppendorf ThermoMixer at 1,000 rpm at 56°C for 10 min. Genomic DNA was extracted using a QIAamp Fast DNA Tissue kit (Qiagen) following the manufacturer's instructions. MeDIP-Seq was carried out as described previously (Ficz et al., 2011). Briefly, genomic DNA was sonicated to yield 150–600 bp fragments, and adaptors for paired-end sequencing (Illumina) were ligated using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs). Immunoprecipitations were carried out using 500 ng DNA per sample, 1.25 μg anti-5mC antibody (Eurogentec Cat# BI-MECY-0100, RRID:AB_2616058) or mouse immunoglobulin G (IgG) control and 10 μL Dynabeads coupled with M-280 sheep anti-mouse antibody (Invitrogen). Pulled down DNA was amplified for 12 cycles (meDIP) or 15 cycles (IgG control)

with adapter-specific indexed primers. Final clean-up and size selection was carried out with AMPure-XP SPRI beads (Beckman Coulter). Libraries were quantified and assessed using the Kapa Library Quantification Kit (Kapa Biosystems) and Bioanalyzer 2100 System (Agilent). Indexed libraries were sequenced (50-bp paired-end) on an Illumina HiSeq 2500 sequencer. Raw fastq data were trimmed with TrimGalore (v0.6.6), using default parameters, and unique reads mapped to the *Mus musculus* GRCm38 genome assembly using Bowtie2 (v2.4.1). Data analysis was carried out using SeqMonk software (www.bioinformatics.babraham.ac.uk).

2.6 RNA-sequencing

Whole male placentas at E10.5 were homogenized using lysing matrix D beads. RNA library preparation and sequencing was performed by Cambridge Genomic Services, Department of Pathology, University of Cambridge. The concentration and purity of RNA was determined by a SpectroStar spectrophotometer (BMG LABTECH) and the RNA integrity was determined by an Agilent Tapestation Bioanalyzer (Aligent Technologies LDA United Kingdom Ltd.). Libraries were prepared using 200 ng of total RNA and TruSeq stranded mRNA Library Preparation kit (Illumina). A unique index sequence was added to each RNA library to allow for multiplex sequencing. Libraries were pooled and sequenced on the Illumina NextSeq500 platform with 75 bp single-end reads. Sequencing was performed in duplicate to provide >18 million reads per sample. To monitor sequencing quality control, 1% PhiX Control (Illumina) spike-in was used. Quality control of Fastq files was performed using FastQC and fastq_screen. Sequences were trimmed with Trim Galore! and aligned to GRCm38 mouse genome using STAR aligner. Alignments were processed using custom ClusterFlow (v0.5dev) pipelines and assessed using MultiQC (0.9. dev0). Gene quantification was determined with HTSeq- Counts (v0.6.1p1). Additional quality control was performed with rRNA and mtRNA counts script, feature counts (v 1.5.0- p2) and qualimap (v2.2). Differential gene expression was performed with DESeq2 package (v1.22.2, R v3.5.2). Read counts were normalised on the estimated size factors.

2.7 Transposable element analysis

To include transposon-derived reads that do not map uniquely, the meDIP-seq datasets were re-aligned using the default settings of bowtie2 to assign reads with multiple equally best alignments to one of those locations at random. Average methylation levels over pro-viral, full-length elements were generated after merging Repeatmasker annotations for *RLTR4_Mm* and *RLTR4_MM-int* elements. RNA-seq data was analysed using SQuIRE (Yang et al., 2019), which assigns multimapping reads using an expectation-maximisation algorithm and provides both subfamily-level and single copy-level information. Differential expression analysis was performed using SQuIRE's Call function.

3 Results

3.1 Global analysis of the *Mtrr*$^{gt/gt}$ placenta methylome

First, we analysed the extent to which impaired one-carbon metabolism affected the placental methylome and ascertained whether there was an impact on fetal growth. Our initial focus was on *Mtrr*$^{gt/gt}$ placentas of conceptuses derived from *Mtrr*$^{gt/gt}$ intercrosses (Figure 1A). We carried out high-throughput sequencing of immunoprecipitated methylated DNA (meDIP-seq) from C57Bl/6J control and *Mtrr*$^{gt/gt}$ placentas at E10.5. *Mtrr*$^{gt/gt}$ placentas were divided into two phenotypic groups including those from fetuses that were phenotypically normal (PN) or were FGR based on crown-rump length (Padmanabhan et al., 2013; Wilkinson et al., 2021). Placentas from C57Bl/6J mice were controls since the *Mtrr*^{gt} allele was backcrossed into the C57Bl/6J genetic background (Padmanabhan et al., 2013). However, we previously identified four regions of structural variation between C57Bl/6J and the *Mtrr*^{gt} line (Blake et al., 2021). To avoid false discovery of changes in DNA methylation during the meDIP-seq data analysis, these regions were excluded bioinformatically along with the 20 Mb region of 129P2Ola/Hsd genomic sequence surrounding the gene-trapped *Mtrr* allele (Bertozzi et al., 2021; Blake et al., 2021), that remained after eight backcrosses (Padmanabhan et al., 2013).

At the global level, we found that the distribution of meDIP-seq reads across different genomic features were not significantly different between C57Bl/6J control and *Mtrr*$^{gt/gt}$ placentas even when phenotypic severity was considered (Supplementary Figure S1A). Furthermore, meDIP-seq datasets from individual placentas did not cluster by *Mtrr* genotype or fetal growth phenotype when data store similarity tools were implemented (Supplementary Figure S1B). As global DNA methylation patterns were similar between experimental groups, we next ascertained differences in DNA methylation at individual loci compared to control placentas. DMRs were defined using the EdgeR function embedded within Seqmonk software (www.bioinformatics.babraham.ac.uk) with default settings ($p < 0.05$, with multiple testing correction) assessing 500 bp contiguous regions. The resulting DMRs were further filtered for regions that displayed a log_2 fold change (FC) > 1 in DNA methylation compared to controls. Only a few DMRs were present in *Mtrr*$^{gt/gt}$ placentas (i.e., PN: 13 DMRs; FGR: 9 DMRs), though both hyper- and hypomethylated regions were observed (Figures 1B, C; Supplementary File S1). The low number of DMRs caused by the *Mtrr*^{gt} allele suggested that DNA methylation changes were subtle regardless of fetal growth phenotypes or were "hidden" by our analysis of whole placentas as individual cell types might be differently affected.

Despite the low number of total DMRs, three key findings emerged (explored further below). Firstly, only one placental DMR associated with the misexpression of a protein-coding gene (*Cxcl1*; Figures 1D, E). Secondly, we identified two hypermethylated DMRs located within the *En2* gene in *Mtrr*$^{gt/gt}$ placentas that were common to PN and FGR conceptuses (Figures 1B, C). Since the *En2* DMRs were also identified in mature spermatozoa from *Mtrr*$^{gt/gt}$ males and in *Mtrr*$^{gt/gt}$ embryos at E10.5 (Blake et al., 2021), they were flagged for further analysis in the context of development and epigenetic inheritance. Lastly, of the 12 hypomethylated DMRs that were identified (i.e., 3 shared DMRs in PN and FGR

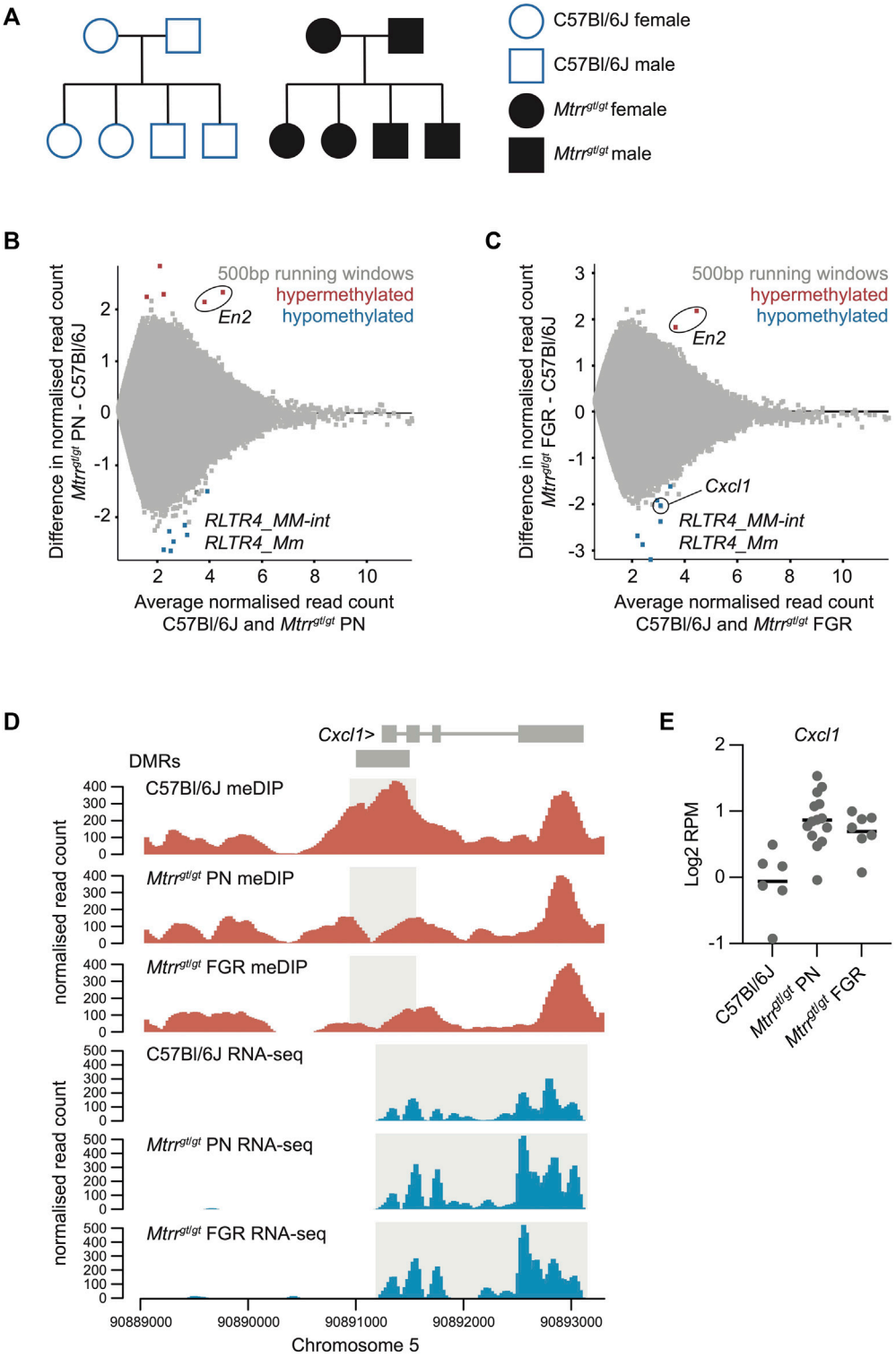

FIGURE 1
Analysis of the *Mtrr*$^{gt/gt}$ placental methylome. **(A)** Schematization of the C57Bl6J control pedigree (blue outline, white fill) and *Mtrr*$^{gt/gt}$ pedigree (black outline, black fill) used in this study. Square, males; Circles, females. **(B,C)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in **(B)** C57Bl/6J placentas and *Mtrr*$^{gt/gt}$ placentas from phenotypically normal (PN) fetuses, and **(C)** C57Bl/6J placentas and *Mtrr*$^{gt/gt}$ placentas associated with fetal growth restriction (FGR). Hypermethylated (red) and hypomethylated (blue) differentially methylated 500 bp regions (DMR) were identified using EdgeR. **(D)** Data tracks showing normalized meDIP-seq (red) and RNA-seq (blue) reads across the *Cxcl1* locus on mouse chromosome 5 in C57Bl/6J, *Mtrr*$^{gt/gt}$ PN and *Mtrr*$^{gt/gt}$ FGR placentas. DMR and transcript expression are highlighted in light grey. **(E)** Graph showing *Cxcl1* transcript expression (log_2RPM) ascertained by RNA-seq in C57Bl/6J, *Mtrr*$^{gt/gt}$ PN, and *Mtrr*$^{gt/gt}$ FGR placentas at E10.5. In all cases data was normalized to the largest data store. For meDIP-seq: C57Bl/6J, *N* = 8 placentas; *Mtrr*$^{gt/gt}$ PN, *N* = 7 placentas; *Mtrr*$^{gt/gt}$ FGR, *N* = 7 placentas. For RNA-seq: C57Bl/6J, *N* = 6 placentas; *Mtrr*$^{gt/gt}$ PN, *N* = 14 placentas; *Mtrr*$^{gt/gt}$ FGR, *N* = 7 placentas.

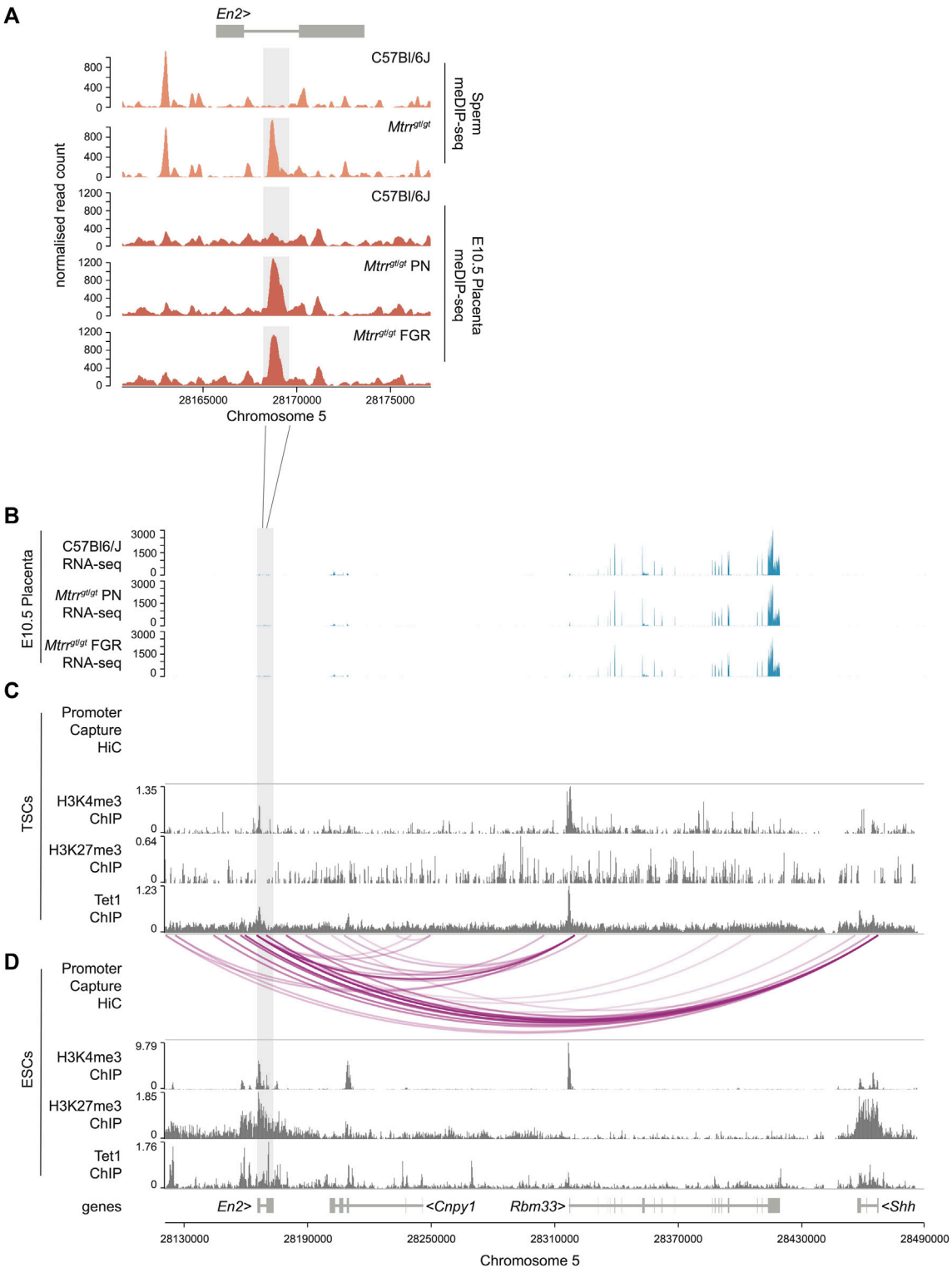

FIGURE 2
En2 DMR as a potential regulator of developmentally important genes in ESCs and not TSCs. **(A)** Data tracks showing normalized meDIP-seq read counts across the *En2* gene in spermatozoa from C57BL/6J and *Mtrr*$^{gt/gt}$ mice (orange) and placentas from C57Bl/6J and *Mtrr*$^{gt/gt}$ conceptuses at E10.5 (red). Placentas were associated with either phenotypically normal (PN) or fetal growth restricted (FGR) fetuses. The *En2* DMR is highlighted in light grey. **(B)** RNA-seq data tracks showing gene expression (blue) in the genomic region near to the *En2* DMR (light grey) in placentas at E10.5 from C57Bl/6J and *Mtrr*$^{gt/gt}$ conceptuses. Placentas from PN and FGR fetuses were considered. **(C,D)** Data tracks showing complete promoter capture HiC-based interactions (purple lines) and H3K27me3, H3K4me3 and Tet1 ChIP-seq peaks (dark grey) at the *En2* locus and downstream genes within **(C)** wildtype mouse trophoblast stem cells (TSCs) and **(D)** wildtype mouse embryonic stem cells (ESCs). See also Supplementary File S3 for data sources. For sperm meDIP-seq: C57Bl/6J, *N* = 8 males; *Mtrr*$^{gt/gt}$, *N* = 8 males. For placenta meDIP-seq: C57Bl/6J, *N* = 8 placentas; *Mtrr*$^{gt/gt}$ PN, *N* = 7 placentas; *Mtrr*$^{gt/gt}$ FGR, N = 7 placentas.

placentas, 5 DMRs in PN placentas only, 4 DMRs in FGR placentas only), ten were associated with endogenous retroviruses (ERVs; Supplementary File S1). Strikingly, the majority of these DMRs (7/10) overlapped with ERV1 elements of the RLTR4 subclass (Figures 1B, C) with implications for genetic stability. Further exploration into the importance of these findings was explored below.

3.2 Potential canonical regulation of placental *Cxcl1* expression by DNA methylation

To explore whether altered placental DNA methylation caused by the *Mtrr*$^{gt/gt}$ genotype had a gene regulatory effect, we carried out RNA-seq on *Mtrr*$^{gt/gt}$ placentas at E10.5 associated with PN and FGR fetuses. Using DESeq, the RNA-seq data was assessed for differentially expressed genes that were within 2 kb of a DMR (identified in *Mtrr*$^{gt/gt}$ placentas) and had transcript levels with a log_2FC > 0.6 compared to control placentas. The *Cxcl1* gene [chemokine (C-X-C motif) ligand 1] was the only dysregulated gene identified in this context. We observed that hypomethylation at the DMR located in the promoter of *Cxcl1* was associated with a modest upregulation of *Cxcl1* transcripts (Figures 1D, E). This finding exemplifies canonical regulation of a gene by DNA methylation. Since *Mtrr*$^{gt/gt}$ placentas from both PN and FGR fetuses displayed hypomethylation at the *Cxcl1* DMR and upregulation of *Cxcl1* transcripts (Figures 1D, E), these molecular changes were likely insufficient to drive the fetal growth phenotype. Yet, CXCL1 is important for decidual angiogenesis to promote maternal blood flow into the implantation site (Ma et al., 2021). Therefore, dysregulation of *Cxcl1* mRNA in *Mtrr*$^{gt/gt}$ placentas might have implications for fetoplacental development beyond fetal growth.

3.3 *En2* DMR as a potential regulator of developmentally important genes

The only two hypermethylated DMRs identified in *Mtrr*$^{gt/gt}$ placentas were found within the *En2* gene. Therefore, their functional importance was explored. These two 500 bp DMRs were in fact contiguous and represented one single 1 kb region in the single *En2* intron (Figure 2A). The *En2* gene encodes a homeobox transcription factor that, when knocked out in mice, leads to autism-spectrum disease-like behaviours (Cheh et al., 2006; Brielmaier et al., 2012; Provenzano et al., 2014) that are accompanied by cerebellar foliation defects (Joyner et al., 1991) and loss of GABAergic interneurons in somatosensory and visual cortical areas (Sgado et al., 2013; Allegra et al., 2014). Indeed, *En2* mRNA is expressed in multiple regions of the developing brain (Davis et al., 1988) and is involved in neurogenesis (Lee et al., 1997). Low levels of *En2* transcripts were reported by RNA-seq in the ectoplacental cone (Bastian et al., 2021) (a population of trophoblast progenitor cells in the mouse placenta). However, our RNA-seq data from whole C57Bl/6J control placentas at E10.5 showed that *En2* transcripts were very lowly expressed (Figure 2B) and thus, *En2* might be considered as an unexpressed gene in the placenta at this developmental stage. Importantly, hypermethylation of the *En2* DMR in *Mtrr*$^{gt/gt}$ placentas was not associated with a change in *En2* transcript levels (Figure 2B) indicating that the *En2* DMR is an unlikely regulator of *En2* gene expression in the placenta.

To investigate a broader regulatory role of the *En2* DMR in the placenta, we explored histone methylation (e.g., H3K4me3 and H3K27me3) enrichment and potential interactions of the DMR with neighbouring genes. To do this, we analysed published H3K4me3 and H3K27me3 ChIP-seq datasets and promoter capture Hi-C datasets from wildtype mouse trophoblast stem cells (TSCs) (Schoenfelder et al., 2018). TSCs are an *in vitro* model of undifferentiated trophoblast cells of the placenta (Tanaka et al., 1998), and these datasets represent the most suitable available for analysis. No enrichment of H3K4me3 or H3K27me3 modifications was evident at the *En2* DMR in TSCs and no complete DMR-promoter interactions were evident in TSCs within the genomic region assessed (Figures 2B, C). Accordingly, genes downstream of the *En2* DMR were also expressed at normal levels in *Mtrr*$^{gt/gt}$ placentas (Figure 2B) indicating that hypermethylation of this region had little to no effect on cis regulation of gene expression in the placenta.

The hypermethylated *En2* DMR is prevalent in different tissue types including mature spermatozoa of *Mtrr*$^{gt/gt}$ males and *Mtrr*$^{gt/gt}$ embryos and placentas at E10.5 (Blake et al., 2021; this study). Given that the *En2* gene is important for the development of embryonic lineages [e.g., neurogenesis (Lee et al., 1997)], the potential regulatory importance of the *En2* DMR was explored outside of the placenta. Additional ChIP-seq and promoter capture Hi-C datasets from wildtype mouse ESCs (Schoenfelder et al., 2018) were analysed in the region proximal to the *En2* DMR. The data revealed that the *En2* DMR had hallmarks of a regulatory locus in ESCs since it was bivalently marked by the enrichment of repressive H3K27me3 and active H3K4me3 modifications (Figure 2D) in a manner that poises this region for activation upon cell differentiation (Macrae et al., 2023). The genomic region defined by the *En2* DMR in ESCs was also enriched for the DNA demethylating enzyme TET1 (Figure 2D), which typically co-localises with polycomb complexes and contributes to keeping unmethylated enhancers and promoters methylation-free (Parry et al., 2021). Furthermore, promoter capture Hi-C experiments in ESCs (Schoenfelder et al., 2018) revealed a potential interaction of the *En2* DMR with the promoters of nearby genes, including *Cnpy1* (canopy FGF signalling regulator 1), *Rbm33* (RNA binding motif 33), and the developmental regulator *Shh* (sonic hedgehog) (Figure 2D). These data contrasted the TSCs data, which showed no such DMR-promoter interactions (Figure 2C). Therefore, we hypothesised that ectopic hypermethylation of the En2 DMR specifically within *Mtrr*$^{gt/gt}$ embryos might affect expression of surrounding genes with developmental consequences. Further analysis of the developmental role of the *En2* DMR in the embryo is required, particularly in the context of abnormal one-carbon metabolism.

3.4 Hypomethylation and ectopic expression of ERVs indicates epigenetic instability in the *Mtrr*^{gt} mouse line

We identified ten hypomethylated DMRs in *Mtrr*$^{gt/gt}$ placentas at E10.5 that were associated with ERV elements (Supplementary File S1). Specifically, seven of these overlapped with RLTR4 elements of the ERV1 subfamily (separately annotated as *RLTR4_Mm* and *RLTR4_MM-int* for the LTRs and internal region, respectively; Figures 1B, C). RLTR4 elements are relatively young retrotransposons that are closely related to murine leukemia virus and that, at least in some mouse strains, remain transpositionally

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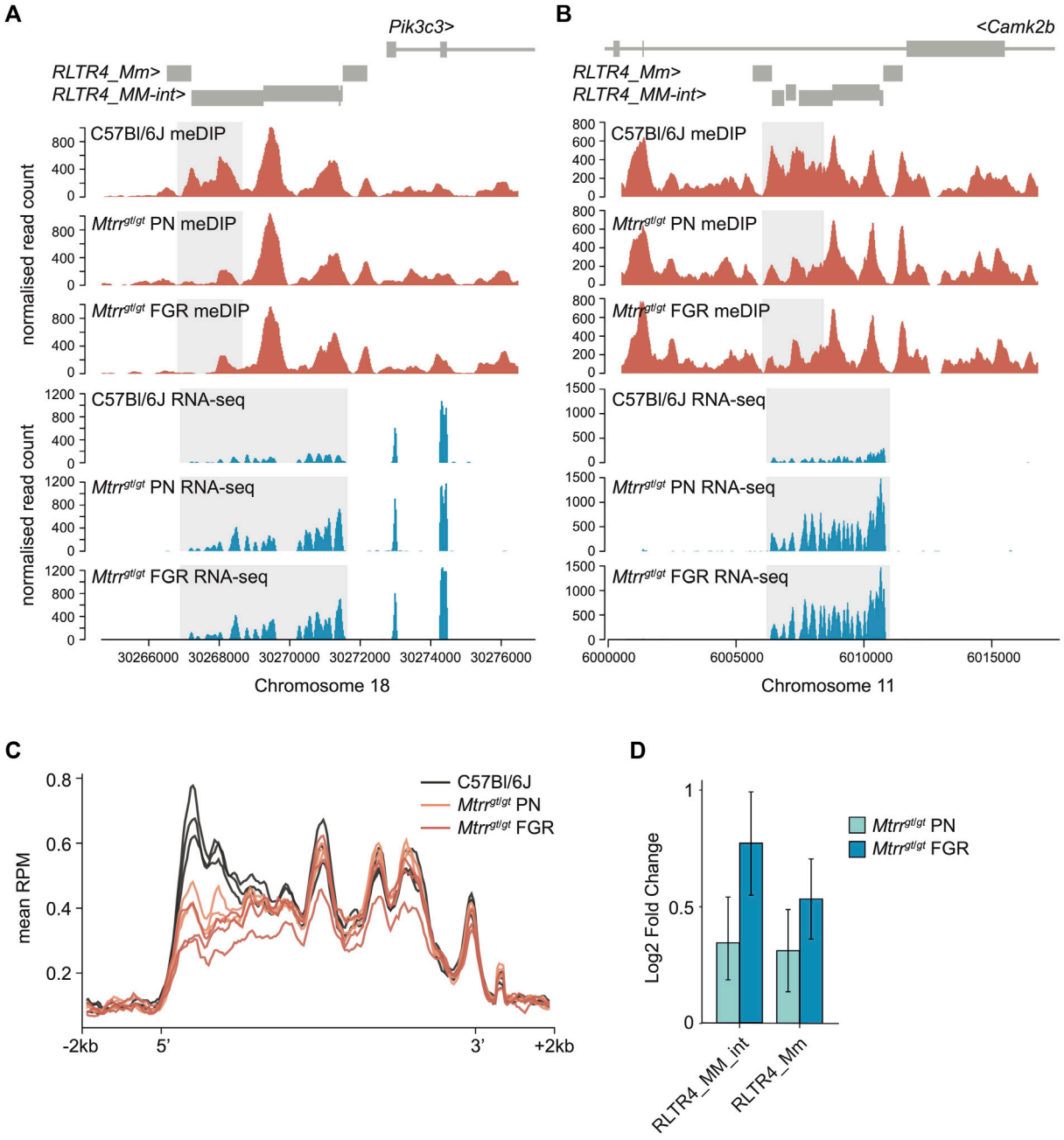

FIGURE 3
Analysis of DNA methylation and transcript expression at ERV subfamily in *Mtrr*$^{gt/gt}$ placentas. **(A,B)** Data tracks showing normalized meDIP-seq (red) and RNA-seq (blue) reads across full-length ERVs comprising *RLTR4_Mm* and *RLTR4_MM-int* elements on mouse **(A)** chromosome 18 associated with the *Pik3c3* gene and **(B)** chromosome 11 associated with the *Camk2b* gene in placentas of C57Bl/6J and *Mtrr*$^{gt/gt}$ conceptuses at E10.5. Placentas from phenotypically normal (PN) and fetal growth restricted (FGR) fetuses were assessed. Differentially methylated region (DMR) and transcript expression are highlighted in light grey. **(C)** Graph representing the average meDIP-seq reads mapping across all full-length RLTR4 elements ± 2 kb in the genome in individual placentas from C57Bl/6J (black), *Mtrr*$^{gt/gt}$ PN (orange) and *Mtrr*$^{gt/gt}$ FGR (red) fetuses at E10.5. **(D)** Enrichment of *RLTR4_Mm* and *RLTR4_MM-int* expression in placentas from *Mtrr*$^{gt/gt}$ PN (light blue) and *Mtrr*$^{gt/gt}$ FGR (dark blue) fetuses at E10.5 relative to C57Bl/6J control placentas as determined by RNA-seq. For meDIP-seq: C57Bl/6J, *N* = 8 placentas; *Mtrr*$^{gt/gt}$ PN, *N* = 7 placentas; *Mtrr*$^{gt/gt}$ FGR, *N* = 7 placentas. For RNA-seq: C57Bl/6J, *N* = 6 placentas; *Mtrr*$^{gt/gt}$ PN, *N* = 14 placentas; *Mtrr*$^{gt/gt}$ FGR, *N* = 7 placentas.

active (Maksakova et al., 2006). Some transposable elements (e.g., IAPs) are highly methylated and resistant to epigenetic reprogramming to avoid genomic transposition (Kobayashi et al., 2012). It is unclear whether this is the case for RLTR4 elements. The RLTR4 elements that associated with placental DMRs in this study typically displayed a pro-viral, full-length configuration, rather than being solo LTRs or other isolated fragments. Furthermore, six out of seven of the RLTR4-associated DMRs mapped to two discrete

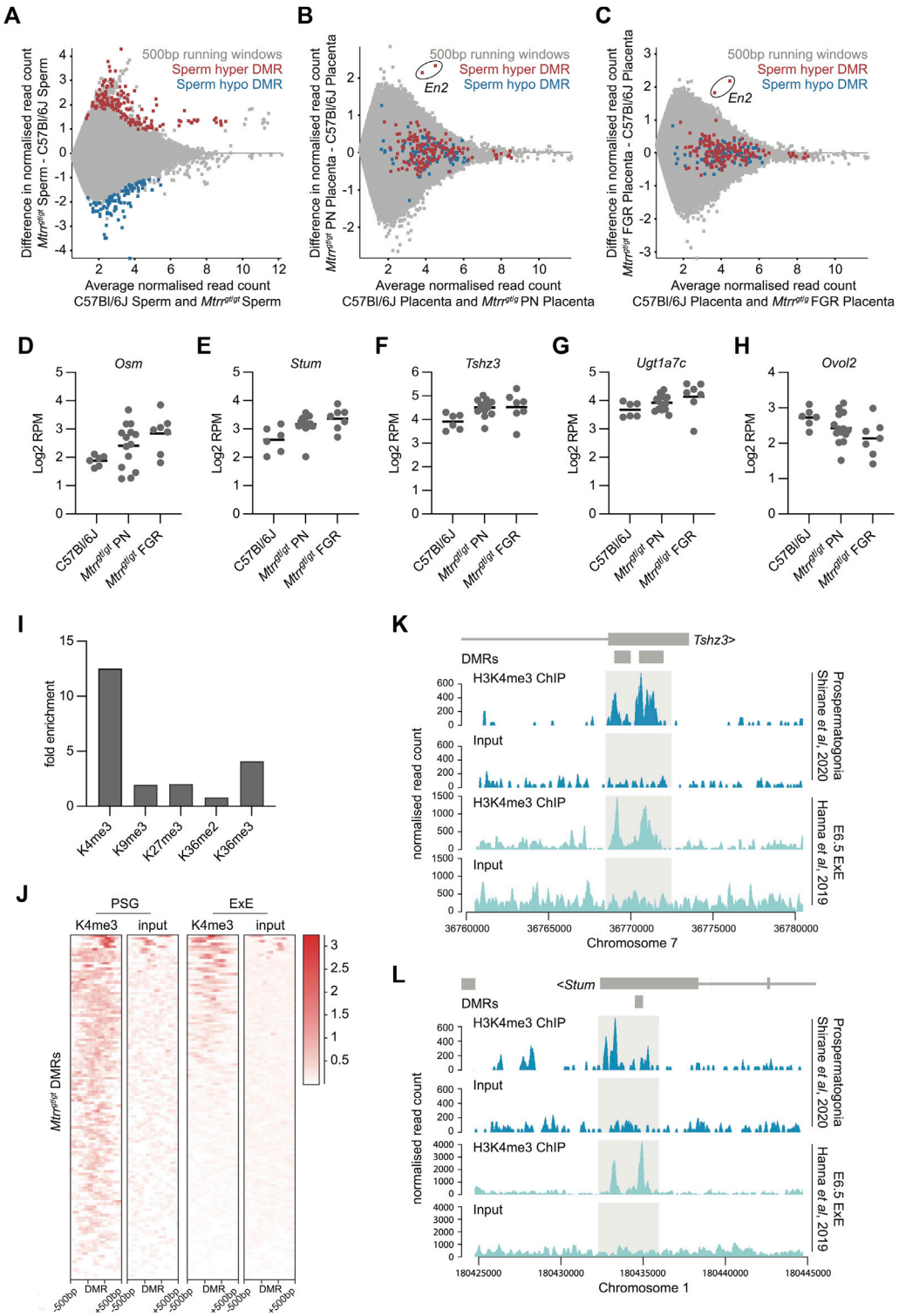

FIGURE 4
Spermatozoa DMRs in *Mtrr*$^{gt/gt}$ males were normalized in *Mtrr*$^{gt/gt}$ placentas yet associated with transcriptional dysregulation. **(A)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in spermatozoa from C57Bl/6J and *Mtrr*$^{gt/gt}$ males. Hypermethylated (red) and hypomethylated (blue) differentially methylated 500 bp regions (DMRs) were identified using EdgeR. **(B)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in placentas from C57Bl/6J and *Mtrr*$^{gt/gt}$ phenotypically normal fetuses at E10.5. The genomic regions where spermatozoa DMRs from *Mtrr*$^{gt/gt}$ males were identified are highlighted on the placenta data. Hypermethylated spermatozoa DMRs (red), hypomethylated spermatozoa DMRs (blue). The *En2* DMRs are indicated. **(C)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in placentas from C57Bl/6J fetuses and *Mtrr*$^{gt/gt}$ fetal growth restricted (FGR) fetuses at E10.5. The genomic regions where spermatozoa DMRs from *Mtrr*$^{gt/gt}$ males were identified are highlighted on the placenta data. Hypermethylated spermatozoa DMRs (red), hypomethylated spermatozoa DMRs (blue). The *En2* DMRs are indicated. **(D–H)** Graphs showing placental transcript expression (log_2RPM) of genes that were associated with spermatozoa DMRs including **(D)** *Osm*, **(E)** *Stum*, **(F)** *Tshz3*, **(G)** *Ugt1a7c*, and **(H)** *Ovol2*. Data was ascertained by RNA-seq of placentas from C57Bl/6J and *Mtrr*$^{gt/gt}$ conceptuses at E10.5. Placentas from phenotypically normal (PN) and fetal growth restricted (FGR) fetuses were assessed. **(I)** Enrichment for specific histone modifications in wildtype prospermatogonia ascertained by ChIP-seq at the 500 bp regions defined as DMRs in spermatozoa of *Mtrr*$^{gt/gt}$ males. Enrichment determined
(*Continued*)

FIGURE 4 (Continued)
relative to the baseline genome. **(J)** Probe alignment plot showing H3K4me3 enrichment ascertained by ChIP-seq from wildtype prospermatogonia and extraembryonic ectoderm (ExE) at E6.5 compared to input controls in regions identified as spermatozoa DMRs (± 500 bp) in $Mtrr^{gt/gt}$ males. **(K,L)** Data tracks showing normalized H3K4me3 ChIP-seq reads and input controls for prospermatogonia (dark blue) and extraembryonic ectoderm (ExE) at E6.5 (light blue) in the regions surround the **(K)** *Tshz3* and **(L)** *Stum* sperm DMRs from $Mtrr^{gt/gt}$ males. Light grey boxes highlight H3K4me3 peaks. Small dark grey boxes indicate the DMRs. See also Supplementary File S3 for data sources. For spermatozoa meDIP-seq: C57Bl/6J, *N* = 8 males; $Mtrr^{gt/gt}$, *N* = 8 males. For placenta meDIP-seq: C57Bl/6J, *N* = 8 placentas; $Mtrr^{gt/gt}$ PN, *N* = 7 placentas; $Mtrr^{gt/gt}$ FGR, *N* = 7 placentas. For RNA-seq: C57Bl/6J, *N* = 6 placentas; $Mtrr^{gt/gt}$ PN, *N* = 14 placentas; $Mtrr^{gt/gt}$ FGR, *N* = 7 placentas.

genomic loci on mouse chromosomes 11 or 18, including in regions that were intragenic (and antisense) to *Camk2b* or were upstream of the gene *Pik3c3*, respectively (Figures 3A, B). Remarkably, a loss of DNA methylation at these DMRs corresponded with ectopic expression of the RLTR4 element in $Mtrr^{gt/gt}$ placentas, independent of the fetal growth phenotype (Figures 3A, B). However, no expression changes in the associated protein-coding genes were observed (Figures 3A, B). Therefore, the genomic regions demarcated by these DMRs appear to require methylation to repress the ERV element activity and not to regulate cis gene expression in the placenta.

Due to their repetitive nature and evolutionary young age, the mapability of short sequencing reads to RLTR4 elements is low. Therefore, to fully appreciated the dysregulation of DNA methylation at RLTR4 elements in $Mtrr^{gt/gt}$ placentas, the placental meDIP-seq and RNA-seq datasets from C57Bl/6J and $Mtrr^{gt/gt}$ placentas at E10.5 were re-mapped to include non-unique reads by using random assignment (bowtie2) for meDIP-seq data and an expectation-maximisation algorithm [SQuIRE (Yang et al., 2019)] for RNA-seq data. When considered globally, the remapped data revealed consistent DNA hypomethylation at the 5′ end of RLTR4 full-length elements in $Mtrr^{gt/gt}$ placentas at E10.5 (Figure 3C). This pattern of DNA hypomethylation was associated with transcript enrichment of global *RLTR4_Mm* and *RLTR4_MM-int* elements in $Mtrr^{gt/gt}$ placentas compared to controls (Figure 3D). Differential expression analysis of individual elements uncovered significant upregulation of twenty-four *RLTR4_Mm* or *RLTR4_MM-int* elements that converged upon 15 full-length loci (Supplementary File S2). None of these methylation changes associated with altered expression of nearby protein-coding genes in $Mtrr^{gt/gt}$ placentas. Furthermore, methylation and transcriptional dysregulation at *RLTR4_Mm* and *RLTR4_Mm-int* elements was unlikely to regulate fetal growth since the RLTR4 elements were similarly affected in $Mtrr^{gt/gt}$ placentas associated with PN and FGR fetuses (Figure 3D). Overall, these data reinforced the hypothesis that epigenetic instability is inherent to the $Mtrr^{gt}$ mouse line (Padmanabhan et al., 2013; Blake et al., 2021) with implications for genetic stability and phenotype establishment beyond FGR.

3.5 Mature germ cell DMRs in $Mtrr^{gt/gt}$ males are reprogrammed in the placenta but correspond to gene misexpression

In our previous study of epigenetic inheritance in the $Mtrr^{gt}$ mouse line (Blake et al., 2021), we found that a small number of candidate DMRs identified in mature spermatozoa (obtained from the cauda epididymis and vas deferens) were not recapitulated in embryos or placentas at E10.5 when interrogated by bisulfite pyrosequencing. Here, we aimed to validate this finding on a genome-wide scale by comparing our spermatozoa (Blake et al., 2021) and placenta meDIP-seq datasets from control and $Mtrr^{gt/gt}$ mice. First, we harmonised DMR calling between datasets by reanalysing the spermatozoa meDIP-seq datasets according to our analysis of the placenta meDIP-seq data. Hypermethylated spermatozoa DMRs in $Mtrr^{gt/gt}$ males that were previously identified in very highly methylated regions in control spermatozoa and described as false positives (Blake et al., 2021), were also clearly identifiable by the current analysis. Accordingly, we screened out these DMRs using whole genome bisulphite sequencing data to quantify absolute methylation levels across all DMRs (Sun et al., 2018). Similar to our candidate-based approach (Blake et al., 2021), DNA methylation patterns in nearly all genomic regions identified as spermatozoa DMRs in $Mtrr^{gt/gt}$ males were normal in $Mtrr^{gt/gt}$ placentas at E10.5 compared to control placentas (Figures 4A–C). This finding occurred regardless of the $Mtrr^{gt/gt}$ fetal growth phenotype. The only exception was the common hypermethylated *En2* DMR that appeared in both spermatozoa and placentas (Figures 4B, C). Conversely, the placental DMRs that overlap with RLTR4 elements were normally methylated in sperm of $Mtrr^{gt/gt}$ males (relative to control spermatozoa) suggesting that germline transposon silencing is maintained and unlikely to play a key role in epigenetic inheritance mechanisms in the $Mtrr^{gt}$ mouse line. Overall, these results indicated that placenta and spermatozoa DMRs in $Mtrr^{gt/gt}$ mice were tissue-specific and that most of the spermatozoa DMRs were effectively reprogrammed in the pre-implantation embryo or during placental development, notwithstanding the shared $Mtrr^{gt/gt}$ genotype of the parental and offspring generations. These data might negate DNA methylation as a mechanistic factor in epigenetic inheritance within the $Mtrr^{gt}$ mouse line. Instead, altered heritability of other epigenetic factors, such as histone modifications or small non-coding RNA content in germ cells, might be an alternative or additional mechanism.

Previously, our locus-specific analysis showed that some genes associated with spermatozoa DMRs were misexpressed in somatic tissues despite reprogramming of DNA methylation at these sites (Blake et al., 2021). Therefore, we questioned the extent to which this association occurred in the wider placental genome. Using DESeq, the $Mtrr^{gt/gt}$ placental RNA-seq dataset was assessed for differentially expressed genes that were within 2 kb of a sperm DMR from $Mtrr^{gt/gt}$ males and had transcript levels with a $log_2FC > 0.6$ compared to control placentas. Five misexpressed genes (i.e., *Stum* (mechanosensory transducer mediator; membrane protein), *Tshz3* (teashirt zinc finger family member 3; transcription factor), *Ovol2* (ovo like zinc finger 2; transcription factor), *Osm* (oncostatin m;

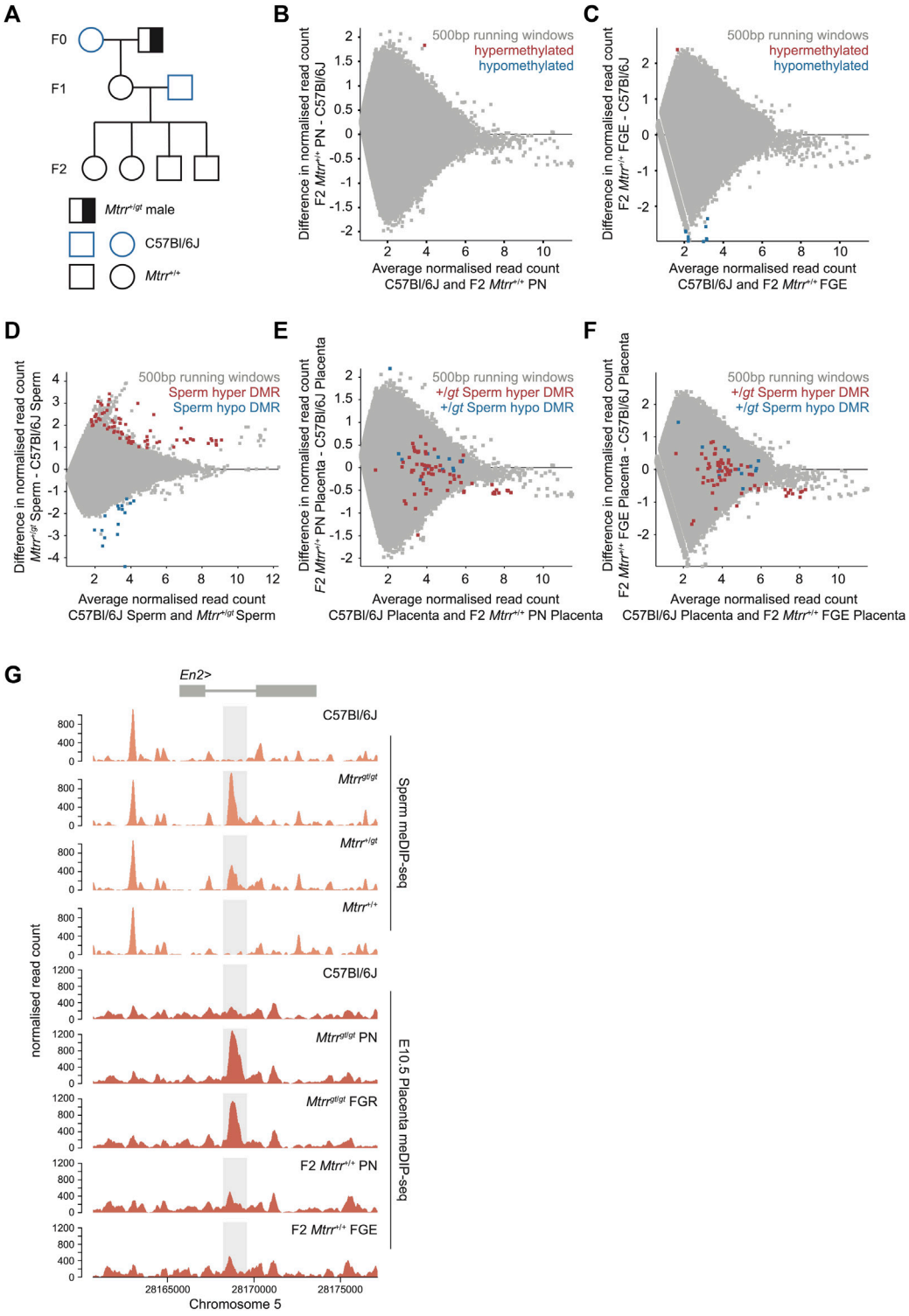

FIGURE 5
Spermatozoa DNA methylation patterns are not transgenerationally inherited in the *Mtrr*^{gt} mouse line. **(A)** *Mtrr*$^{+/gt}$ maternal grandfather pedigree used in this study. Squares, males; circles females; blue outline, C57Bl/6J mouse line; black outline, *Mtrr*^{gt} mouse line; white fill, *Mtrr*$^{+/+}$; half black/half white fill; *Mtrr*$^{+/gt}$. F0, parental generation; F1, first filial generation; F2, second filial generation. **(B)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in placentas at E10.5 from C57Bl/6J conceptuses and F2 *Mtrr*$^{+/+}$ conceptuses derived from F0 *Mtrr*$^{+/gt}$ maternal grandfathers. Placentas from phenotypically normal (PN) fetuses were assessed. Hypermethylated (red) and hypomethylated (blue) differentially methylated regions (DMRs) were determined relative to control placentas using EdgeR. **(C)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in placentas at E10.5 from C57Bl/6J conceptuses and F2 *Mtrr*$^{+/+}$ fetal growth enhanced (FGE) conceptuses derived from F0 *Mtrr*$^{+/gt}$ maternal grandfathers. Hypermethylated (red) and hypomethylated (blue) DMRs were determined using EdgeR. **(D)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in spermatozoa from C57Bl/6J and F0 *Mtrr*$^{+/gt}$ males. Hypermethylated DMRs (red), hypomethylated DMRs (blue). **(E)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in placentas from C57Bl/6J and F2 *Mtrr*$^{+/+}$ PN fetuses at E10.5.
(*Continued*)

FIGURE 5 (Continued)
The genomic regions where spermatozoa DMRs from F0 *Mtrr*$^{+/gt}$ males were identified are highlighted on the placenta data. Hypermethylated spermatozoa DMRs (red), hypomethylated spermatozoa DMRs (blue). **(F)** MA plot of log_2 normalized meDIP-seq read counts of 500 bp contiguous regions in placentas at E10.5 from C57Bl/6J fetuses and F2 *Mtrr*$^{+/+}$ FGE fetuses. The genomic regions where spermatozoa DMRs from F0 *Mtrr*$^{+/gt}$ males were identified are highlighted on the placenta data. Hypermethylated sperm spermatozoa m DMRs (red), hypomethylated spermatozoa DMRs (blue). **(G)** Data tracks across the *En2* gene showing normalized meDIP-seq read counts in spermatozoa (orange) from C57BL/6J males and *Mtrr*$^{gt/gt}$, *Mtrr*$^{+/gt}$ and *Mtrr*$^{+/+}$ males together with meDIP read counts in placentas at E10.5 (red) associated with C57Bl/6J fetuses, *Mtrr*$^{gt/gt}$ PN and FGR fetuses, and F2 *Mtrr*$^{+/+}$ PN and FGE fetuses. The *En2* DMR is highlighted in light grey. In all cases data was normalized to the largest data store. For spermatozoa meDIP-seq: C57Bl/6J, *N* = 8 males, F0 *Mtrr*$^{+/gt}$, *N* = 8 males. For placenta meDIP-seq: C57Bl/6J, *N* = 8 placentas; F2 *Mtrr*$^{+/+}$ PN, *N* = 8 placentas; F2 *Mtrr*$^{+/+}$ FGE, *N* = 3 placentas.

cytokine), and *Ugt1a7c* (UDP glucouronosyltransferase 1 family, polypeptide A7C; enzyme in glucouronidation pathway) met these criteria but only in *Mtrr*$^{gt/gt}$ placentas with FGR (Figures 4D–H). The occurrence of transcriptional disruption despite normal DNA methylation reinforced our hypothesis that abnormal one-carbon metabolism influences other epigenetic mechanisms.

Next, we explored whether histone modifications were present in the developing germline at regions demarcated by spermatozoa DMRs to better understand the broader epigenetic context of these regions. To do this, ChIP-seq datasets were analysed for histone mark enrichment in developing wildtype male germ cells (i.e., prospermatogonia) (Shirane et al., 2020) at specific genomic regions defined by spermatozoa DMRs from *Mtrr*$^{gt/gt}$ males. First, we found that 103 out of 252 DMRs (40.9%) overlapped with an H3K4me3 peak in wildtype prospermatogonia, the majority which were located within gene bodies (Supplementary Table S1). This value represented a 12-fold enrichment compared to the baseline genome (i.e., only 3.3% of 500 bp regions across the whole genome overlapped with H3K4me3 peaks) and was substantially more enriched than the other histone modifications at the same locations (Figure 4I). Since H3K4me3 is typically associated with active transcription (Howe et al., 2017), it was an ideal candidate to further explore as an underlying inherited epigenetic mark associated with transcriptional disruption in the placenta. Therefore, we assessed whether wildtype trophoblast progenitor cells at E6.5 (i.e., extraembryonic ectoderm) displayed H3K4me3 enrichment at genomic locations identified as spermatozoa DMRs using a published ChIP-seq dataset (Hanna et al., 2019). Indeed, a substantial subset of these genomic regions was also enriched for H3K4me3 in extraembryonic ectoderm (Figure 4J). Remarkably, four out of five dysregulated genes in *Mtrr*$^{gt/gt}$ placentas that were associated with a spermatozoa DMR in *Mtrr*$^{gt/gt}$ males (i.e., *Stum*, *Tshz3*, *Ovol2*, *Ugta7c*) were among those enriched for H3K4me3 in both prospermatogonia and extraembryonic ectoderm (Figure 4K–L; Supplementary Figure S2). We infer from these data that the *Mtrr*^{gt} allele potentially disrupts histone marks, such as H3K4me3, in developing and/or mature germ cells leading to altered patterns of the same histone mark in the early conceptus with implications for gene regulation. Indeed, we found that 59.0% of H3K4me3 peaks identified in extraembryonic ectoderm were also found in prospermatogonia (using MACS peak calling function embedded in SeqMonk software). This finding more broadly supports a role for H3K4me3 in inheritance of epimutations from germ cells to the placenta. Future mechanistic experiments should focus on multigenerational patterns of H3K4me3 in the *Mtrr*^{gt} mouse line.

3.6 Mature male germ cell DMRs in *Mtrr*$^{+/gt}$ mice are not multigenerationally inherited

Our previous locus-specific analyses in F2 *Mtrr*$^{+/+}$ placentas indicated significant alteration of DNA methylation patterns caused by either a maternal grandfather or maternal grandmother *Mtrr*^{gt} allele (Padmanabhan et al., 2013). Here, a transgenerational mechanism was explored in the *Mtrr*$^{+gt}$ maternal grandfather pedigree (Figure 5A) using a genome-wide approach to identify the locations of spermatozoa DMRs from F0 *Mtrr*$^{+/gt}$ males (Blake et al., 2021) and determine whether the placental methylome and transcriptome was altered in these regions two generations later in the F2 *Mtrr*$^{+/+}$ grandprogeny. The following matings were performed to generate this pedigree (Figure 5A): F0 *Mtrr*$^{+/gt}$ males were mated with C57Bl/6J control females, and the resulting F1 *Mtrr*$^{+/+}$ females were selected for mating with C57Bl/6J males to generate F2 *Mtrr*$^{+/+}$ conceptuses. F2 *Mtrr*$^{+/+}$ conceptuses were rigorously phenotyped at E10.5, and the placentas from PN and FGE fetuses were examined. First, the broader methylome of whole F2 *Mtrr*$^{+/+}$ placentas was assessed via meDIP-seq. When compared with C57Bl/6J controls, there were no significant differences in the distribution of meDIP reads across genomic features (Supplementary Figure S1C) and no clustering of biological replicates according to phenotype or pedigree (Supplementary Figure S1D) indicating similar global methylation among experimental groups. Few DMRs were identified by the meDIP-seq analysis including one hypermethylated DMR in F2 *Mtrr*$^{+/+}$ placentas from PN fetuses (Figure 5B) and 11 DMRs (10 hypomethylated, 1 hypermethylated) in FGE-associated F2 *Mtrr*$^{+/+}$ placentas (Figure 5C). Closer analysis revealed that 9 out of 10 of the hypomethylated DMRs from F2 *Mtrr*$^{+/+}$ FGE placentas were clustered in two locations on chromosomes 14 and 17, which are frequently susceptible to mapping artefacts in our datasets and so were excluded. The remaining three placental DMRs from F2 *Mtrr*$^{+/+}$ placentas were in nondescript genomic regions (Supplementary File S1). Importantly, the RLTR4 elements identified in *Mtrr*$^{gt/gt}$ placentas (Figure 3) exhibited normal levels of DNA methylation and transcript expression in the F2 *Mtrr*$^{+/+}$ placentas relative to control placentas (Supplementary Figure S3). This result suggested that the changes in DNA methylation described in *Mtrr*$^{gt/gt}$ placentas are intrinsically associated with the *Mtrr*^{gt} allele and are unlikely to be transgenerationally inherited or caused by genetic differences between the C57Bl/6J control and *Mtrr*^{gt} mouse lines.

When the meDIP-seq datasets from spermatozoa of F0 *Mtrr*$^{+/gt}$ males (Figure 5D) (Blake et al., 2021) were compared to placentas of F2 *Mtrr*$^{+/+}$ conceptuses (PN and FGE) at E10.5, there was no DMR overlap (Figures 5E, F). This finding reinforces our hypothesis that specific DMRs in the *Mtrr*^{gt} mouse line are not inherited from germline to somatic cells over multiple generations. This was even the case at the *En2* DMR, which was present in spermatozoa from F0 *Mtrr*$^{+/gt}$

males and not in F2 *Mtrr*$^{+/+}$ placentas (Figure 5G). In this context, the spermatozoa and placenta methylome data revealed that the dosage of the *Mtrr*^{gt} allele in mice correlated with the degree of hypermethylation at the *En2* DMR (Figure 5G). Therefore, the *En2* locus was particularly responsive to *Mtrr*-driven disruption of one-carbon metabolism. Altogether, these data further separate the transmission of specific differential methylation patterns via the germline from fetal growth phenotype inheritance in the *Mtrr*^{gt} mouse line.

4 Discussion

Despite its well-studied role in development and disease, the molecular function of one-carbon metabolism is complex and not well understood. Here, we used the *Mtrr*^{gt} mouse line to explore the epigenetic role of one-carbon metabolism by assessing the placental methylome in association with fetal growth phenotypes. In doing so, we identified several genomic regions in *Mtrr*$^{gt/gt}$ placentas with altered DNA methylation including in a gene promoter that conceivably regulates *Cxcl1* gene expression in a canonical manner, in a presumptive developmental regulatory region located within the *En2* gene, and in a subset of RLTR4 transposable elements. While unlikely to underlie the fetal growth phenotypes, it is possible that these DNA methylation changes are functionally relevant in other tissue types and/or for driving other phenotypes. For instance, CXC chemokine expression from peripheral blood mononuclear cells correlates with folate and homocysteine levels in human subjects (Holven et al., 2002). Alternatively, knocking out the mouse gene *Mtfhr* to disrupt folate metabolism causes cerebellar patterning defects that are associated with downregulation of *En2* gene expression (Chen et al., 2005). Ultimately, our findings support widespread epigenetic instability in the *Mtrr*^{gt} mouse line.

Our previous locus-specific analyses indicated that *Mtrr*$^{gt/gt}$ placentas or wildtype placentas exposed to a maternal grandparental *Mtrr*^{gt} allele are epigenetically unstable (Padmanabhan et al., 2013; Bertozzi et al., 2021; Blake et al., 2021). Yet, we identified fewer placenta DMRs by meDIP-seq than were expected despite using standard analysis parameters that yielded many spermatozoa DMRs in *Mtrr*$^{gt/gt}$ mice (e.g., 13 placenta DMRs vs. 252 spermatozoa DMRs). It is possible that placental DNA methylation is less sensitive than male germ cells to impaired one-carbon metabolism. DNA in trophoblast cells is globally hypomethylated compared to other cell types (Senner et al., 2012) and changes in DNA methylation might be less striking in this context. The use of whole placentas that contain multiple cell types (e.g., trophoblast cell subtypes, fetal vascular endothelium, and maternal decidua and immune cells) with their own DNA methylation and transcriptional signatures (Vento-Tormo et al., 2018; Andrews et al., 2023) might confound our analysis to some extent. The placental DMRs that we identified are likely present throughout the tissue, while other undetected DMRs may be confined to a single cell type and not appreciated in our analysis. Assaying the placenta at earlier developmental time points when the trophoblast progenitor population is more homogeneous may be informative. Alternatively, single cell-based sequencing methods may uncover additional DMRs in *Mtrr*$^{gt/gt}$ placentas at E10.5 that correlate with cell-type specific transcriptional dysregulation and phenotypes.

Transposable elements, which make up ~40% of the mammalian genome (Goodier and Kazazian, 2008), are heavily methylated to suppress transposition causing deleterious mutation. In this study, we observed hypomethylation and ectopic expression of several RLTR4 elements in *Mtrr*$^{gt/gt}$ placentas, which might have profound consequences to genomic stability during development. While whole genome sequencing revealed that *de novo* mutation rates are similar in control and *Mtrr*$^{gt/gt}$ mice (Blake et al., 2021), it is still possible that increased transposition might occur in this context, generating structural variation with implications for phenotype inheritance. Since spermatozoa from *Mtrr*$^{gt/gt}$ males showed normal RLTR4 DNA methylation, we propose that DNA methylation was poorly maintained in early embryogenesis or in placenta development to cause hypomethylation at these sites. Although RLTR4 elements were the only transposons identified by meDIP-seq in this study, DNA methylation patterns of variably methylated intracisternal A particle (VM-IAP) retrotransposons are also considerably shifted in *Mtrr*$^{gt/gt}$ mice as determined by bisulfite pyrosequencing (Bertozzi et al., 2021). KRAB-ZPFs are known to regulate VM-IAPs (Bertozzi et al., 2020), and mechanistically, the *Mtrr*^{gt} locus contains a cluster of 129P2Ola/Hsd-derived KRAB zinc finger proteins (ZFPs) in an otherwise C57Bl/6J background because of the mutagenesis process (Bertozzi et al., 2021). However, the KRAB-ZFP clusters within the *Mtrr* locus do not appear to regulate RLTR4 expression (Wolf et al., 2020). Others have shown that paternal *Mthfr* deficiency in mice causes hypomethylation of L1Md subfamily of LINE-1 retrotransposons (Karahan et al., 2021). Altogether, these data highlight the importance of one-carbon metabolism in maintaining epigenetic stability at early developmental stages when deleterious transposition events could have profound consequences.

While the mechanistic understanding of epigenetic inheritance remains in its infancy, several candidate epigenetic factors have been identified (e.g., chromatin modifications, small non-coding RNA content in germ cells) (Blake and Watson, 2016; Hanna et al., 2019). Our data provides genome-wide evidence that nearly all spermatozoa DMRs caused by the *Mtrr*^{gt} allele were epigenetically reprogrammed in the placenta and were not transgenerationally inherited. This contrasts with another study that demonstrates transgenerational inheritance of directed epimutations of DNA methylation in mouse obesity genes along with an obesity phenotype, despite evidence that these epimutations are reprogrammed in primordial germ cells (Takahashi et al., 2023). The lack of DMR inheritance in the *Mtrr*^{gt} mouse line suggests that there might be paradigm-specific effects. However, there are clues that spermatozoa DMRs caused by an *Mtrr*^{gt} allele might still play a role in epigenetic inheritance since they are associated locus-specific disruption of transcription in *Mtrr*$^{gt/gt}$ placentas (this study) and in F2 *Mtrr*$^{+/+}$ embryos and adult livers (Blake et al., 2021) despite being reprogrammed to normal tissue-specific methylation levels. This association evokes a role for other epigenetic mechanisms aside from DNA methylation in epigenetic inheritance mechanisms. We observed enrichment for the activating H3K4me3 histone mark in developing wildtype male germ cells and trophoblast specifically at genomic locations defined by spermatozoa DMRs in *Mtrr*$^{gt/gt}$ mice

including loci associated with *Mtrr*$^{gt/gt}$ placental gene misexpression. Others have shown a similar association in a mouse model of paternal *Mthfr* deficiency (Karahan et al., 2021).

We propose a model whereby impaired one-carbon metabolism alters H3K4me3 deposition in developing male germ cells, which then drives the changes in DNA methylation through modified access of DNA methyltransferases. In the pre-implantation embryo when DNA methylation is reprogrammed, a subset of abnormal H3K4me3 marks may persist, driving further changes in establishing aberrant *de novo* DNA methylation patterns or in gene expression in early cell lineages of the placenta and/or embryo. The most drastic epigenetic changes likely lead to altered lineage decisions and developmental phenotypes. Since a wide spectrum of phenotypes are observed in *Mtrr*^{gt} mouse line, disrupting one-carbon metabolism might cause stochastic epigenetic changes across the genome, affecting different cell types in different individuals. The most striking data that reinforces a potential role for histone H3K4me in epigenetic inheritance comes from a study whereby wildtype mice were fed a folate-deficient diet. Mature spermatozoa from folate-deficient males displayed alterations in histone H3K4me3 patterns specifically at developmental genes and putative enhancers, a subset of which were retained in the F1 8-cell embryos and were associated with gene misexpression (Lismer et al., 2020). We did not observe any discernable changes in H3K4me3 enrichment in spermatozoa or 8-cell embryos derived from folate-deficient males specifically within the genomic regions identified as spermatozoa DMRs from *Mtrr*$^{gt/gt}$ males. This may be due to the differences in the mouse models employed, with our genetic approach causing a more severe metabolic effect than dietary deficiency. Regardless, this finding suggests that genomic hotspots regulated by one-carbon metabolism are unlikely and that the epigenome is differently or stochastically affected in these models. To fully understand the mechanisms involved in epigenetic inheritance, histone methylation should be explored as an inherited epigenetic mechanism in the *Mtrr*^{gt} mouse model.

Overall, this study together with our previously published work (Padmanabhan et al., 2013; Bertozzi et al., 2021; Blake et al., 2021) indicate that one-carbon metabolism is required for the maintenance of epigenetic stability in the placenta and the germline. The widespread effect of disrupting one-carbon metabolism on the epigenome provides some explanation towards the complex molecular role of folate metabolism during development. Instability of the epigenome can alter transcriptional pathways and genomic stability, with substantial downstream effects on developmental outcome. Single-cell sequencing technology and a broader analysis of epigenetic mechanisms (e.g., histone marks and chromatin structure) together with DNA methylation will enable the identification of complex epigenome-phenotype relationships that persist over multiple generations in context of the *Mtrr*^{gt} mouse line.

Data availability statement

The original data presented in the study are deposited in the GEO repository, accession number GSE233482. The published datasets presented in this study can be found in online repositories. The names of the repository and the accession numbers can be found in Supplementary File S3.

Ethics statement

The animal study was reviewed and approved by University of Cambridge Animal Welfare and Ethical Review Body.

Author contributions

CS and EW conceptualised the study and designed the experiments. EW performed the dissections and phenotyped the conceptuses. CS and ZD generated the placenta meDIP libraries. MP, CS, and MB designed and performed the bioinformatics analyses. CS, MB, and EW collected and analysed the data, and interpreted the results. EW and CS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1209928/full#supplementary-material

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The influence of early environment and micronutrient availability on developmental epigenetic programming: lessons from the placenta

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DNA methylation is the most commonly studied epigenetic mark in humans, as it is well recognised as a stable, heritable mark that can affect genome function and influence gene expression. Somatic DNA methylation patterns that can persist throughout life are established shortly after fertilisation when the majority of epigenetic marks, including DNA methylation, are erased from the pre-implantation embryo. Therefore, the period around conception is potentially critical for influencing DNA methylation, including methylation at imprinted alleles and metastable epialleles (MEs), loci where methylation varies between individuals but is correlated across tissues. Exposures before and during conception can affect pregnancy outcomes and health throughout life. Retrospective studies of the survivors of famines, such as those exposed to the Dutch Hunger Winter of 1944-45, have linked exposures around conception to later disease outcomes, some of which correlate with DNA methylation changes at certain genes. Animal models have shown more directly that DNA methylation can be affected by dietary supplements that act as cofactors in one-carbon metabolism, and in humans, methylation at birth has been associated with peri-conceptional micronutrient supplementation. However, directly showing a role of micronutrients in shaping the epigenome has proven difficult. Recently, the placenta, a tissue with a unique hypomethylated methylome, has been shown to possess great inter-individual variability, which we highlight as a promising target tissue for studying MEs and mixed environmental exposures. The placenta has a critical role shaping the health of the fetus. Placenta-associated pregnancy complications, such as preeclampsia and intrauterine growth restriction, are all associated with aberrant patterns of DNA methylation and expression which are only now being linked to disease risk later in life.

KEYWORDS

epigentics, DNA methylation, placenta, imprinting, metastable epialles, DOHaD

1 Introduction

According to the Developmental Origins of Health and Disease hypothesis, environmental exposures in early life affects later life risk. Part of this connection may come through DNA methylation, patterns of which are known to change under different conditions. The placenta is important for mediating the connection between mother and fetus, both able to respond to the environment itself and controlling the environment of the fetus. Many pregnancy complications are linked to placenta function and birth outcomes can have a large effect on later disease risk. This review will summarise current knowledge on the effect of early environmental exposure on later disease risk, especially where this may be mediated by DNA methylation. We will highlight the unique nature of the placenta epigenome and its potential as a connection between environment and health.

1.1 Epigenetic processes in regulating transcription

Epigenetic marks are heritable DNA modifications that can influence gene expression without changing the DNA sequence. These include chemical modifications of DNA bases, post-translational histone modifications and chromatin structure, and their configuration can be affected by a variety of environmental exposures.

1.2 Histone modifications

Histones wrapped with DNA form the nucleosome, that can alter gene accessibility by forming transcriptionally inactive heterochromatin or transcriptionally active euchromatin (Li et al., 2007). Expression can be controlled by reversible post-translational modifications on histone amino acid tails, with complex cross-talk between modifications (Kouzarides, 2007). For example, lysine 9 or 27 acetylation on histone 3 (H3K9ac or H3K27ac) weakens DNA-histone interactions, and so opens chromatin to facilitate transcription, whereas trimethylation of the same lysines is associated with heterochromatin formation (Quina et al., 2006).

1.3 Cytosine methylation in the human genome

5-methylcytosine (5 mC) is well established as a stable, heritable DNA methylation mark that affects gene expression and genome stability. It is created by the addition of a methyl group to the 5-carbon atom of the cytosine ring (Moore et al., 2013). This modification is important for many processes, including tissue-specific gene regulation, imprinting and X-chromosome inactivation, working in combination with histone modifications (Mikkelsen et al., 2007; Moore et al., 2013). In promoters and intergenic regions, it is generally associated with transcriptional silencing through recruiting gene suppressor proteins, promoting heterochromatin and disrupting transcription factor binding (McMahon et al., 2017; Kaluscha et al., 2022). In bodies of highly expressed genes, high levels of 5 mC stop promiscuous transcription initiation (Neri et al., 2017).

Most 5 mC is found on CpG dinucleotides (a cytosine base next to a guanine), up to 80% of which are methylated in mammalian genomes (Lister et al., 2009). These CpG dinucleotides are often clustered into CpG islands, which are associated with 70% of known gene promoters, where methylation can silence gene expression (Mohn et al., 2008; Illingworth et al., 2010). They are also enriched in repetitive elements, satellite DNA and transposable elements to help maintain genome stability (Vera et al., 2008; Lister et al., 2009; Si et al., 2009).

In humans, 5 mC is created and maintained by DNA methyltransferases (DNMTs) using S-adenyl methionine (SAM) as a methyl donor. DNMTs include: DNMT3a and 3b for *de novo* methylation in embryos and germ cells, with the non-catalytic DNMT3L as a cofactor (Bourc'his and Bestor, 2004; Kaneda et al., 2004), and DNMT1 for maintenance during DNA replication and repair (Hermann et al., 2004; Mortusewicz et al., 2005). Methylation can be removed passively through replication without methylation maintenance, or actively, through several intermediates catalysed by Ten-Eleven-Translocation (TET) proteins (Hackett et al., 2013). One of these intermediates, 5-hydroxymethylcytosine (5hmC), has been proposed to also play a regulatory role as its distribution shows strand and sequence bias. 5hmC is enriched in regulatory elements and promoters in embryonic stem cells and occasionally in placenta (Hernandez Mora et al., 2018), as well as in actively transcribed genes in neuronal cells (Mellén et al., 2012; Yu et al., 2012).

1.4 Non-cytosine methylation

Another suggested regulatory modification is methylation to the adenine base (m6dA), which is a known epigenetic mark in bacteria, protists (Wion and Casadesús, 2006), and eukaryotes such as *C. elegans* (Greer et al., 2015). In humans, m6dA is a common RNA modification but is also detected on DNA, where it has been associated with actively transcribed genes and risk of tumorigenesis (Xiao et al., 2018). However, suggestions for a human DNA adenine methylase or demethylase have failed to be replicated (Xie et al., 2018; Musheev et al., 2020) and other studies suggest DNA m6dA in humans arises purely through nucleotide salvage from RNA degradation (Liu et al., 2020; Musheev et al., 2020).

1.5 Methyl donors: the one-carbon metabolism pathway

The synthesis of both post-translational protein methylation, including histones, and DNA methylation is dependent on one-carbon metabolism, along with many other cellular processes (Ducker and Rabinowitz, 2017). The methyl group is donated from S-adenosylmethionine (SAM), leaving S-adenosyl homocysteine (SAH) (Figure 1). Many components of this pathway must come from the diet, both methyl donors, including folate, choline and betaine, and cofactors, including vitamins B-2, B-6, and B-12 (James et al., 2018). Studies in animal models

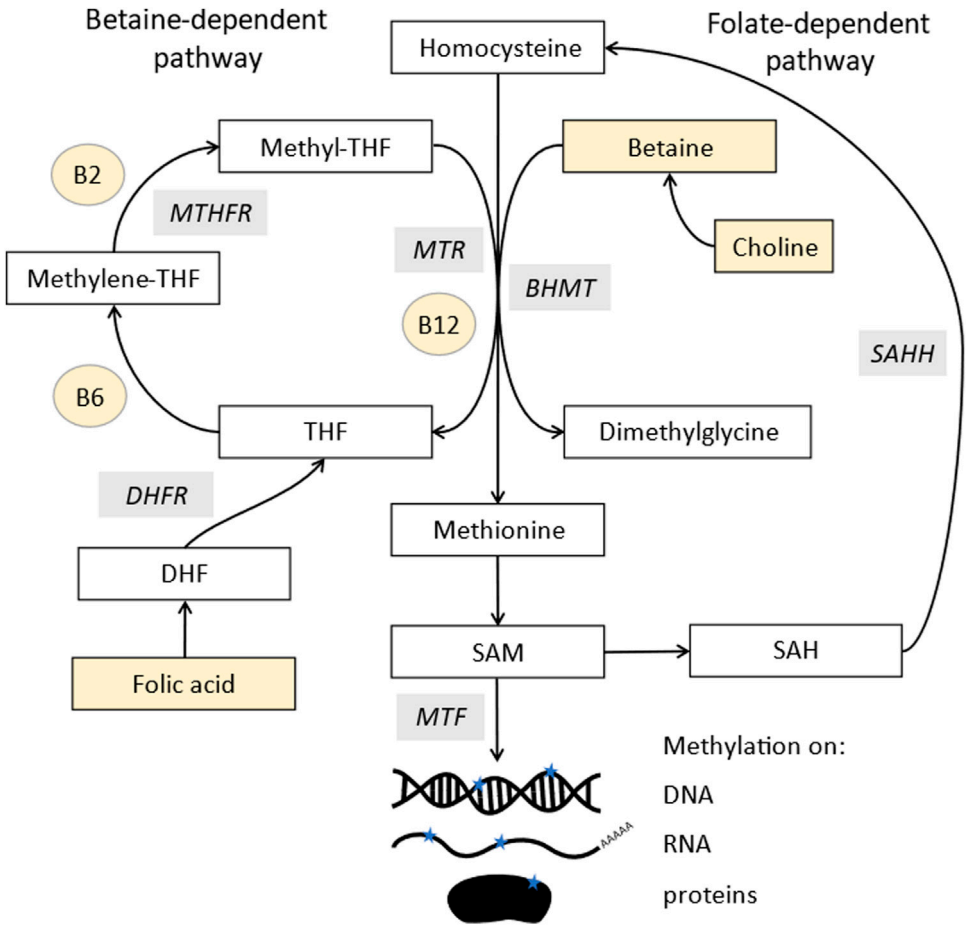

FIGURE 1
Methylation pathway. Homocysteine (top middle) is converted into methionine by two pathways: methionine synthase (MTR), which uses vitamin B12 as a cofactor and acquires a methyl group from the conversion of 5-methyltetrahydrofolate (methyl-THF) into tetrahydrofolate (THF). MethyL-THF is acquired from dietary folates which is converted from dihydrofolate (DHF) by dihydrofolate reductase (DHFR) and 5,10- methylenetetrahydrofolate (5,10MTHF) by methylene tetrahydrofolate reductase (MTHFR). Methionine can also acquire a methyl group from betaine via a reaction involving betaine homocysteine methyl-transferases (BHMT). Methionine is further converted to s-adenosylmethionine (SAM), the major methyl donor for all methyl transferases (MTF), which add methyl groups to DNA, RNA, lipids, and proteins. SAM is recycled via s-adenosylhomocysteine (SAH) which is converted to homocysteine in a reversible reaction by s-adenosylhomocysteine hydrolase (SAHH). Components highlighted in yellow are derived from the diet.

demonstrate that supplementing these nutrients can alter DNA methylation patterns (Waterland and Jirtle, 2003; Sinclair et al., 2007).

SAM is derived from methionine, which comes from methylated homocysteine. There are two enzymes that can make methionine, most commonly methionine synthase, which requires vitamin B12 as a cofactor. It uses 5-methyltetrahydrofolate as a methyl donor, whose synthesis is folate dependent and also links into purine biosynthesis. The second enzyme, betaine-homocysteine methyltransferases (BHMT), has two isoforms, of which one is expressed only in the liver and kidneys, with the other (BHMT2) expressed more widely (Pajares and Pérez-Sala, 2006). This uses betaine as a methyl donor, which comes either from choline or direct dietary supplementation.

The donation of the methyl group from SAM is catalysed by methyl transferases, which are inhibited by SAH. Therefore, methylation stops if the process of converting SAH back to homocysteine and methionine and SAM is inhibited. Ways to measure methylation potential therefore include the SAM:SAH ratio as well as levels of the pathway components (Mason, 2003).

1.6 Methylation life cycle

DNA methylation can change dynamically throughout life but there are many loci where it remains consistent. For these loci, there are two major reprogramming events to consider. The first is in the primordial germ cells that give rise to the sperm and oocyte, where DNA is passively demethylated during initial formation of the germ cells to remove any previous marks (Hackett et al., 2013; Guo et al., 2015). The DNA can then be remethylated to establish parent-of-

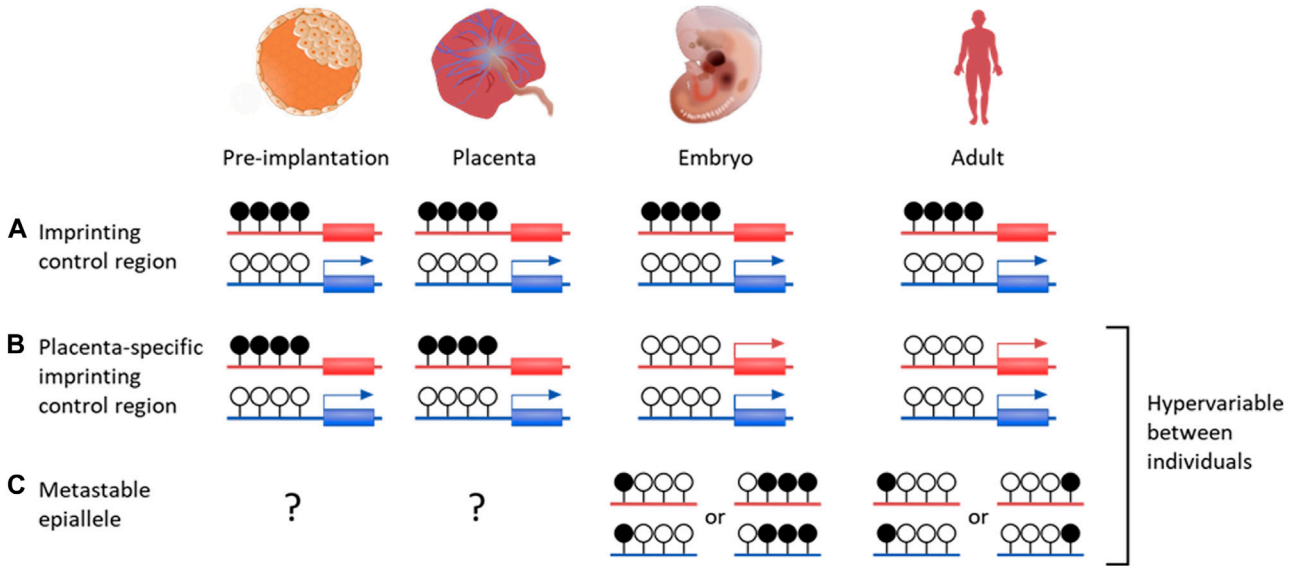

FIGURE 2
DNA methylation profiles of various genetic features that are important for placenta development and growth. Canonical genomic imprinting is associated with regions of allelic methylation inherited from one gamete. **(A)** Oocyte-derived methylation is faithfully maintained throughout development on the maternal allele, resulting in paternal expression. **(B)** An example of a germline-derived, placenta-specific imprint. During pre-implantation development these regions are indistinguishable from canonical imprinted DMRs. Upon implantation, embryonic tissues become demethylated through to adulthood, while allelic methylation is maintained solely in the placenta. **(C)** While establishment of metastable epialleles during early development is currently unknown, these intervals are particularly vulnerable to environmental influences. Once systematically methylated, they are stable over time, despite showing inter-individual variability. Blue genes are paternally expression, red genes are maternally expressed. Black filled lollipops are methylated CpG intervals, while unfilled are unmethylated.

origin (PoO) specific methylation, which according to studies in mice occurs before birth in sperm but not until maturation for oocytes (Hiura et al., 2006; Guo et al., 2014; 2015; Gahurova et al., 2017).

The next major reprogramming stage occurs after fertilisation, where a wave of demethylation before the blastocyst stage results in the lowest developmental level of genome methylation (Weaver et al., 2009). Parental DNA from sperm begins almost entirely methylated, except in CpG islands, but at this stage most of its methylation is actively removed (Guo et al., 2014; Shen et al., 2014; Smith et al., 2014). In contrast, maternal, oocyte-derived DNA is methylated mostly at active gene bodies and maintains more methylation (Smallwood et al., 2011). It is demethylated passively by dilution through replication (Hirasawa et al., 2008; Shen et al., 2014).

Differential maintenance of methylation at this stage has the potential to create changes in gene expression. This can create metastable epialleles, loci with variable methylation between, but not within, individuals. Regions that maintain PoO specific methylation through this process create germline differentially methylated regions (gDMRs), with the potential to be imprinted.

Where differential methylation is maintained, alleles are protected against demethylation by ZFP57 and ZFP445, which targets DNMT1 (Li et al., 2008; Quenneville et al., 2011; Takahashi et al., 2019). The opposite alleles are kept unmethylated by many different factors, either acting together or on different genes, including CFP1, SP1 or CTCF (CCTC-binding factor) (Macleod et al., 1994; Fedoriw et al., 2004).

1.7 Imprinting

Imprinted genes are found in therian (eutherian and marsupial) mammals and are defined by differential expression between maternal and paternal alleles, which can be specific to developmental stage, tissue or isoform (Renfree et al., 2013). Incorrect imprinting can have long term effects, such as imprinting diseases (Peters, 2014; Monk et al., 2019). Imprinting is generally a result of allele-specific repression from gDMRs, with most imprinted genes found within regions containing an imprinting control region (ICR) (Figure 2) (Spahn and Barlow, 2003; Schulz et al., 2010). As well as directly controlling expression, imprinted DMRs are often associated with repressive histone modifications, such as H3K9me3 on methylated alleles (Monk et al., 2006; Court et al., 2014).

There are over 120 confirmed imprinted genes in humans, of which most are associated with maternally methylated regions (Okae et al., 2014; Humanimprints.net; Geneimprint, 2023). Some gDMRs maintain allelic methylation throughout life but the majority are transient, surviving the pre-implantation demethylation then becoming entirely methylated or unmethylated after implantation (Proudhon et al., 2012). It is possible these have a function as ICRs in the pre-implantation embryo, or in the placenta, where many transient gDMRs are maintained (Figure 2) (Sanchez-Delgado et al., 2016).

Examples of non-canonical imprinting are so far restricted to mice. In these cases, imprinted genes have no detectable methylation differences between parental gametes. Instead, they have the histone modification H3K27me3 in the oocyte, which later leads to maternal

allele methylation in extra-embryonic tissues (including placenta) (Inoue et al., 2017).

1.8 Metastable epialleles

MEs are loci with variable methylation between individuals, but consistent cross-tissue methylation within individuals, suggesting the methylation is established early in development before separation of the germ layers. They are often associated with transposable elements, which evolve fast and often give rise to new cis-regulatory DNA elements or even new exons and genes (Imbeault et al., 2017; Bourque et al., 2018).

The Agouti viable yellow (A^{vy}) mouse allele is a well characterised example of a mammalian metastable epiallele associated with a transposable element, which causes a yellow coat colour when expressed (Dickies, 1962; Duhl et al., 1994). This allele was created by insertion of an Intracisternal A-particle (IAPs), a Class II endogenous retrovirus (ERV) with protein-coding sequences between long terminal repeats (LTRs) (Cole et al., 1981). The insertion created a cryptic promoter in the LTR which drives expression when unmethylated and crucially the epigenetic states, including DNA methylation and histone profiles, are variable between individuals (Duhl et al., 1994; Morgan et al., 1999; Kazachenka et al., 2018). The phenotype is heritable, with maternal inheritance causing an incomplete shift in yellow coats in the litter (Morgan et al., 1999). The pattern in phenotype can also be shifted by environment, for example, a preconception diet rich in methyl donors and cofactors *in utero* leads to increased methylation and fewer offspring with yellow coats (Wolff et al., 1998; Cooney et al., 2002; Waterland and Jirtle, 2003; Cropley et al., 2006).

Subsequent studies have found other variably methylation IAPs, which vary between but not within individuals (Kazachenka et al., 2018). Most are not heritable and many are not changed by environmental exposures like bisphenol A or methyl donor supplementation (Bertozzi et al., 2021). Sites also seem to function independently, as methylation is generally not correlated across different sites within individuals but is found at consistent levels across populations at each site (Kazachenka et al., 2018).

2 Developmental origins of health and disease

The Developmental Origins of Health and Disease (DOHaD) hypothesis states that environmental exposures in early life affect the risk of adverse health outcomes later in life (Barker, 1990). Epidemiological studies have shown many associations between early environment and later disease outcomes in humans.

There are several possible mechanisms that could link early environmental exposures to lifelong health of which DNA methylation changes, causing gene expression changes, are a leading candidate (Fleming et al., 2018). When investigating these cases, the time period around conception may be a critical window because, as previously discussed, shortly after fertilisation patterns of DNA methylation are established that can persist throughout life.

2.1 Early life exposures and health

Adverse birth outcomes can be caused by many different factors, including a wide variety of environmental exposures. Both maternal obesity and preconception physical activity are associated with risk of preeclampsia (Aune et al., 2014; Marchi et al., 2015; Poston et al., 2016). Alcohol and caffeine intake have been linked to reduced birthweight (Konje and Cade, 2008; Popova et al., 2021), and tobacco smoke exposure to worse birth outcomes, including reduced fetal growth (Peterson and Hecht, 2017). These adverse birth outcomes can subsequently be linked to later life disease, as discussed previously with placenta-related outcomes.

Important exposures often occur over long time periods in humans, so the critical windows of sensitivity are easier to separate in animal models. These studies show short term effects during pregnancy, such as placental growth being affected by a preconception zinc deficiency in rodents (Tian et al., 2014). Longer term effects include increased risks of offspring hypertension and adiposity from a preimplantation low protein diet in rodents (Kwong et al., 2000; Watkins et al., 2008a; Watkins et al., 2008b). These models suggest the blastocyst is capable of sensing nutrient status and altering the phenotype of placental development (Eckert et al., 2012; Watkins et al., 2015).

In humans, famines occurring over well-defined dates allow the study of exposures in specific developmental windows. One well-studied example is the Dutch Hunger Winter, a severe famine in 1944-45. Prenatal exposure to this famine increased the risk of diabetes, schizophrenia and other diseases in later life (Lumey et al., 2011).

Many examples of studies on the effect of early life nutrition and health outcomes are from a Sub-Saharan population in rural Gambia that experiences two distinct seasons with very different environmental, especially nutritional, exposures. In the rainy/hungry season, food availability is lower and energy status is poorer than in the dry/harvest season (Prentice et al., 1981). Babies born in the rainy season are more likely to have intrauterine growth restriction (Ceesay et al., 1997). They are also 10 times more likely to die prematurely, which may be linked to infection (Moore et al., 1997; 1999), as babies born in the hungry season have altered T cell immunity, with lower $CD3^+$ and $CD4^+$ lymphocyte counts (Ngom et al., 2011).

Micronutrition supplementation studies can test the association between nutritional environment and health more directly, for example, folic acid is known to reduce neural tube defects by up to 70% when taken in the months before and after conception and may also reduce the risk of small for gestational age births (Mastroiacovo and Leoncini, 2011; Hodgetts et al., 2015; Gao et al., 2016). The UK based UPBEAT trial used an intervention during pregnancy that reduced processed and snack food consumption and found a decrease in infant adiposity at 6 months of age (Patel et al., 2017).

An alternative approach to isolating early life environment is the study of assisted reproductive technologies (ART), such as *in-vitro* fertilisation, which provide a different environment only around conception. These show increased risk of high blood pressure in children and altered heart shape and chamber size in infants (Ceelen et al., 2008; Valenzuela-Alcaraz et al., 2013).

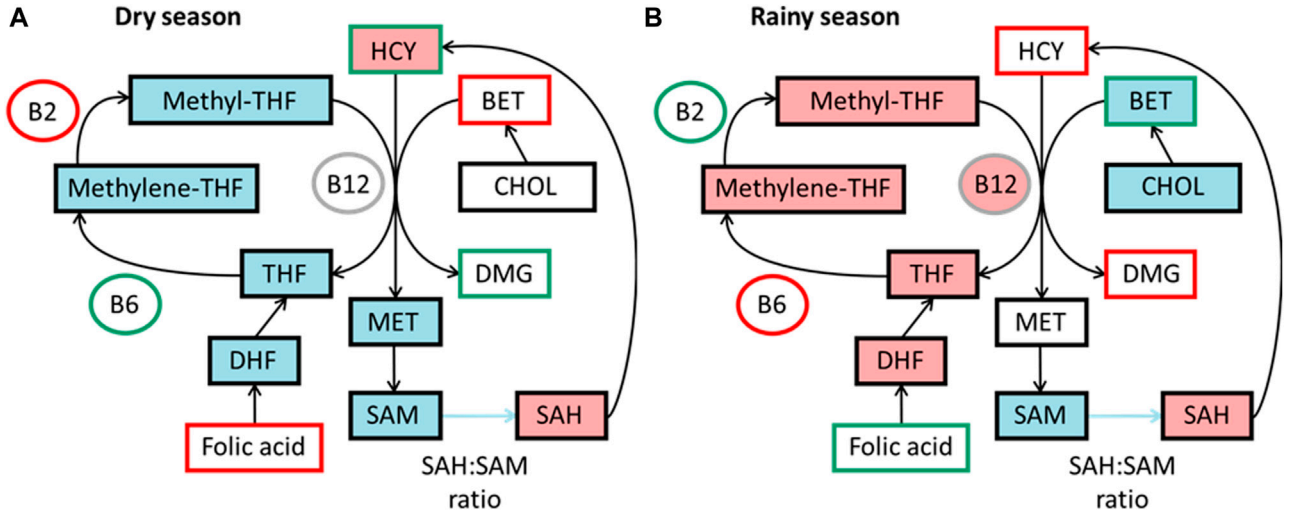

FIGURE 3
Simplified summaries of metabolic pathways highlighting seasonal differences reported from studies in The Gambia. Comparative levels between seasons in diet as measured by blood biomarkers and components predicting DNA methylation in each season, data from James et al. (2019) **(A)** Components outlined in green are higher in dry season and components outlined in red are lower. Components highlighted in blue are positive predictors of DNA methylation and pink negative predictors in dry season. **(B)** Components outlined in green are higher in rainy season and components outlined in red are lower. Components highlighted in blue are positive predictors of DNA methylation and pink negative predictors in rainy season. BET, betaine; B12, vitamin B-12; B2, vitamin B-2; B6, vitamin B6; CHOL, choline; DHF, dihydrofolate; DMG, dimethyl glycine; HCY, homocysteine; MET, methionine; methylene-THF, N5,10-methylene tetrahydrofolate; methyl-THF, N5-methyl tetrahydrofolate; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine; THF, tetrahydrofolate.

2.2 Early life environment and methylation

As discussed, methylation could be a mechanism for the link between early environmental exposures and health. In humans, ART has been linked to an increased risk of imprinting disorders (Lazaraviciute et al., 2014) and changes in methylation and histone modifications at imprinted genes (Choux et al., 2020). However, another study found no variability in 25 imprinted DMRs and allelic expression in placenta or cord blood in spontaneously conceived and assisted pregnancies (Camprubí et al., 2013).

Many environmental factors are known to affect methylation, for example, there are changes in methylation at repetitive regions with fetal bisphenol A exposure (Faulk et al., 2016) and changes in methylation at 12 CpGs in blood and 12 in buccal epithelial cells with prenatal exposure to phthalates (England-Mason et al., 2022). Aflatoxin B1 exposure *in utero* in The Gambia was associated with differential methylation at 71 CpG sites in infant white blood cells, including in growth factor and immune-related genes (Hernandez-Vargas et al., 2015).

2.3 Early life nutrition and methylation

Nutrition exposure may be key to investigating methylation variability, due to the direct links of micronutrients into the 1C pathway that provides methyl groups required for DNA methylation. Again, animal studies can provide more detailed exposure timing and insight into molecular mechanisms. In mouse studies of periconceptional maternal obesity, fetal growth rate is directly affected by alterations in methylation at the ribosomal DNA promoter which controls the levels of ribosomal RNA (Denisenko et al., 2016; Holland et al., 2016).

In humans, data from the Dutch Hunger Winter shows adults whose mothers were exposed to the famine only before and during the periconceptional period have modestly decreased DNA methylation in the *IGF2* gene (Heijmans et al., 2008). Further studies have shown exposure to this famine in early gestation affects methylation in many regions, mostly annotated as regulatory and with nearby genes expressed in early developmental phases (Tobi et al., 2014).

Identification of MEs in humans is complex, but advances in molecular techniques have suggested their existence in the human genome and identified putative links with environment and disease. They are a useful tool when exploring the link between environment, methylation and health because they are established very early in development and are hypervariable. They can also be studied in easily available samples, such as fetal blood obtained from the umbilical cord.

The levels of micronutrients essential for the 1C cycle in diet can affect methylation levels (Waterland and Jirtle, 2003; Sinclair et al., 2007). In The Gambia, micronutrients related to the 1C cycle vary across the seasons, as measured by maternal blood biomarkers (Dominguez-Salas et al., 2013). Studies have found higher concentration of methyl donors in the rainy season, potentially giving a higher methylation potential (Figure 3).

Non-imprinted DMRs in cord blood, placental tissue or peripheral blood from Gambian infants have also been associated with birthweight, and 25 DMRs in infant blood at 12 months are associated with length for age (Quilter et al., 2021). A meta-analysis

found DNA methylation in neonatal blood has been linked with many pregnancy exposures and is associated with birthweight through childhood, but not adulthood. There is also an overlap between CpGs related to birthweight and intrauterine exposures (Küpers et al., 2019).

These changes were first associated with significant changes in methylation at seven infant MEs, with increased levels of homocysteine and B6 and decreased levels of B2 correlating with higher methylation (Dominguez-Salas et al., 2014). A model found different biomarkers predicted methylation between seasons, with vitamin B-2 and methionine as positive predictors in the dry season, and choline and vitamin B-6 as positive and folate and vitamin B-12 as negative predictors in the rainy season (Figure 3) (James et al., 2019). The different seasonal effects suggest a switch between the betaine dependent pathway in the rainy season to the folate dependent pathway in the dry season.

In further studies, children conceived in the Gambian rainy season showed consistently increased methylation at multiple MEs (Waterland et al., 2010; Khulan et al., 2012; Silver et al., 2022). Methylation changes extend beyond MEs; Silver et al. (Silver et al., 2022) identified 259 CpGs that showed a robust signature of differential methylation between seasons of conception (SoC-CpGs) across two independent cohorts. In one of these cohorts methylation measured in blood in early and mid-childhood showed that the seasonality effect was attenuated in the older cohort.

Many SoC-CpGs overlapped with MEs identified in previous studies and many have been associated with sex (Kessler et al., 2018). They were also enriched for CpGs previously identified to be methylated on PoO specific alleles (Zink et al., 2018), which were also enriched for placenta-specific oocyte gDMRs (Sanchez-Delgado et al., 2016).

One example of a non-coding RNA gene responsive to SoC is *VTRNA2-1*, which is also a putative ME (Silver et al., 2015). This tumour suppressor gene has been linked to cancer and there is evidence that it is maternally imprinted (Romanelli et al., 2014; Silver et al., 2015).

The EMPHASIS (Epigenetic Mechanisms linking Preconceptional nutrition and Health Assessed in India and sub-Saharan Africa) study conducted two randomised controlled trials that used different micronutrient interventions taken preconception and during pregnancy. Six differentially methylated CpGs were found in Gambian children, of which four were around the endothelial cell-specific molecule 1 (*ESM1*) gene (Saffari et al., 2020). Another randomised controlled trial found sex-specific epigenetic changes in Gambian cord and infant blood from periconceptional micronutrient supplementation (Khulan et al., 2012). In these children, girls had reduced methylation at *IGFR2* and boys at *MEG3/GTL2*, but these differences disappeared by 9 months (Cooper et al., 2012).

One of the genes with increased methylation associated with periconceptional micronutrient supplementation and conception in the Gambia rainy seasons is *PAX8* (paired-box 8) (Waterland et al., 2010; Silver et al., 2015; Saffari et al., 2020). It is a master regulator of thyroid gene expression, and its methylation has been associated with thyroid function in Gambian children (Candler et al., 2021). Another example is *POMC*, where methylation has been separately shown to reduce *POMC* transcription and is associated with obesity in children (Kuehnen et al., 2012; Kühnen et al., 2016).

3 Human placenta

3.1 Placenta and disease

The placenta is a transient organ that acts as a physical connector and barrier between maternal and fetal blood, mediating nutrient and gas exchange, and waste removal between the embryo and the mother. It also functions as an endocrine organ, producing hormones including human chorionic gonadotropin (hCG) (Lopata et al., 1997). Placenta development and structure is highly species-specific; the human placenta displays deep trophoblast invasion which is unique to great apes (Imakawa et al., 2015). Correct functioning is vital for appropriate fetal development, for example, increased glucose transfer in gestational diabetes increases fetal adiposity and macrosomia, which subsequently is associated with disease in later life (Pettitt et al., 1993; Tint et al., 2020).

Many pregnancy complications are linked with placental development defects, including preeclampsia, fetal growth restriction, recurrent miscarriage and still-birth (Brosens et al., 2011). These conditions contribute to a high proportion of maternal and neonatal morbidity and mortality, especially in sub-Saharan Africa (Graham et al., 2016).

Intrauterine growth retardation (IUGR) affects 10%–15% pregnancies and is a subset of small for gestational age, which is defined as birth weight below the 10th percentile (Harkness and Mari, 2004). Many IUGR cases are caused by placental insufficiency. In these cases, there is deficient remodeling of uterine spiral arteries that supply the placenta creating a lower villous volume and surface area for maternal-fetal exchange, and dysregulation of many genes (Burton and Jauniaux, 2018). Babies with IUGR have a high risk of hypertension, type 2 diabetes and heart disease later in life (Curhan et al., 1996; Rich-Edwards et al., 1997).

Preeclampsia affects 3%–5% of pregnancies in the developed world and forms part of the hypertensive disorders that cause 12% of maternal deaths during and after pregnancy (WHO, 2005). It is characterised by hypertension and proteinuria in the second half of pregnancy (Roberts and Cooper, 2001) and is also associated with defective uterine spiral artery remodeling. Its effects range from mild to multiorgan failure and it is caused by low perfusion in the placenta (Brosens et al., 2011).

Overall placenta morphology has also been associated with later disease risk. For example, in the Helsinki Birth Cohort that experienced prenatal famine exposure, placenta thickness has been associated with risk of sudden cardiac death (Barker et al., 2012). Another study linked longer and more oval placentas in this cohort with colorectal cancer (Barker et al., 2013).

3.2 The unique state of the placenta epigenome

The placenta is hypomethylated overall, with around 3% cytosine bases methylated compared to 4% in somatic tissue (Fuke et al., 2004). Global placenta methylation increases from 2.8% in first trimester to 3.1% in term placentas, unlike global methylation in somatic tissues which decreases with age (Fuke et al., 2004). The location of methylation also changes with gestational age,

suggesting that genes act at specific stages (Novakovic et al., 2011; Camprubí et al., 2013). Methylation changes with gestational age are accompanied by a change in gene expression levels in 25% of placenta expressed genes (Sitras et al., 2012; Uusküla et al., 2012). Most imprinted genes show reduced expression with advancing gestational age independent of allelic methylation (Monteagudo-Sánchez et al., 2019; Pilvar et al., 2019).

The hypomethylation in placenta is not confined to specific genomic features but does include repetitive elements, including transposable elements, as well as large (>100 kb), partially methylated domains (PMDs) that are largely stable throughout gestation (Schroeder et al., 2013; Schroeder and Lasalle, 2013). Repetitive elements are highly methylated in somatic tissue but variably methylated in placenta and highly species specific, which may allow them to act as drivers for placenta evolution. There are multiple placenta-specific promotors derived from TEs, which includes endogenous retroviruses, including for the *CYP19A1*, *NOS3* and *PTN* genes (Dunn-Fletcher et al., 2018). ERV families have been identified with regulatory potential that are close to trophoblast-specific expressed genes (Sun et al., 2021; Frost et al., 2023). In human trophoblast stem cells, genetic editing showed these elements act as enhancers (Frost et al., 2023).

HERV-W is a co-opted human ERV envelope gene which illustrates how these elements can exhibit placenta-specific function. One of its copies produces Syncitin-1, which is important for syncytiotrophoblast fusion, a multinucleated cell layer around placenta villi (Blond et al., 1999; Mi et al., 2000). Its 5′ UTR contains a LTR with a trophoblast-specific enhancer and a promoter region with a CpG island, whose methylation controls syncitin-1 expression (Matoušková et al., 2006; Gao et al., 2012). *HERV-W* also highlights how these elements can be important for pregnancy outcomes as it is hypomethylated and expressed in placenta, with lower expression in preeclampsia and IUGR placentas (Ruebner et al., 2013).

Placenta methylation at gene promoters is highly variable between individuals, with increased variability in term compared to first trimester placentas (Novakovic et al., 2011). However, there is also variability across a placenta depending on the sampling site (Janssen et al., 2015) which may reflect cellular composition.

Placental methylation at many genes has been associated with pregnancy outcomes. Placentas from pregnancies with preeclampsia have higher global DNA methylation and within this, blood pressure increases correlate with methylation increases (Kulkarni et al., 2011). Studies looking at preterm infants found lower SAM:SAH ratio and global methylation (Khot et al., 2017). Sinclair et al. (2007) found 15 loci where methylation associated with birthweight, of which four had corresponding changes in transcript levels in placenta.

In recent years, genome-wide DNA methylation values have been used to show discrepancies between chronological and biological age. Such "epigenetic clocks" use algorithms to calculate biological age on the basis of hundreds to thousands of CpG sites across the genome (Horvath, 2013). By calculating multi-organ clocks it is possible to predict clinically useful biomarkers associated with age-related disease and mortality (Lu et al., 2019). Whilst a pan-tissue epigenetic clock cannot reliably estimate gestational age, Lee et al. (2019) generated bioinformatic pipelines to generate placenta-derived epigenetic clocks that can track gestational age.

Deviations from normal placenta aging have been reported for several pregnancy complications. Accelerated epigenetic aging has been linked to early onset preeclampsia (Mayne et al., 2017), and lower fetal weight (Tekola-Ayele et al., 2019), while deceleration has been associated with maternal weight gain during pregnancy (Workalemahu et al., 2021). Together this indicates that both epigenetic age acceleration and deceleration are associated with distinct risk and protective factors, with studies from many laboratories are trying to identify distinct, tissue and cell-type specific trajectories to predict pregnancy outcomes.

3.3 Placenta-specific imprinting

Although the placenta is hypomethylated overall compared to somatic tissue, it exclusively maintains PoO methylation and expression at many transient gDMRs (Figure 2). In humans, over 150 maternally methylated DMRs have been identified in the placenta, around half of which have confirmed paternal expression (Yuen et al., 2011; Barbaux et al., 2012; Sanchez-Delgado et al., 2015; Hamada et al., 2016; Hanna et al., 2016; Sanchez-Delgado et al., 2016). Placenta-specific imprinting is highly polymorphic between individuals in humans and poorly conserved between humans and mice, though it may be more similar in other primates (Hanna et al., 2016; Sanchez-Delgado et al., 2016).

Hanna et al. (2016) found a monoallelic methylation in 50% of heterozygous samples at selected DMRs, looking just at trophoblast and villi cells to remove differences from cell composition, and there was significantly less variability at the same sites in somatic tissues. Complementary studies have showed that of 104 placenta-specific gDMRs studied, 52% intervals possess low methylation and biallelic expression in multiple term biopsies (Sanchez-Delgado et al., 2016; Monteagudo-Sanchez et al., 2019), although the frequency was variable between loci. It is unknown if this variation arises from the interactions with in cis genetic variants, environmental exposures, or is truly stochastic.

One well characterised transiently imprinted gene in mice is *Zdbf2* (zinc finger DBF-type containing 2) (Duffié et al., 2014). Generally, imprinting is poorly conserved between humans and mice, but the methylation pattern is the same at *Zdbf2*. The locus has a maternal pre-implantation gDMR, causing paternal expression of an alternative long isoform Liz (Duffié et al., 2014). This leads to creation of a paternal secondary DMR, which blocks the repressive H3K27me3 mark allowing paternal expression from the canonical *Zdbf2* promoter. In somatic tissue, the maternal gDMR is lost after implantation, leaving the paternal sDMR and *Zdbf2* expression. In extraembryonic lineages, including the placenta, the maternal gDMR and Liz expression are maintained instead (Kobayashi et al., 2009; Duffie et al., 2014). In humans, decreases in placental *ZDBF2* expression have been associated with intrauterine growth restriction (Monteagudo-Sánchez et al., 2019).

The impact of imprinted genes in placenta has been well studied in mice. Mouse models have demonstrated that generally deletions of genes which are paternally expressed, including *Igf2*, *Peg1* and *Peg3*, reduce placental size and increase the incidence of IUGR (DeChiara et al., 1991; Lefebvre et al., 1998). The opposite happens with deletions on maternally expressed genes, such as *Grb10* and

Phlda2, causing fetal over-growth (Charalambous et al., 2003; Salas et al., 2004).

In humans, IUGR has been associated with placental expression of the imprinted *GPR1-AS1* and *ZDBF2* genes (Monteagudo-Sánchez et al., 2019). It is also associated with increases in *HYMA1* expression, and, in girls only, lower *PLAGL1* expression (Iglesias-Platas et al., 2014). *PLAGL1* is a transcription factor and its expression changes impact a network of genes downstream.

4 Placenta-specific effects of environment on health

The placenta is particularly interesting in the context of DOHaD because early environment can influence placental function, impacting development and pregnancy outcomes. Further, the placenta has a large impact on fetal development and placenta-related adverse pregnancy outcomes can influence later life disease. Placental function can be influenced by many environmental exposures, such as maternal smoking and obesity (Reijnders et al., 2019; Scott et al., 2022). Increased physical activity before and during early pregnancy can reduce the incidence of preeclampsia (Aune et al., 2014). ART is associated with increased risk of IUGR or premature birth (Camprubí et al., 2013).

Adequate nutrition in the first trimester and periconceptional folic acid supplementation are associated with lower resistance of the uterine and umbilical arteries in second and third trimesters, in which high resistance can be associated with preeclampsia and fetal growth restriction risk (Reijnders et al., 2019). The effect of the previously discussed Dutch Famine has also been studied, with famine exposure reducing placenta weight, birthweight and increasing preterm births, stillbirths and neonatal death (Susser and Stein, 1994; Lumey, 1998). A study in Gambia found that periconceptional multiple micronutrient supplementation improved placenta vascular function (Owens et al., 2015).

A systematic analysis of the effects of micronutrient supplementation looked at six placenta-related outcomes: preeclampsia, small for gestational age, low birthweight, preterm birth, stillbirth and maternal death (Kinshella et al., 2021). No single factor affected all six, but one or more were affected by vitamins C and E, vitamin D, vitamin D and calcium co-supplementation, calcium, iron and/or folic acid, zinc and multiple micronutrient supplementation. In another review, 14 of 25 nutritional factors reviewed were significantly associated with preeclampsia incidence, including vitamin D deficiency and high serum iron (Kinshella et al., 2022).

4.1 Placenta-specific effects of environment on methylation

In rodents, placenta methylation is affected by maternal diet. A gestational high-fat diet leads to placenta global hypomethylation and changes in methylation at enzymes involved in epigenetic machinery and expression (Gallou-Kabani et al., 2010). In mice, folate deficiency leads to lower overall placental methylation (Mahajan et al., 2019). There are also changes at many genes in the transcriptome and methylome, and the genes vary with sex, even being involved in different biological functions (Gabory et al., 2012).

Knowledge on the effect of diet on human placental methylation is limited, but changes in methylation linked to many other maternal exposures have been more widely studied. For example, ART has been linked to methylation changes in placenta, specifically reduced methylation at the *H19* and *MEST* DMRs with an increase in *H19* expression (Nelissen et al., 2013). In another study, ART placentas had increased expression of *INSIG1* and *SREBF1*, linked to cholesterol metabolism, with decreased methylation (Lou et al., 2014). A recent review looked at other exposures associated with methylation changes in placentas both globally and at specific sites, including air pollution, maternal smoking, bisphenol A and trace metals (Mortillo and Marsit, 2022). They observed that the effects are generally stronger when the exposure occurs in the first trimester. In other studies, maternal depression and anxiety have also been linked to a decrease in overall placenta methylation levels, with some changes in gene expression (Chen et al., 2014).

There are some studies on the effects of maternal diet on placental methylation in humans, for example, prenatal vitamin intake has been associated with a small reduction in placental methylation, including at sites associated with neuronal developmental pathways (Dou et al., 2022). Looking at specific components, folate has been widely studied during pregnancy, as its deficiency is associated with low birth weight, preterm birth, spontaneous abortion and birth outcomes including neural tube defects (Tamura and Picciano, 2006; Fekete et al., 2010; Greenberg et al., 2016; Hoffbrand et al., 2014). One-carbon components, including folate, have been studied in complicated pregnancies to see how they affect DNA methylation. There are three components important for folate transport through the placenta: Three main components participate in this process: folate receptor α (*FRα)*, reduced folate carrier (*RFC*) and proton-coupled folate transporter (*PCFT*) (Solanky et al., 2010; Castano-Morena et al., 2020). *FRα* mRNA was lower in preterm placentas compared to term, which correlated with increased methylation on the fetal side (chorionic plate). The increased methylation in preterm placentas also correlated with higher folate and lower B12 concentrations in the cord blood (Piñuñuri et al., 2020). The syncytiotrophoblasts in IUGR placentas had lower folate update and reduced levels of *RFC* but not *FR-α* (Chen et al., 2018).

In preterm placentas, expression of MAT2A and AHCY, enzymes involved in producing SAM, is higher in preterm placentas, and a SAH:SAM ratio, giving a lower methylation potential (Khot et al., 2017). These studies highlight how maternal nutrition can have an effect on methylation through one-carbon cycle components. However, not much is known still about how the overall methylation changes are acting to alter the function of the placenta.

5 Future directions

The placental epigenome is hypomethylated and hypervariable so has the potential for creating large inter-individual differences. This gives it great potential as a tissue to investigate the effects of environmental exposures on DNA methylation. So far, there has been limited study on the effects non-nutritional exposures and no study on the correlation in methylation at MEs between embryonic and extra-embryonic tissues. If MEs in placenta and somatic tissue are matched, this could suggest MEs are established before the

trophectoderm lineage separates. Many of the MEs identified as responsive to season of conception in The Gambia overlap placenta DMRs which could suggest a mechanistic link, perhaps an issue with residual DMRs rather than somatically acquired. However, this is unlikely to explain all MEs, those that are not related to gDMRs must have a different mechanism of variability.

MEs are also known to disappear with age, so a readily obtainable early life tissue such as the placenta is an excellent place to look. This would also diversify the tissues used, as MEs are commonly identified only in cord blood. It is possible that more variation may be maintained in placenta as it is only needed for 9 months so is not regulated as highly as somatic tissue, or the unique epigenetic landscape may play an important role. Perhaps if there is a correlation, the placenta epigenome and transcriptome would serve as a good biomarker for later life disease. There is need for more work on MEs in humans to determine the effects of genetic variation and their relationship to environmental exposures and health.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Methylation is maintained specifically at imprinting control regions but not other DMRs associated with imprinted genes in mice bearing a mutation in the *Dnmt1* intrinsically disordered domain

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Differential methylation of imprinting control regions in mammals is essential for distinguishing the parental alleles from each other and regulating their expression accordingly. To ensure parent of origin-specific expression of imprinted genes and thereby normal developmental progression, the differentially methylated states that are inherited at fertilization must be stably maintained by DNA methyltransferase 1 throughout subsequent somatic cell division. Further epigenetic modifications, such as the acquisition of secondary regions of differential methylation, are dependent on the methylation status of imprinting control regions and are important for achieving the monoallelic expression of imprinted genes, but little is known about how imprinting control regions direct the acquisition and maintenance of methylation at these secondary sites. Recent analysis has identified mutations that reduce DNA methyltransferase 1 fidelity at some genomic sequences but not at others, suggesting that it may function differently at different loci. We examined the impact of the mutant DNA methyltransferase 1 P allele on methylation at imprinting control regions as well as at secondary differentially methylated regions and non-imprinted sequences. We found that while the P allele results in a major reduction in DNA methylation levels across the mouse genome, methylation is specifically maintained at imprinting control regions but not at their corresponding secondary DMRs. This result suggests that DNA methyltransferase 1 may work differently at imprinting control regions or that there is an alternate mechanism for maintaining methylation at these critical regulatory regions and that maintenance of methylation at secondary DMRs is not solely dependent on the methylation status of the ICR.

KEYWORDS

genomic imprinting, DNA methylation, DMNT1, epigenetics, differentially methylated regions

1 Introduction

Genomic imprinting results in parent of origin-specific monoallelic expression of approximately 150 genes in mammals (Morison et al., 2005; https://www.geneimprint.com/site/genes-by-species.Mus+musculus). Parent of origin-specific DNA methylation at imprinting control regions (ICRs) is established during gametogenesis, inherited at fertilization, maintained throughout development, and serves as the primary imprinting mark; as such, it is responsible for distinguishing the parental alleles from each other and regulating their expression accordingly (Barlow and Bartolomei, 2014). Differential methylation of ICRs is therefore essential for establishing imprints, and recent studies have further proven the importance of maintaining differential methylation at ICRs in order to retain monoallelic imprinted expression patterns. Epigenetic editing resulting in methylation of the typically unmethylated maternal tandem repeats within the *Dlk1-Dio3* IG-DMR led to paternalization of the maternal allele, including the acquisition of methylation across the IG-DMR and concomitant silencing of *Meg3* (Kojima et al., 2022). Conversely, targeting TET1 activity to the tandem repeats with the IG-DMR on the typically methylated paternal IG-DMR maternalized the paternal allele as evidenced by decreased methylation across this locus and expression of *Meg3* from the typically silent paternal allele (Kojima et al., 2022).

In addition to the primary, or gametic, differentially methylated regions (DMRs) that function as ICRs and are essential for regulating imprinted expression, some imprinted genes also acquire distinct regions of differential methylation during post-implantation development (Tremblay et al., 1997; Hanel and Wevrick, 2001; Takada et al., 2002; Bhogal et al., 2004; Gagne et al., 2014; Guntrum et al., 2017). Acquisition of parent of origin-specific methylation at these secondary, or somatic, DMRs is dependent on the epigenetic state of the corresponding primary DMR, although the exact mechanisms driving methylation acquisition at secondary DMRs are not well understood (Saito et al., 2018; Hara et al., 2019). For example, epigenetic alteration of methylation at the IG-DMR or targeted deletion of IG-DMR sequences directly influences the methylation state of the corresponding secondary DMR located at the *Gtl2* (*Meg3*) promoter (Aronson et al., 2021; Kojima et al., 2022), highlighting the relationship between these two elements. While differential methylation of the primary DMRs is essential for establishing the parent of origin epigenotype at each imprinting cluster, the subsequent acquisition of parent of origin-specific methylation at secondary DMRs appears to be important for maintaining parent of origin-specific expression of individual loci (Stöger et al., 1993; Constância et al., 2000; Bhogal et al., 2004; Kobayashi et al., 2009; Kagami et al., 2010; John and Lefebvre, 2011; Nakagaki et al., 2014; Aronson et al., 2021; Kojima et al., 2022).

Despite the demonstrated importance of differential methylation at secondary DMRs in the regulation of the individual imprinted genes with which they are associated, DNA methylation is less consistent at secondary DMRs than at primary DMRs (Tremblay et al., 1997; Hanel and Wevrick, 2001; Takada et al., 2002; Yatsuki et al., 2002; Arnaud et al., 2003; Coombes et al., 2003; Ono et al., 2003; Nowak et al., 2011; Woodfine et al., 2011; Arand et al., 2012; Gagne et al., 2014; Guntrum et al., 2017; Nechin et al., 2019). Investigation of DNA methylation patterns at secondary DMRs revealed high levels of methylation asymmetry (Guntrum et al., 2017; Nechin et al., 2019), which may be a result of TET activity at these loci which would lead to 5-hydroxymethylcytosine enrichment and subsequent active or passive demethylation (Valinluck and Sowers, 2007; Tahiliani et al., 2009; He et al., 2011; Ito et al., 2011; Kohli and Zhang, 2013). Despite the high levels of methylation asymmetry observed at secondary DMRs, overall levels of DNA methylation remain consistent across development, consistent with the hypothesis that the epigenetic profile at primary DMRs directs methylation acquisition at secondary DMRs throughout development (Bhogal et al., 2004; Gagne et al., 2014; Guntrum et al., 2017; Nechin et al., 2019).

The establishment and maintenance of DNA methylation is achieved by DNA methyltransferases (Dnmts). Dnmt3a and Dnmt3b function as *de novo* methyltransferases while Dnmt1 functions as the maintenance methyltransferase (Li and Zhang, 2014). Dnmt1 plays a critical role in maintaining global methylation, and complete loss of *Dnmt1* activity is embryonic lethal (Li et al., 1992). Dnmt1 has also been shown to be responsible for maintaining methylation at primary DMRs associated with imprinted genes, including during the genome-wide demethylation that occurs during pre-implantation development (Howell et al., 2001; Hirasawa et al., 2008; Liu et al., 2022). Mutation of *Dnmt1* supports the hypothesis that it may function differently at different genomic locations. Dissection of *Dnmt1* via mutational analysis has identified specific regions of the Dnmt1 protein that are essential for maintaining non-imprinted but not imprinted methylation patterns and *vice versa* (Borowczyk et al., 2009; Shaffer et al., 2015). These mutations are located in the intrinsically disordered domain (IDD) of Dnmt1, suggesting that different sequences within this region may influence Dnmt1 activity at different targets within the mouse genome (Shaffer et al., 2015).

Herein, we describe our investigation of the *Dnmt1* P allele. The P allele is a mutation in the mouse *Dnmt1* IDD that replaces six codons with the corresponding rat sequence (Shaffer et al., 2015). Work by Shaffer and others (Shaffer et al., 2015) illustrated that $Dnmt1^{P/P}$ is lethal, likely due to a dramatic reduction in global DNA methylation. Despite the overall reduction in DNA methylation globally and at IAP sequences, methylation was relatively well maintained at primary DMRs associated with imprinted loci (Shaffer et al., 2015). We compared DNA methylation levels in $Dnmt1^{P/P}$ mutant embryos across development to determine whether methylation is also maintained better at secondary DMRs, whose methylation status is dependent on the methylation state of the corresponding primary DMR. Our results illustrate that methylation at secondary DMRs associated with imprinted genes is dramatically reduced in $Dnmt1^{P/P}$ mutants, supporting the hypothesis that methylation is maintained differently at different sequences and that different factors may be responsible for maintaining methylation at primary vs. secondary DMRs.

2 Materials and methods

2.1 Mice

Sv/129 mice heterozygous for the *Dnmt1* P allele mutation (Shaffer et al., 2015) were obtained from Dr. Mellissa Mann

(Magee-Womens Research Institute, Pittsburg, PA). Natural matings between heterozygous pairs were used to generate *Dnmt1*$^{+/+}$, *Dnmt1*$^{P/+}$ and *Dnmt1*$^{P/P}$ embryos, which were collected at 9.5, 12.5, 15.5 and 18.5 days post coitum (dpc). Natural matings were also used to generate offspring in order to maintain the *Dnmt1* P allele in the colony. Ethical approval for procedures involving animals was granted by the Bryn Mawr College Institutional Animal Care and Use Committee, PHS Welfare Assurance Number A3920-01.

2.2 Genotyping

Genotypes were determined using a PCR-based assay described by Shaffer *et al.* (Shaffer et al., 2015). Briefly, DNA was extracted from embryo or 3–4 weeks mouse tails using proteinase K digestion and genomic DNA was purified using a Genomic DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, cat#D4011). PCR using oligonucleotides flanking the P allele mutation was followed by restriction digestion with *Ava*I, and wild type vs. mutant P alleles were distinguished by agarose gel electrophoresis (wild type allele, 627 bp; P allele, 447 + 180 bp). Chi-square goodness of fit tests were conducted in Microsoft Excel, using the raw number of *Dnmt1*$^{+/+}$, *Dnmt1*$^{P/+}$ and *Dnmt1*$^{P/P}$ embryos or pups collected at each developmental stage, to determine whether the observed values deviated significantly from the Mendelian ratios expected from crosses between heterozygous pairs.

2.3 DNA purification, template preparation and bisulfite sequence analysis

Genomic DNA was isolated from 9.5, 12.5, 15.5 and 18.5 dpc embryo heads following proteinase K digestion and a series of phenol/chloroform extractions as described previously (Davis et al., 1999). Purified DNA was subjected to bisulfite mutagenesis using an EZ DNA Methylation-Direct kit (Zymo Research, Irvine, CA, cat#D5020). Mutagenized DNA was subjected to nested or semi-nested PCR amplification; primers, PCR annealing temperatures and expected second round PCR product size for each locus analyzed are detailed in Supplementary Table S1. Resulting amplicons were purified from agarose gels using a Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA, cat#D4002) and quantified using a Qubit 3.0 Fluorometer (ThermoFisher Scientific, cat#Q33216). Equimolar amounts of PCR product from multiple loci were combined with a minimum of 50 ng from each amplicon and submitted to Azenta (South Plainfield, NJ) for NextGeneration-based amplicon sequencing. Sequence reads were uploaded to a Galaxy Instance hosted at Bryn Mawr College, paired and processed using fastp, mapped to known target sequences, and analyzed for non-CpG bisulfite conversion efficiency as well as for the presence of cytosines vs. thymines in a CpG context (Afgan et al., 2018). The bisulfite conversion efficiency was >99% for all datasets used in this analysis.

2.4 Analysis of downsampled sequences

Downsampling of NGS data was performed using Galaxy tools to obtain 20–25 sequencing reads for each locus analyzed. Percent methylation for each strand was calculated and the raw data from each allele in *Dnmt1*$^{+/+}$ and *Dnmt1*$^{P/P}$ embryos was ranked and assessed for statistically significant differences using a Mann-Whitney U test (http://vassarstats.net/utest.html).

3 Results

3.1 The *Dnmt1* P allele alters sequences uniquely present in *Mus* and *Rattus*

The amino terminal intrinsically disordered domain of Dnmt1, residues 92–391, includes a 160 amino acid region unique to eutherian mammals proposed to play a role in mammalian-specific methylation processes such as genomic imprinting (Borowczyk et al., 2009; Liu et al., 2017). Previous research indicated that different portions of this domain may influence the catalytic activity of Dnmt1 at different sequences (Borowczyk et al., 2009). While the primary sequence of Dnmt1 is highly conserved across species (Supplementary Figures S1A–C), Shaffer *et al.* (Shaffer et al., 2015) identified a 10 amino acid region present in mouse and rat that is not present in humans and suggested that this region may be responsible for species specific methylation. Further analysis illustrated that the mouse-rat region is specific to *Mus* and *Rattus* genera, as it is not present in other rodents, including the closely related deer mouse (*Peromyscus leucopus* and *Peromyscus maniculatus*) (Figure 1; Supplementary Figures S1D). The *Dnmt1* P allele, which substituted the mouse codons specifying LESHTV for the rat codons specifying PEPLSI, is embryonic lethal, displaying dramatically reduced levels of global DNA methylation, indicating that this region does not function similarly in *Mus* vs. *Rattus* (Shaffer et al., 2015).

3.2 The *Dnmt1* P allele is neonatal lethal

Embryos homozygous for the *Dnmt1* P allele have dramatically reduced levels of global methylation but methylation was observed to be better maintained at primary DMRs associated with imprinted genes (Shaffer et al., 2015). Since the methylation at secondary DMRs associated with imprinted genes is dependent on the methylation of the corresponding primary DMR (Saito et al., 2018; Hara et al., 2019), we wanted to determine whether *Dnmt1*$^{P/P}$ mutant embryos would also retain most of their methylation at secondary DMRs as a consequence of the methylation profile at the associated primary DMR or whether the preferential retention of methylation at primary DMRs is unique in *Dnmt1*$^{P/P}$ mutant embryos, suggesting either that Dnmt1 functions differently at these sequences or that the maintenance of methylation at primary DMRs can be achieved with other DNA methyltransferases.

We collected and genotyped embryos derived from natural matings between *Dnmt1*$^{P/+}$ mice at 9.5, 12.5, 15.5 and 18.5 dpc; at least four litters were collected at each developmental stage. While there was some deviation from the expected 1:2:1 Mendelian ratio at each developmental stage, none of the differences were significant

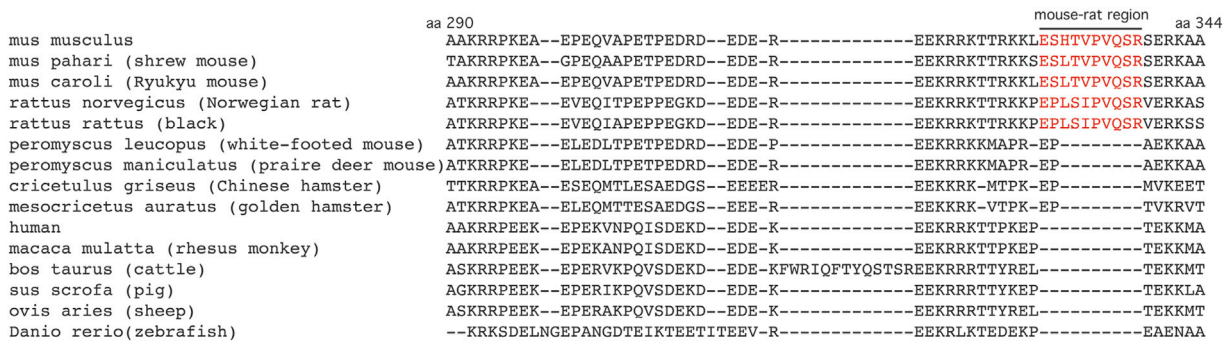

FIGURE 1
Alignment of Dnmt1 amino acid sequences 290–344. An 8–10 amino acid sequence (red) within the Dnmt1 intrinsically disordered domain is present in *Mus musculus, Mus pahari, Mus caroli, Rattus norvegicus* and *Rattus rattus*, but is not found in other rodents or non-rodent species. Sequences were aligned using the COBALT multiple alignment tool (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi).

TABLE 1 Genotypes of embryos derived from *Dnmt1*$^{P/+}$ x *Dnmt1*$^{P/+}$ matings. *p* values were calculated using a chi-square goodness of fit test based on the 1:2:1 Mendelian genotype ratio expected from heterozygous parents; no significant differences from the 1:2:1 predicted ratio were detected.

Embryonic stage	+/+	P/+	P/P	*p*-Value
9.5 dpc	8	13	15	0.0639
12.5 dpc	9	14	8	0.8374
15.5 dpc	6	19	8	0.6065
18.5 dpc	15	26	6	0.1368
	38	72	37	0.9633

(Table 1). Furthermore, we did not observe consistent differences in morphology between wild-type, heterozygous or homozygous mutant embryos, suggesting that *Dnmt1*$^{P/P}$ embryos survive throughout gestation (Supplementary Figure S2). In contrast, no *Dnmt1* $^{P/P}$ pups survived beyond 1 day after birth. Three of the 245 pups that were observed following natural matings between *Dnmt1* $^{P/+}$ mice were *Dnmt1* $^{P/P}$, and all three were deceased on postpartum day 1. Of the 242 pups that survived beyond postpartum day 1, all survived into adulthood: 84 of the surviving offspring were wild-type, and 158 were heterozygous for the *Dnmt1* P allele mutation (Table 2). These data indicate that the *Dnmt1* P allele is a neonatal lethal. Our observations of the three dead *Dnmt1* $^{P/P}$ pups indicated that none of them had milk in their stomachs. We hypothesize that *Dnmt1* $^{P/P}$ embryos may survive gestation but are unable to eat and/or breathe after birth and are therefore inviable, and that the majority of the *Dnmt1* $^{P/P}$ pups that were born were consumed by their parents before they were observed on postpartum day 0 or day 1.

3.3 DNA methylation levels are relatively well maintained at primary DMRs associated with imprinted genes in *Dnmt1*$^{P/P}$ mutant embryos as compared to secondary DMRs and non-imprinted sequences

We analyzed methylation levels at primary and secondary DMRs associated with imprinted loci as well as at non-imprinted loci in DNA derived from 9.5, 12.5, 15.5 and 18.5 dpc wild-type and *Dnmt1*$^{P/P}$ siblings (Table 3). Purified genomic embryo DNA was subjected to bisulfite mutagenesis and target loci were amplified by PCR (Supplementary Table S1). Purified amplicons were quantified and pooled at equimolar amounts prior to Next-Generation sequencing. NGS data was analyzed using a Galaxy instance to determine bisulfite mutagenesis efficiency based on non-CpG cytosine conversion to uracil (thymine) and the frequency of cytosine methylation at CpG dinucleotides. Data were obtained from one wild-type and one *Dnmt1*$^{P/P}$ embryo at 9.5, 12.5 and 15.5 dpc, and from two wild-type and two *Dnmt1*$^{P/P}$ embryos at 18.5 dpc.

DNA methylation levels were reduced in *Dnmt1*$^{P/P}$ embryos at all loci examined and at all developmental stages analyzed relative to their wild-type siblings. At primary DMRs, the amount of methylation detected in *Dnmt1*$^{P/P}$ DNA was between 80% and 100% of the wild-type value at 87% of the sequences analyzed (Figure 2A; Supplementary Table S2). While all primary DMRs except the *Airn* ICR consistently exhibited reduced levels of DNA methylation in *Dnmt1*$^{P/P}$ mutant embryos, the difference in methylation between *Dnmt1*$^{+/+}$ and *Dnmt1*$^{P/P}$ embryos was generally less than 16%, suggesting either that the P allele form of Dnmt1 functions reasonably well at these sequences or that an alternative mechanism for maintaining methylation at these sequences exists. Furthermore,

TABLE 2 Genotypes of viable pups derived from *Dnmt1*$^{P/+}$ x *Dnmt1*$^{P/+}$ matings. *p* values were calculated using a chi-square goodness of fit test based on the 1:2:1 Mendelian genotype ratio expected from heterozygous parents vs. the expected 1:2 ratio for a recessive lethal.

Days post-partum	+/+	P/+	P/P	*p*-Value (1:2:1)	*p*-Value (1:2)
P21-28	84	158	0	2.65×10^{-18}	0.6494

10.3389/fcell.2023.1192789

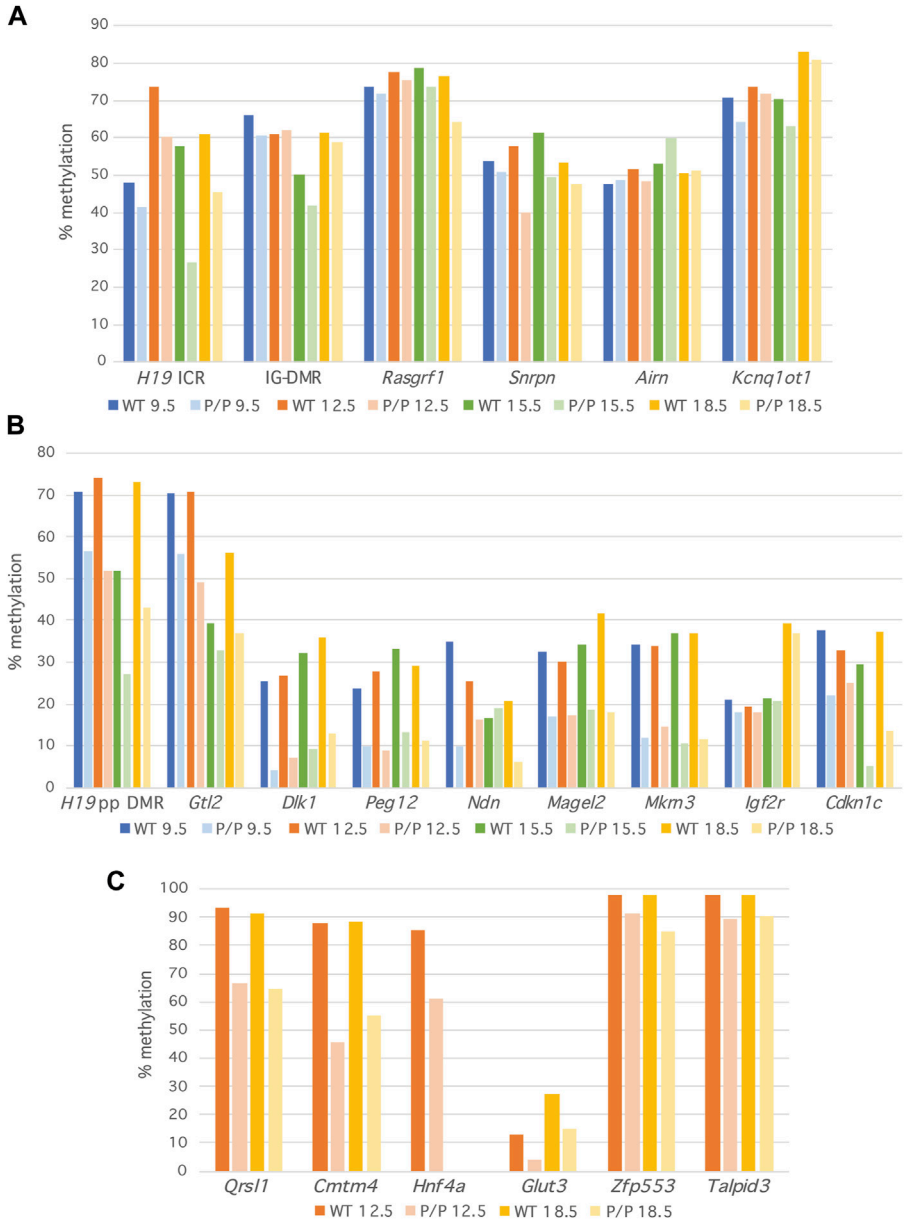

FIGURE 2
Methylation levels are minimally reduced at primary DMRs in *Dnmt1*$^{P/P}$ (P/P) embryos as compared to their wild-type siblings. Percent methylation derived at each locus from NGS data; 9.5 dpc (blue), 12.5 dpc (orange), 15.5 dpc (green) and 18.5 dpc (yellow). Data were obtained from a single wild-type or *Dnmt1*$^{P/P}$ mutant embryo at each developmental stage. **(A)** Primary DMRs. **(B)** Secondary DMRs. **(C)** Non-imprinted loci.

TABLE 3 Primary and secondary DMRs analyzed within different imprinting clusters.

Imprinting cluster	Primary DMR	Secondary DMR(s)
Igf2	*H19* ICR	*H19*-pp (promoter proximal)
Dlk1	IG-DMR	*Gtl2, Dlk1*
	Rasgrf1	
Pws	*Snrpn*	*Peg12, Ndn, Magel2, Mkrn3*
Igf2r	*Airn*	*Igf2r*
Kcnq1	*Kcnq1ot1*	*Cdkn1c*

the amount of methylation observed at primary DMRs in wild-type and *Dnmt1*$^{P/P}$ embryos was consistent in biological replicates (Supplementary Figure S3A) and throughout the embryonic stages analyzed (Figure 2A; Supplementary Table S2). The observation that there is some loss of methylation at primary DMRs in *Dnmt1*$^{P/P}$ embryos suggests that methylation is imperfectly maintained at these sequences during early embryonic development. However, as additional loss of methylation was not observed in *Dnmt1*$^{P/P}$ mutant embryos as development progressed, whatever deficit the Dnmt1 P allele has in maintaining methylation occurs early and does not accumulate. The *H19* ICR displayed more dramatic differences in DNA methylation between the wild-type and *Dnmt1*$^{P/P}$ samples than

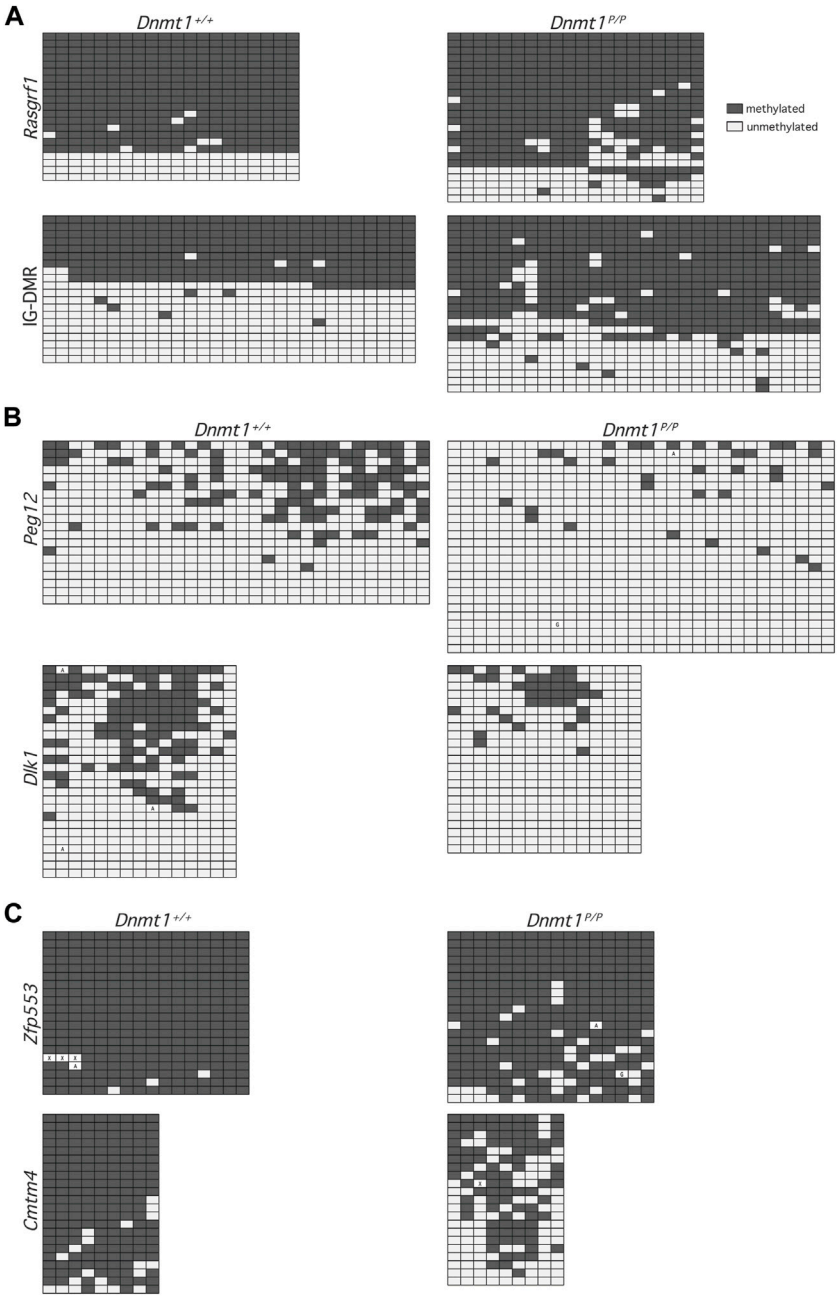

FIGURE 3
Methylation patterns across representative downsampled NGS sequences. Each row represents methylation data obtained at CpG dinucleotides within a single sequence: methylated (filled), unmethylated (open). Boxes containing an A or G represent PCR-induced error and indicate the nucleotide observed at that position; boxes containing an X represent undetermined sequence. Data were obtained from 12.5 dpc *Dnmt1*$^{+/+}$ (left) and *Dnmt1*$^{P/P}$ (right) embryos. **(A)** Primary DMRs *Rasgrf1* and IG-DMR. **(B)** Secondary DMRs *Peg12* and *Dlk1*. **(C)** Non-imprinted loci *Zfp553* and *Cmtm4*.

the other primary DMRs analyzed, accounting for three of the four primary DMR samples where methylation maintenance was below 80% in *Dnmt1*$^{P/P}$ mutant embryos (Supplementary Table S2). This could be attributed to the fact that the *H19* ICR sample sizes were consistently very small and as a result the data obtained for this locus may not as accurately reflect the DNA methylation patterns present (Supplementary Table S3). Similarly, the observation that the amount of methylation at *Airn* is higher in *Dnmt1*$^{P/P}$ samples as compared to their wild-type siblings is likely an artifact associated with the small sample size. We consistently detected methylation levels around 70% for two of the primary DMRs analyzed, *Rasgrf1* and *Kcnq1ot1*, a higher value than would be expected based on their known parent of origin-specific methylation patterns (Figure 2A; Figure 3A). We believe this is likely due to biased amplification of the methylated allele at these loci, which appears to be occurring at the same frequency in the wild-type and *Dnmt1*$^{P/P}$ embryos (Figure 3A). Methylation levels at the remaining DMRs associated with imprinted loci were detected at expected frequencies in wild-type embryos.

To assess methylation levels at additional primary DMRs in wild-type vs. *Dnmt1*$^{P/P}$ embryos, we analyzed the 15.5 dpc RRBS data generated by Shaffer *et al.* (Shaffer et al., 2015). 17 of the 22 ICRs we analyzed were not represented in the tiles generated in their analysis, including all six of the primary DMRs targeted in our study. The RRBS data illustrated that methylation was well maintained at the primary DMRs associated with *Nespas/GnasXL*, *Inpp5f* and *Peg13*, with the percent methylation in *Dnmt1*$^{P/P}$ embryos being 89, 87% and 86% the level detected in wild-type embryos, respectively. Two ICRs, *Fkbp6* and *Cdh15*, showed more variation between wild-type and *Dnmt1*$^{P/P}$ embryos, with methylation differences of 24%.

In contrast to what was observed at primary DMRs, DNA methylation levels were dramatically reduced at secondary DMRs in *Dnmt1*$^{P/P}$ embryos (Figure 2B; Supplementary Figure S3B). At most secondary DMRs, the level of methylation in *Dnmt1*$^{P/P}$ embryos varied from 13% to 85% of the amount observed in their wild-type siblings. One notable exception to this finding was observed at *Igf2r*, which displayed a minimal reduction in methylation detected in wild type vs. *Dnmt1*$^{P/P}$ embryos. This difference could be attributed to the fact that while methylation is acquired at most secondary DMRs by 9.5 dpc, the secondary DMR associated with *Igf2r* acquires methylation during late gestation (Stöger et al., 1993; Bhogal et al., 2004; Gagne et al., 2014; Guntrum et al., 2017; Nechin et al., 2019). In support of this hypothesis, the average amount of methylation observed at *Igf2r* was approximately two-fold higher in 18.5 dpc embryos than in embryos collected at earlier developmental stages (Figure 2B; Supplementary Figure S3B). Excluding the *Igf2r* results, methylation levels in *Dnmt1*$^{P/P}$ embryos were below 80% the value observed in wild-type embryos at 84% of the sequences analyzed and below 50% in 49% of the sequences analyzed (Supplementary Table S2), considerably less than what was observed at the corresponding primary DMRs. Similar to what was observed at primary DMRs, methylation levels were relatively consistent across development suggesting that for the most part, methylation levels did not change in wild type nor in *Dnmt1*$^{P/P}$ embryos once it was acquired during early post-implantation development.

Shaffer *et al.* (Shaffer et al., 2015) illustrated a global loss of DNA methylation in *Dnmt1*$^{P/P}$ mice by analyzing methylation levels using methylation-sensitive Southern blots to examine methylation levels at IAP elements as well as LUMA assays to examine methylation levels across the genome. We took a targeted approach to examine DNA methylation levels at non-imprinted, single copy sequences. We analyzed methylation at two loci reported to have tissue-specific DNA methylation patterns, *Glut3* and *Hnf4a* (Yagi et al., 2008; Ganguly et al., 2014) as well as four ZFP57-bound loci displaying strain-specific methylation in embryonic stem cells: *Zfp553, Qrsl1, Cmtm4* and *Talpid3* (Strogantsev et al., 2015). All of the non-imprinted loci showed a reduction in the amount of DNA methylation present in *Dnmt1*$^{P/P}$ embryos as compared to their wild type siblings (Figure 2C), but the extent to which DNA methylation was lost varied between loci. Methylation was reasonably well maintained at *Zfp553* and *Talpid3*, but was dramatically reduced at *Glut3, Hnf4a, Cmtm4* and *Qrsl1*. Examination of these sequences using the CpG Island Finder (Gardiner-Garden and Frommer, 1987; EMBOSS, 2023), and the UCSC Genome Browser (UCSC Genome Browser) illustrated that CpG density varies at the loci examined in our study. All six primary DMRs and all nine secondary DMRs contain CpG islands or are CpG-rich. In contrast, the regions of *Zfp553*, *Glut3* and *Hnf4a* analyzed are CpG-rich, but the methylated regions of *Cmtm4*, *Qrsl1* and *Talpid3* examined in this study are CpG-poor (Supplementary Figure S4A). Therefore, CpG density does not correlate with the ability of the *Dnmt1* P allele to maintain methylation.

We further investigated these loci to determine whether *Zfp553* and *Talpid3*, non-imprinted sequences that retain methylation in *Dnmt1*$^{P/P}$ mutant embryos, share any features with primary DMRs that might make them resistant to methylation loss. While *Zfp553* and *Talpid3* display methylation in embryonic stem cells that is presumably gametic in origin (Strogantsev et al., 2015), so do *Qrsl1* and *Cmtm4*, which show dramatic loss of methylation in *Dnmt1*$^{P/P}$ mutant embryos (Figure 2C). Furthermore, Shaffer and others (Shaffer et al., 2015) illustrated that methylation is lost at gametically methylated IAP elements in *Dnmt1*$^{P/P}$ mutant embryos. Together, these data suggest that gametic inheritance of methylation does not predict a sequence's ability to retain methylation in the presence of the P allele form of Dnmt1. We additionally assessed each locus for other chromatin features, including euchromatin vs. heterochromatin status, enhancer vs. promoter vs. transcription unit status, the presence of the transcriptionally permissive histone modifications H3K4me1, H3K4me3 and H3K27ac, and the presence of histone modifications associated with transcriptional repression and/or DNA methylation, H3K9me3, H3K36me3 and H3K27me3, in 12.5 dpc mouse midbrain using the UCSC Genome Browser (Supplementary Figure S4B). While both *Zfp553* and *Talpid3* are enriched for H3K36me3, the only primary DMR containing this modification is *Airn*. Based on these analyses, there was no apparent association between a particular chromatin signature and the ability of the corresponding sequence to retain methylation in *Dnmt1*$^{P/P}$ mutant embryos. Of note, many of the DMRs associated with imprinted loci displayed both permissive and repressive modifications, consistent with the fact that the parental alleles have opposing epigenetic states.

3.4 DNA methylation loss is randomly distributed across individual sequences in *Dnmt1*$^{P/P}$ embryos

The lower levels of DNA methylation observed in *Dnmt1*$^{P/P}$ embryos could be due to loss of methylation across a subset of DNA strands, loss of methylation at specific sequences within the DNA, or non-specific loss of methylation across all DNA strands. To distinguish between these possibilities, we extracted a subset of the NGS sequences obtained and analyzed the DNA methylation profiles of individual sequences. Analysis of the extracted primary DMR sequences for *Rasgrf1* and the *Dlk1-Dio3* IG-DMR showed that the methylation profile amongst alleles obtained from *Dnmt1*$^{P/P}$ embryos was not significantly different than it was in their wild-type

siblings (p values = 0.1802, 0.4839), and further illustrated that methylation was generally well maintained across the sequences analyzed (Figure 3A; additional data not shown). In contrast, the methylation patterns observed at the secondary DMRs associated with *Dlk1* and *Peg12* in wild type vs. *Dnmt1*$^{P/P}$ embryos showed significant loss of methylation across each locus (p values = 0.0041, 0.0088), with inconsistent methylation remaining (Figure 3B; additional data not shown). Similar trends were observed at non-imprinted loci that showed either modest or dramatic differences in methylation between wild type and *Dnmt1*$^{P/P}$ embryos (Figure 3C). Methylation was better maintained at *Zfp553* in *Dnmt1*$^{P/P}$ embryos and was distributed evenly across the sequences analyzed, although the loss of methylation between wild type and *Dnmt1*$^{P/P}$ embryos was significant (p-value = 0.0005). *Cmtm4* displayed a dramatic and dispersed loss of methylation in *Dnmt1*$^{P/P}$ embryos (p-value = <0.0001). Overall, these data suggest that methylation is poorly maintained across loci in *Dnmt1*$^{P/P}$ embryos rather than lost entirely from specific sequences.

4 Discussion

DNA methyltransferases carry out both *de novo* and maintenance methylation, with Dnmt1 primarily functioning as the maintenance methyltransferase and *de novo* methylation resulting from the activity of Dnmt3a and Dnmt3b (Li and Zhang, 2014; Hervouet et al., 2018). The fidelity of Dnmt1 in maintaining methylation patterns by methylating newly synthesized daughter strands has been estimated to be 95%–96% (Ushijima et al., 2003; Laird et al., 2004; Vilkaitis et al., 2005), yet high levels of hemimethylation have been observed at some genomic loci, including secondary DMRs associated with imprinted genes, suggesting inconsistent maintenance of methylation at these sequences (Guntrum et al., 2017; Nechin et al., 2019). We previously found that 30%–50% of the CpG dyads in secondary DMRs are hemimethylated, suggesting that DNA methylation is passively and/or actively lost at these sequences, possibly due to 5-hydroxymethylcytosine enrichment at these sequences (Guntrum et al., 2017; Nechin et al., 2019) (TDavis lab, data not shown). Despite the fact that hemimethylation should result in reduced methylation levels following subsequent rounds of DNA replication, methylation levels remain constant at secondary DMRs throughout development, leading us to propose that methylation at these loci may be lost due to reduced Dnmt1 fidelity and restored by *de novo* methylation via Dnmt3a/3b. Indeed, despite the established roles of Dnmt1 and the Dnmt3 family proteins, evidence suggests that Dnmt3 enzymes may function cooperatively with Dnmt1 to maintain methylation at repetitive and CpG-rich sequences (Liang et al., 2002; Chen et al., 2003; Jones and Liang, 2009; Liu et al., 2022). While Dnmt1 appears to be sufficient for maintaining DNA methylation in ES cells at many primary DMRs associated with imprinted genes, Dnmt3a and 3b contribute to maintenance methylation at several ICRs including *H19* and IG-DMR and Dnmt3b has been shown to be necessary for maintaining methylation at *Rasgrf1* (Hirasawa et al., 2008; Liu et al., 2022). Liu and others (Liu et al., 2022) further suggested that Dnmt3a and 3b may be more important than Dnmt1 for maintaining methylation at approximately half of the secondary DMRs analyzed in their study.

The suggestion that Dnmt3a/3b may function cooperatively with Dnmt1 in maintaining methylation raises a question as to what directs Dnmt3a and/or Dnmt3b to methylate secondary DMRs and other loci throughout development. We hypothesized that primary DMRs signal the *de novo* acquisition of methylation at secondary DMRs in the same imprinting cluster, and that this activity occurs both during the initial acquisition of methylation at secondary DMRs during post-implantation development and throughout the remainder of development. Several lines of evidence support this hypothesis, best illustrated at the *Dlk1-Dio3* imprinting cluster. Analysis of methylation and gene expression patterns in patients with IG-DMR and *MEG3*-DMR microdeletions illustrate the hierarchical way in which DNA methylation is established across this imprinting cluster (Kagami et al., 2010; Beygo et al., 2015). Furthermore, deletion of the tandem repeat in the paternally-inherited IG-DMR, its replacement with CpG-free sequences and targeted demethylation of the repeat via epigenetic editing in mice all resulted in loss of methylation at the IG- and *Gtl2*-DMRs and concomitant loss of imprinting at both maternally- and paternally-expressed imprinted genes (Saito et al., 2018; Hara et al., 2019; Aronson et al., 2021; Kojima et al., 2022). These experiments demonstrated that the methylation status of the tandem repeat within the ICR is necessary both to establish and maintain parental epitypes and expression profiles across this cluster.

If the methylation status at the primary DMR is the primary driver of the methylation status at the corresponding secondary DMRs within the same imprinting cluster, then methylation should be maintained equally well at primary and secondary DMRs in *Dnmt1*$^{P/P}$ mutant mice, but our results did not support this hypothesis. Despite the fact that embryos homozygous for the *Dnmt1* P allele maintain methylation relatively well at primary DMRs associated with imprinted genes (data herein and (Shaffer et al., 2015)), we found that methylation was dramatically reduced at secondary DMRs in *Dnmt1*$^{P/P}$ embryos as compared to their wild-type siblings. Therefore, while we cannot exclude the possibility that the Dnmt3 proteins work cooperatively with Dnmt1 in maintaining methylation at secondary DMRs, their action cannot compensate for the mutant Dnmt1 protein. This could be because while wild-type Dnmt1 interacts with Dnmt3a/b (Kim et al., 2002; Qin et al., 2011), mutant Dnmt1 is unable to do so either because of its disrupted structure and/or its low concentration (Shaffer et al., 2015). Failure of such an interaction could impact the maintenance of methylation that requires the coordinated activity of both Dnmt1 and Dnmt3 proteins. To further explore whether the Dnmt3 proteins play a role in maintaining methylation at imprinted loci, it will be important to assess whether either or both of these enzymes localize to primary and/or secondary DMRs in wild-type and *Dnmt1*$^{P/P}$ mutant embryos at developmental stages after methylation is initially established.

The *Dnmt1* P allele has dramatically different effects at different loci within the mouse genome. This mutation is located within an N-terminal intrinsically disordered domain that interacts with at least 8 different proteins that may play roles in regulating Dnmt1 activity both broadly and at specific sequences (Liu et al., 2017). In support of this hypothesis, different mutations within the IDD impact DNA methylation in different ways, with some

mutations affecting methylation at ICRs but having no effect on non-ICR methylation while other mutations have the opposite effect (Borowczyk et al., 2009; Shaffer et al., 2015). The ability of different, expressed, IDD-deleted forms of Dnmt1 protein to selectively maintain DNA methylation at imprinted vs. non-imprinted sequences supports the idea that IDD-mediated protein-protein interactions provide specificity to Dnmt1 activity (Borowczyk et al., 2009). Shaffer and others (Shaffer et al., 2015) suggested that the region altered in the P allele, which is specific to *Mus* and *Rattus*, might be important for methylation of species-specific sequences. The P allele mutation results in a local increase in the intrinsic disorder score which likely impacts the way in which Dnmt1 interacts with other proteins (Liu et al., 2017) and may therefore affect the ability of Dnmt1 to interact efficiently with proteins that generally guide it to hemimethylated DNA, such as UHRF1 and MeCP2 (Kimura and Shiota, 2003; Bostick et al., 2007; Sharif et al., 2007; Zhang et al., 2011). While alteration of these sequences may disrupt protein-protein interactions and Dnmt1 activity, Shaffer *et al.* also illustrated that Dnmt1 protein levels are dramatically reduced in $Dnmt1^{P/P}$ mutant mid-to late gestation embryos and suggested that failure of the P allele mutant Dnmt1 protein to interact with other proteins may lead to its degradation, compromising its ability to methylate newly replicated sequences (Shaffer et al., 2015). Since global methylation is significantly decreased in $Dnmt1^{P/P}$ mid-to late gestation embryos, but ICR methylation is maintained ((Shaffer et al., 2015) and data herein), it is possible that Dnmt1 activity at ICRs is less dependent on the region disrupted by the P allele because the mechanism by which Dnmt1 maintains methylation at these sequences is different than the mechanism it uses to more generally maintain methylation across the mouse genome. Alternatively, Dnmt1 may have a higher affinity for ICR sequences, resulting in its activity primarily being directed to those genomic regions even when protein levels are low. It is also possible that methylation at primary DMRs is maintained in an alternate way in $Dnmt1^{P/P}$ individuals, perhaps through the action of Dnmt3a/3b. In support of this hypothesis, Thakur and others (Thakur et al., 2016) demonstrated the ability of a Dnmt3a isoform to restore methylation at primary DMRs in *Dnmt3a/3b* knock-out ES cells.

Given the dispersed pattern of methylation at imprinted and non-imprinted loci in $Dnmt1^{P/P}$ embryos, we suggest that methylation fidelity is reduced in the presence of this mutation because the mutant Dnmt1 fails to faithfully recognize hemimethylated sequences and methylate the newly synthesized complement, thereby leading to an overall loss of methylation. Preliminary data from our lab illustrates an increase in hemimethylation in sequences derived from $Dnmt1^{P/P}$ embryos: we found significantly more hemimethylation at the IG-DMR in $Dnmt1^{P/P}$ 12.5 dpc embryos as compared to their wild-type siblings (18.34% vs. 12.89%, $p = 0.0407$; data not shown). Additional analyses will be necessary to further test this hypothesis.

Despite the dramatic loss of global methylation in $Dnmt1^{P/P}$ mice, embryonic development appears to progress relatively normally although $Dnmt1^{P/P}$ individuals are unable to survive after birth, presumably as a consequence of altered gene expression patterns. While DNA methylation at promoters correlates with gene silencing (Li and Zhang, 2014), the precise amount of promoter DNA methylation required to achieve silencing at individual loci has not been studied in detail and is likely not generalizable. It is known that loss of Dnmt1 activity has a dramatic impact on imprinted gene expression (Li et al., 1993; Caspary et al., 1998; Nakagaki et al., 2014), but in these mutants methylation is dramatically reduced at both primary and secondary DMRs, complicating the ability to determine how loss of methylation specifically at secondary DMRs, without altering their primary sequence, impacts imprinted gene expression. The differential effects of the P allele on methylation levels at primary vs. secondary DMRs associated with imprinted genes provides an opportunity for assessing the relative importance of methylation at primary vs. secondary DMRs in regulating the expression of individual imprinted genes, and these experiments are currently underway.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA under the following accession numbers SAMN34130389, SAMN34130390, SAMN34130391, SAMN34130392, SAMN34130393, SAMN34130394, SAMN34130395, SAMN34130396, SAMN34130397, SAMN34130398.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Bryn Mawr College.

Author contributions

SR, LG, AA, and CS-L contributed to experimental design and carried out molecular genetic studies and data analysis. TD conceived of the study and experimental design, oversaw molecular genetic studies and data analysis and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Epigenetic control and genomic imprinting dynamics of the Dlk1-Dio3 domain

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Genomic imprinting is an epigenetic process whereby genes are monoallelically expressed in a parent-of-origin-specific manner. Imprinted genes are frequently found clustered in the genome, likely illustrating their need for both shared regulatory control and functional inter-dependence. The *Dlk1-Dio3* domain is one of the largest imprinted clusters. Genes in this region are involved in development, behavior, and postnatal metabolism: failure to correctly regulate the domain leads to Kagami–Ogata or Temple syndromes in humans. The region contains many of the hallmarks of other imprinted domains, such as long non-coding RNAs and parental origin-specific CTCF binding. Recent studies have shown that the Dlk1-Dio3 domain is exquisitely regulated *via* a bipartite imprinting control region (ICR) which functions differently on the two parental chromosomes to establish monoallelic expression. Furthermore, the *Dlk1* gene displays a selective absence of imprinting in the neurogenic niche, illustrating the need for precise dosage modulation of this domain in different tissues. Here, we discuss the following: how differential epigenetic marks laid down in the gametes cause a cascade of events that leads to imprinting in the region, how this mechanism is selectively switched off in the neurogenic niche, and why studying this imprinted region has added a layer of sophistication to how we think about the hierarchical epigenetic control of genome function.

KEYWORDS

Dlk1-Dio3 domain, genomic imprinting, CTCF, chromatin architecture, DNA methylation, long non-coding RNA

Introduction

Genomic imprinting in mammals

Genome function is regulated temporally and tissue specifically through the orchestrated interplay of regulatory factors, genomic features, and epigenetic states. Epigenetic modifications are dynamic during development and across the cell cycle. A hierarchy of successive epigenetic states, including DNA methylation, ensures the creation of healthy individuals. In mammals, extensive epigenetic reprogramming events occur during germ cell development, fertilization, and early embryogenesis (Smith and Meissner, 2013). Although DNA methylation is essential for normal mammalian development, there appear to be multiple ways in which it can regulate and maintain cell fate and function, which remain incompletely understood. Although intensively studied, the association between DNA

methylation and transcription is often correlative, with little experimental evidence to support causal relationships.

One major process in which predictive and causal relationships between DNA methylation and gene expression is more comprehensively understood is that of mammalian genomic imprinting (Bartolomei and Ferguson-Smith, 2011; Ferguson-Smith, 2011). Genomic imprinting is an epigenetically regulated process causing genes to be expressed from one chromosome homolog according to the parent-of-origin. Imprinting is highly conserved in eutherian mammals, and mouse studies have provided insights into the repertoire of developmental and physiological pathways regulated by imprinted genes (Ferguson-Smith and Bourc'his, 2018; Cleaton et al., 2014). Failure to correctly establish and maintain imprints is associated with developmental syndromes, including growth abnormalities, neurological and metabolic disorders, and numerous forms of cancer (Uribe-Lewis et al., 2011; Ishida and Moore, 2013). Imprinted genes are also highly expressed in the developing and adult brain, and are implicated in numerous brain functions, including behavior (Keverne, 1997; Liu et al., 2010; Furutachi et al., 2013; Tsan et al., 2016). Several syndromes that result from dysregulation of imprinted loci involve brain dysfunction, such as Prader–Willi syndrome (PWS) (Aman et al., 2018), Angelman syndrome (Nicholls et al., 1998), Turner syndrome (Bondy, 2006; Lepage et al., 2013), autism (Flashner et al., 2013), bipolar depression (Pinto et al., 2011), and schizophrenia (Ingason et al., 2011; Isles et al., 2016).

Genomic imprinting in laboratory mice is a tractable model for studying epigenetic regulation as the two parentally inherited, genetically identical genomic regions within the same nucleus express a different repertoire of genes in a parental-origin-specific manner. Imprinting is established in the germ cells through the differential deposition of DNA methylation in the two parental germlines (Smallwood et al., 2011). Differential DNA methylation marks (DMRs) at imprinting control regions (ICRs) are maintained in the post-fertilization period and protected from the global methylation erasure in the early embryo by KRAB zinc finger proteins ZFP57 and ZFP445 in order to maintain the epigenetic memory of parental origin (Takahashi et al., 2019). However, the dynamic hierarchy of events initiated by the ICRs that leads to the long-range domain-wide temporal and tissue-specific behavior of imprinted genes is not fully understood.

Studies assessing global as well as locus-specific alterations to ICRs have emphasized that loss of imprinting results in reciprocal effects on imprinted genes with the biallelic expression of some genes within the cluster and biallelic repression at others (Tucker et al., 1996; Li et al., 2008; Demars and Gicquel, 2012; Azzi et al., 2014; Takahashi et al., 2019). Phenotypically, perturbations to individual imprinted genes exert effects in numerous developmental and physiological pathways (Kalish et al., 2014; Tucci et al., 2019). Together, this has led to the prevailing notion in the field that at least some imprinted genes are dosage-sensitive. Deletions or insertions of the genes themselves, and aberrations that disrupt the pattern of imprinted gene expression, like mutations in the ICR or uniparental disomy (UPD), contribute to tumor progression and disease. In addition, the balance between maternally and paternally inherited genes can modulate phenotypes. For instance, PWS patients with maternal UPD or with ICR deletions have increased maternal expression along with a loss of paternal gene expression. These individuals are far more associated with psychotic illnesses than PWS patients with individual paternal gene deletion genotypes (Nicholls et al., 1998; Tucci et al., 2019). Yet, because the intricate epigenetic control at imprinted clusters controls the parent-specific expression of multiple genes, it is difficult to assign the relative contribution of the individual gene dosage to the resulting physiological phenotypes. Utilizing a systemic set of mutants at a single imprinted domain allows us to dissect the relationship between allelic expression, dosage, epigenetic control, and phenotypical outcomes.

Genomic imprinting at the Dlk1-Dio3 domain

One of the largest imprinted clusters in mammals is the 1.2 Mb Dlk1-Dio3 domain. This region is conserved between mice and humans, and is one of the major developmentally regulated mammalian imprinted domains. Failure to correctly imprint genes in this cluster in humans leads to Temple or Kagami–Ogata syndromes, both of which exhibit neurological, developmental, and behavioral impairments (Ioannides et al., 2014; Kagami et al., 2015). In mice, both maternal and paternal UPDs containing this domain lead to prenatal lethality, further illustrating the developmental importance of the correct dosage of genes in the region.

Four protein-coding genes, *Dlk1*, *Rtl1*, *Dio3*, and one isoform of *Begain* (located upstream of *Dlk1* beyond a large LINE1-rich region), are preferentially expressed from the paternal allele (Figure 1A) (Tierling et al., 2009). *Dlk1* encodes delta like non-canonical Notch ligand 1. Paternal loss of the gene leads to partial neonatal lethality, and those animals that survive display post-natal growth retardation, increased adiposity, and skeletal defects (Moon et al., 2002). More recently, mice lacking *Dlk1* have also been shown to be prone to anxiety-like behaviors (García-Gutiérrez et al., 2018). *Rtl1* is a Ty3-gypsy retrotransposon-derived neogene that has evolved a function in placentation in eutherians. Loss of the paternal copy of *Rtl1* causes placental retardation and, in some mouse strains, can cause delayed parturition (Youngson et al., 2005; Sekita et al., 2008; Ito et al., 2015; Kitazawa et al., 2020; Kitazawa et al., 2021). The most distal imprinted gene in the domain, *Dio3*, encodes type 3 iodothyronine deiodinase, which is a negative regulator of thyroid hormone metabolism (Tsai et al., 2002). *Dio3* null mice show partial neonatal lethality and postnatal growth restriction. However, paternal loss of the gene leads to a much milder phenotype, reflecting the less stringent imprinting of this gene (Elena Martinez et al., 2014).

The maternally inherited chromosome expresses multiple imprinted noncoding transcripts, including *Gtl2* (also known as *Meg3*) (Figure 1). *Gtl2* is a long non-coding RNA (lncRNA) that is downregulated or lost in numerous human cancers, including breast and colorectal cancers (Makoukji et al., 2016; Buccarelli et al., 2020). *Gtl2* is also thought to form a long polycistronic transcript along with its associated transcripts *Rian* and *Mirg*, and acts as a host for multiple snoRNAs and miRNAs, including the miR-379/miR-410 cluster, all of which are driven by the *Gtl2* promoter (Cavaillé et al., 2002; Seitz et al., 2004; Tierling et al., 2006). In humans, the miRNAs in this cluster have been shown to be

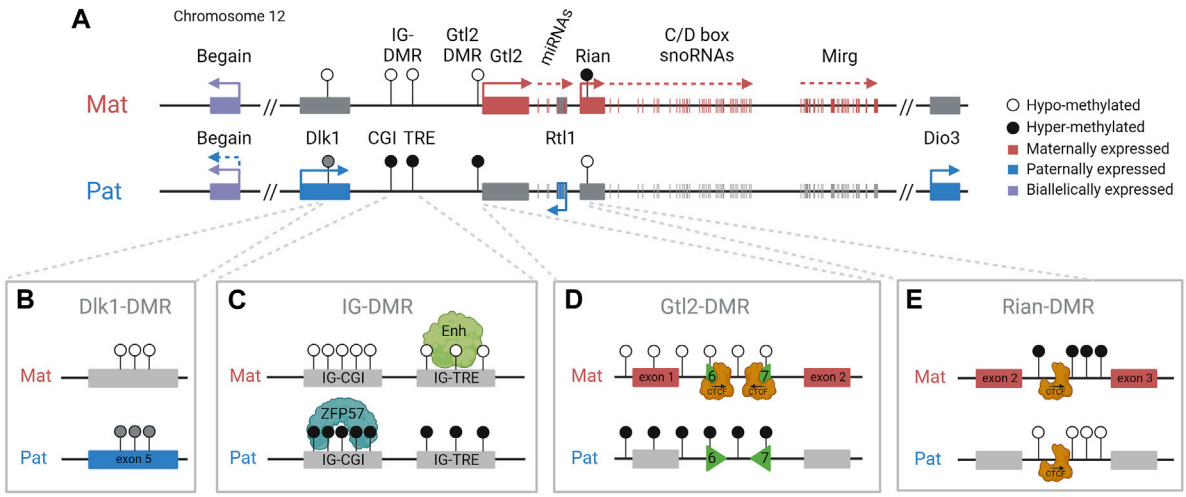

FIGURE 1
Dlk1-Dio3 imprinted domain and key regulatory features. **(A)**. The paternally inherited chromosome expresses *Dlk1*, *Rtl1*, *Dio3*, and one isoform of *Begain*. The maternally inherited chromosome expresses *Gtl2/Meg3*, *antiRtl1*, and arrays of snoRNAs and miRNAs. The IG-DMR is methylated in sperm, is unmethylated in oocytes, and is the imprinting control region for the domain. DMRs are indicated by circles: black (methylated) and white (unmethylated). **(B)**. The somatic Dlk1-DMR, in the last exon of the *Dlk1* gene, is differentially methylated with partial methylation on the paternal chromosome. **(C)**. The IG-DMR contains a CpG island (CGI) that binds ZFP57 on the methylated paternal copy and a transcriptional regulatory element (TRE) that has an enhancer-like function on the unmethylated maternal copy. **(D)**. The Gtl2-DMR contains two differentially methylated CTCF binding sites (CTCF6 and 7) binding CTCF only on the unmethylated maternal chromosome. **(E)**. The Rian-DMR, in the second intron of the *Rian* gene, is methylated in the reverse pattern as the IG-DMR and Gtl2-DMR, methylated on the maternal chromosome, and hypomethylated on the paternal chromosome.

downregulated in the pancreatic islets of donors with type 2 diabetes (Kameswaran et al., 2014). Neonatal mice with a maternal deletion of the entire miR-379/miR-410 cluster are hypoglycemic and show impaired transition from fetal to postnatal metabolism (Labialle et al., 2014). These mice have also been shown to have increased anxiety-related behaviors in adulthood (Marty et al., 2016). Therefore, appropriate expression of genes in this region is essential for the lifelong health of mammals, and understanding the epigenetic regulation of these genes has significant biomedical relevance for a diverse range of processes.

Imprinting at the *Dlk1-Dio3* locus is regulated by the germline-derived intergenic DMR (IG-DMR) (Lin et al., 2003), which is required for regulating parent-specific expression in this locus (Rocha et al., 2008). This DMR is normally methylated in sperm and not methylated in oocytes, and maintains this parent-specific pattern throughout development (Figure 2A). After implantation, secondary somatic DMRs are established in the region: one at the promoter of the *Gtl2* gene (the Gtl2-DMR) and another tissue-specific partial DMR over the fifth exon of *Dlk1*. Both of these somatic DMRs are also hypermethylated on the paternal chromosome (Takada et al., 2000). A third somatic DMR is located in the second intron of *Rian* (known as *MEG8* in humans) (Zeng et al., 2014). This DMR is dependent on the IG-DMR, but in contrast to the other DMRs in the region, it gains secondary methylation on the maternally inherited chromosome (Figure 3). Here, we review the current knowledge on how differential epigenetic landscapes, genetic elements, and transcription are exquisitely coordinated to regulate genome function in this domain.

Regulation and hierarchy of imprinting at the Dlk1-Dio3 region

The IG-DMR spans approximately 5 kb between *Dlk1* and *Gtl2*. Maternal deletion of this ICR region leads to paternalization of the maternal chromosome, and mice die between e16.5 and birth (Lin et al., 2003; Lin et al., 2007). However, on paternal transmission, the deletion has no effect (Figure 2B). The IG-DMR contains a small CpG island comprising seven tandemly repeated sequences, five of which contain a ZFP57 binding motif (Takada et al., 2002). The sequence-specific zinc finger protein, ZFP57, only binds to the methylated paternal chromosome, where it interacts with TRIM28, which in turn recruits the repressive epigenetic machinery, including DNMTs and heterochromatin-associated proteins such as SETDB1 and HP1, thereby maintaining the methylation memory of the germline imprint in early development in an environment where most epigenetic modifications elsewhere are being erased (Quenneville et al., 2011; Messerschmidt et al., 2012).

Recently, the IG-DMR was shown to be a bipartite element comprising two distinct functional elements (Aronson et al., 2021). In addition to the CpG island (IG-CGI) described earlier, it also contains a transcriptional regulation element (IG-TRE) that can bind pluripotency transcription factors in mouse embryonic stem cells (mESCs) and exhibit active enhancer marks (H3K27ac) and nascent transcription (Danko et al., 2015; Luo et al., 2016) (Figure 1C). It was, therefore, suggested that the IG-TRE serves as a putative enhancer, driving the expression of the maternally inherited genes within the domain.

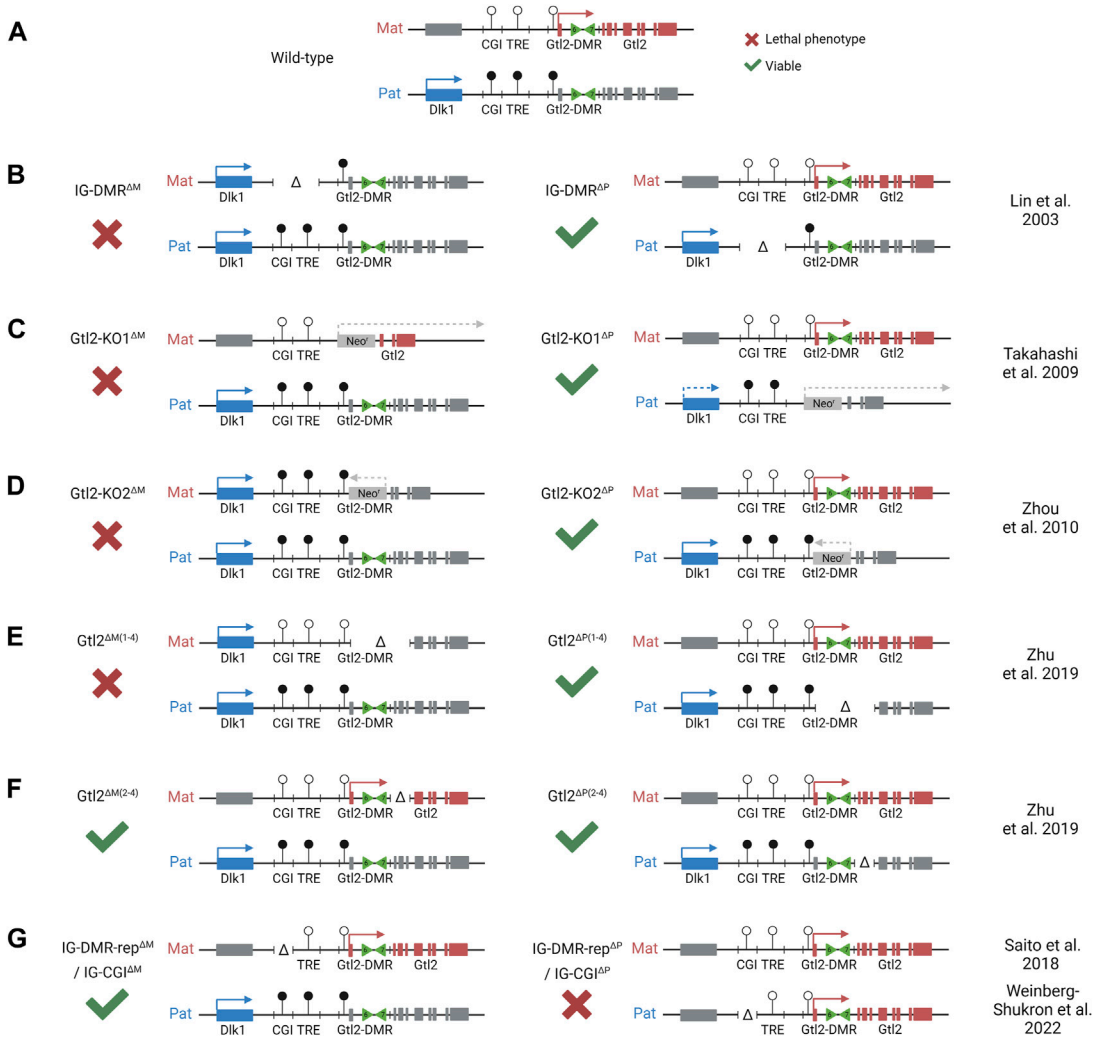

FIGURE 2
Integrated model depicting effects of different mice models with deletions at the Dlk1-Dio3 locus. Colored boxes represent expression from maternal (red) and paternal (blue) alleles. Gray boxes represent allelically repressed genes. Lollipops represent methylated (black) and unmethylated (white) regulatory elements. **(A)**. WT pattern of expression at the Dlk1-Dio3 locus. **(B)**. Maternal deletion of the entire IG-DMR results in a maternal-to-paternal epigenotype switch. **(C)**. Maternal replacement of *Gtl2* exons 1–7 with a forward-facing neomycin resistant cassette results in partial loss of the maternal gene expression. The same paternal substitution results in partial loss of the paternal gene expression. **(D)**. Maternal replacement of *Gtl2* exons 1–6 with a reverse facing neomycin resistant cassette results in loss of the maternal gene expression and activation of the maternal *Dlk1* expression. The same paternal substitution had no effect on the expression of methylation in the region. **(E)**. Maternal deletion of *Gtl2* exons 1–4 (including part of the Gtl2-DMR) results in a maternal-to-paternal epigenotype switch, which is similar to the IG-DMR deletion. However, methylation at the IG-DMR is not affected by this deletion. Paternal deletion has no effect. **(F)**. Neither maternal nor paternal deletion of *Gtl2* exons 2–4 has an effect on the expression of methylation at the Dlk1-Dio3 locus, indicating that the Gtl2-DMR, not the *Gtl2* gene, regulated imprinting at this region. **(G)**. An isolated paternal deletion of the IG-CGI results in a paternal-to-maternal epigenotype switch. Although maternal deletion has no effect, indicating that the IG-CGI is the primary methylation mark on the paternal chromosome, it is dispensable from the maternal chromosome.

Furthermore, these data also indicate that the IG-CGI is required to inactivate the paternal IG-TRE and maintain a repressive chromatin landscape on the paternal chromosome (Stelzer et al., 2016; Kojima et al., 2022; Weinberg-Shukron et al., 2022). Surprisingly, in contrast to the full IG-DMR deletion (maternal to paternal epigenotype switch but no effect upon paternal transmission), an isolated paternally derived deletion of the IG-CGI results in the reciprocal paternal-to-maternal epigenotype switch, with the IG-TRE becoming hypomethylated on the paternal chromosome (Saito et al., 2018) (Figure 2G).

Together, these findings indicate that the key element regulating imprinted expression on the maternal chromosome is the IG-TRE, which promotes activity from the maternally inherited non-coding RNAs, with the unmethylated IG-CGI being irrelevant for that function. In addition, the key element on the paternally inherited chromosome is a germline-methylated IG-CGI that is required for methylation and repression of the IG-TRE.

Some ICRs, including the IG-DMR, have also been shown to bind AFF3, a component of the super elongation complex-like 3

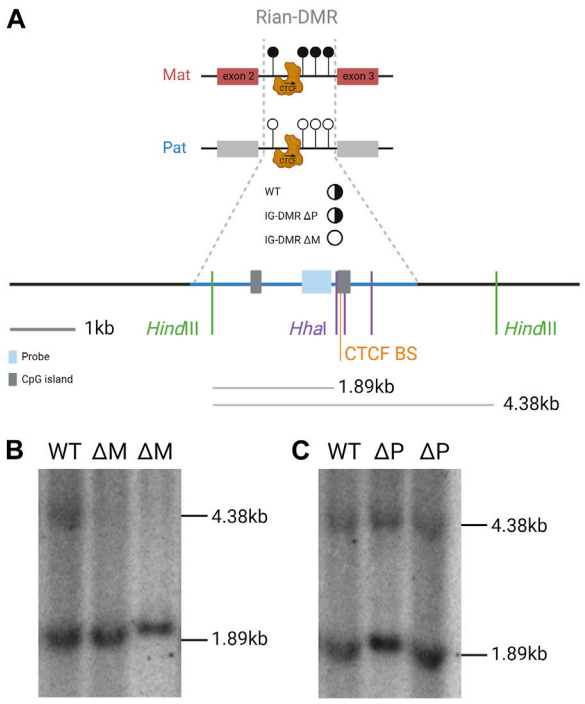

FIGURE 3
Rian-DMR is biallelically unmethylated in e16 embryos that inherit the IG-DMR deletion from their mother. **(A)**. Scale summary of the Southern blot displayed in part b relative to sequence features of the region and the location of the hybridization probe and the digest fragments that are hybridized with the probe. Full black circle, fully methylated; half black half white circle, differentially methylated; white circle, unmethylated. Blue shade, probe; gray shade, CpG islands; blue line, Rian-DMR +/-1 kb. **(B,C)**. Methylation-sensitive restriction-digested Southern blot of genomic DNA from e16 embryos hybridized with a Rian-DMR-specific probe. The genomic DNA was digested with *Hind*III in combination with *Hha*I in all lanes. WT, wild-type embryo; ΔM, IG-DMR maternally transmitted knockout embryo; ΔP, IG-DMR paternally transmitted knockout embryo.

(SEC-L3), on the methylated allele in ESCs where it is thought to interact with the ZFP57/TRIM28 complex (Luo et al., 2016). However, the function of this interaction is not clear as depletion of AFF3 in ESCs leads to decreased expression of the maternally expressed genes in the *Dlk1-Dio3* region, demonstrating that it does not cooperate in protecting the IG-DMR from demethylation. Intriguingly, AFF3 also binds to a second region at the 3' side of the IG-DMR downstream of the IG-TRE. Here, AFF3 is co-bound with ZFP281 but only on the unmethylated maternal copy (Wang et al., 2017). ZFP281 is a zinc finger protein that has previously been reported to act as both a transcriptional activator and a repressor (Wang et al., 2008). In the *Dlk1-Dio3* locus, depletion of ZFP281 from ESCs leads to decreased AFF3 binding at this downstream region, but not at the methylated IG-CGI. As depletion of AFF3 leads to decreased expression of *Gtl2*, *Mirg*, and *Rian*, this suggests that the downstream bound region is relevant for AFF3 function and that it also acts as an enhancer for maternally expressed genes (Wang et al., 2017). Whether this second region is acting in concert with the IG-TRE to control the expression of *Gtl2* and its associated transcripts remains to be established.

The combined results from the IG-DMR and the IG-CGI deletion indicate that the paternal IG-CGI is required to inactivate the paternal IG-TRE and maintain a repressive chromatin landscape on the paternal chromosome; this same element is dispensable on the maternal chromosome that is normally not methylated. On the other hand, the IG-TRE is dominant over the IG-CGI on the maternal chromosome where it is required to establish maternal gene expression and prevent methylation at the Gtl2-DMR. Together, the paradoxical effects imposed by distinct deletions within the IG-DMR represent an attractive experimental framework for dissecting the impact of changes in gene dosage on embryonic phenotypes. Synthesizing the result of the two genetic models shows that normal development cannot occur with biallelic expression of maternal genes and repression of *Dlk1* or with biallelic expression of *Dlk1* and repression of maternal transcripts. In both models, as in WT, monoallelic expression of the genes in this locus is consistent with normal development.

In accordance with that, flipping imprinting on both alleles produced viable offspring, showing that the parental origin of the imprint is irrelevant, provided appropriate balanced gene expression is established and maintained at this locus (Weinberg-Shukron et al., 2022). This has been demonstrated for another imprinted gene as well, where *Zdbf2* dosage, regardless of parental origin, regulates postnatal body weight (Glaser et al., 2022). These studies emphasize the importance of exquisite dosage control by genomic imprinting and the adaptability of this epigenetically regulated mechanism in particular developmental contexts (Liao et al., 2021).

The role of the Gtl2-DMR and Gtl2 lncRNA in regional control

Monoallelic expression of *Gtl2/Meg3* exclusively from the maternally inherited chromosome is first observed in e3.5 blastocysts (Nowak et al., 2011; Sato et al., 2011). This imprinted expression precedes the acquisition of methylation at the *Gtl2* promoter on the paternal chromosome that is not observed until after e5.5. This suggests that transcription from the maternal promoter protects the maternal allele from gaining methylation and that methylation on the paternal chromosome occurs secondary to and in the absence of transcription. Once established, the Gtl2-DMR extends from the promoter into the first intron of the gene. Mouse models deleting the maternal Gtl2-DMR recapitulate the full ICR deletion, with the downregulation of maternally expressed genes, upregulation of paternally expressed genes, and embryos dying *in utero* (Takahashi et al., 2009; Zhou et al., 2010; Zhu et al., 2019) (Figures 2C–E). Furthermore, a patient with a maternal microdeletion of the Gtl2-DMR presented with features similar to UPD(14)Pat or maternal IG-DMR deletion patients (Kagami et al., 2010). Together, these data indicate that the unmethylated Gtl2-DMR on the maternal chromosome, once established, is able to act as an imprinting control region for the entire domain, but it is unclear whether this is by the Gtl2 lncRNA itself or *via* direct *cis*-acting elements within the DMR.

The *Gtl2* gene transcribes a long non-coding RNA whose function as a regulatory transcript continues to be explored, and several different roles have been proposed. *MEG3*, the human

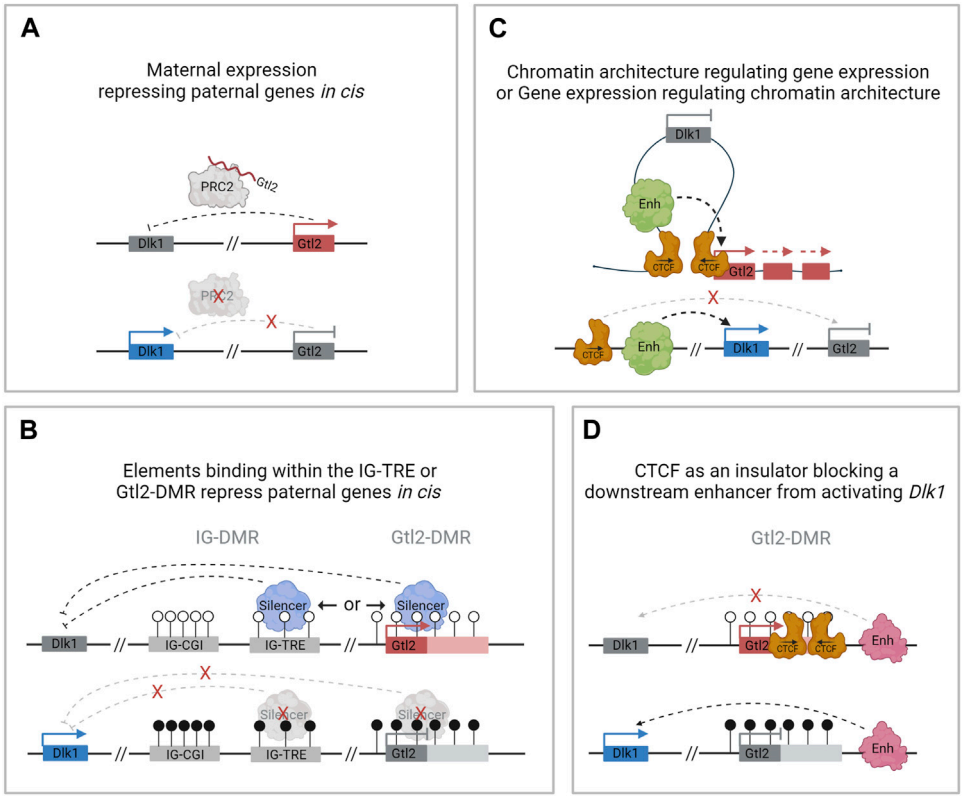

FIGURE 4
Possible mechanisms of regulation at the Dlk1-Dio3 locus by *Gtl2*. **(A)**. *Gtl2* lncRNA facilitates PRC2 recruitment in *cis* and represses paternally expressed genes on the maternally inherited chromosome. **(B)**. The IG-TRE or the Gtl2-DMR harbors elements that directly repress paternal genes in *cis*. **(C)**. Differential CTCF binding at the Gtl2-DMR regulates gene expression by restricting access to shared enhancers. **(D)**. Differentially methylated CTCF binding sites at the Gtl2-DMR function as an insulator, preventing *Dlk1* from being expressed from the maternal chromosome.

ortholog of *Gtl2*, is downregulated in many forms of cancer and, therefore, is believed to function as a tumor suppressor (Makoukji et al., 2016; Buccarelli et al., 2020). The *MEG3* lncRNA has been shown to interact with another tumor suppressor, p53, and influence the expression of p53 target genes (Zhou et al., 2007; Zhu et al., 2015). The lncRNA has also been found to interact with the polycomb repressive complex 2 (PRC2) in both mouse and human cells (Zhao et al., 2010; Mondal et al., 2015). In humans, *MEG3* exon3 is thought to contain the region of interaction with PRC2; this exon is conserved with mouse exon3, suggesting a shared function between the two species. *MEG3* is then thought to recruit PRC2 to its target genes in *trans* though interaction with GA-rich repeat regions and formation of RNA–DNA triplexes (Mondal et al., 2015).

In other imprinted regions, lncRNAs have been shown to silence other genes in the domain in *cis* (Pauler et al., 2012), and in the Dlk1-Dio3 domain, *in vivo* manipulations that activate *Gtl2* on the paternal chromosome result in repression of *Dlk1* (Lin et al., 2003; Li et al., 2008; Weinberg-Shukron et al., 2022). Furthermore, knockdown of the *Gtl2* lncRNA leads to increased expression of *Dlk1* coupled with decreased histone H3K27me3 over the *Dlk1* gene in mouse ESCs (Zhao et al., 2010). This suggests that in mice, the *Gtl2* lncRNA may facilitate PRC2 recruitment *in cis* and that one of its major functions is to repress paternally expressed genes on the maternally inherited chromosome (Figure 4A). However, in human iPSCs lacking *MEG3* expression, no difference was observed in PRC2 occupancy over the *DLK1* promoter, and there were no significant changes in the expression levels of either *DLK1* or *DIO3* compared with iPSCs that express *MEG3* (Kaneko et al., 2014). It should be noted that these experiments were performed *in vitro* in cell lines where *Dlk1* is only weakly expressed.

More recently, mouse models have also caused doubt regarding the idea that the *Gtl2* lncRNA silences the *Dlk1-Dio3* domain *in vivo*. Whereas a deletion model that removes *Gtl2* exons 1–4 leads to the loss of imprinting in the whole domain (Figure 2E), deleting exons 2–4 causes downregulation of *Gtl2* but no change in *Dlk1* expression in e11.5 embryos (Figure 2F) (Zhu et al., 2019). This suggests that the Gtl2-DMR does not solely function to restrict *Gtl2* expression to the maternal chromosome and that the lncRNA does not silence the paternally expressed genes on the maternal chromosome *in cis*. Instead, these observations suggest that the Gtl2-DMR harbors elements that can directly repress the expression of the paternally expressed genes *in cis* (Figure 4B). In agreement with this hypothesis are data from ESC deletions. Sanli et al. (2018) made ESC lines lacking either the *Gtl2* promoter or intron 1. Intriguingly, the loss of intron 1 alone on the maternal chromosome was sufficient to silence *Gtl2* and all the associated maternally expressed non-coding transcripts in ESCs and upon differentiation to NPCs. Furthermore, *Dlk1* expression became biallelic in NPCs upon the

loss of the maternal intron 1, indicating that *Gtl2* intron 1 may be playing a vital role in imprinting control across this domain.

Gtl2 intron 1 is approximately 2.5 kb in length. It contains two CTCF binding sites that are conserved in eutherian mammals and are only able to bind CTCF on the unmethylated maternally inherited chromosome (Lin et al., 2011) (Figure 1D). CTCF plays an important role in genome organization and is frequently found at boundaries of topologically associating domains (TADs) which are self-interacting regions (Merkenschlager and Nora, 2016). Although TADs are thought to be maintained between different tissues, sub-TADs within them are tissue-specific and orchestrate local genomic contacts throughout development (Smith et al., 2016). Data from ESCs indicate that the *Gtl2* CTCFs form the boundary of a parent-of-origin-specific sub-TAD on the maternal chromosome (Llères et al., 2019). Thus, another possible mechanism for establishing differential expression profiles between the two parental chromosomes could be that differential CTCF binding contributes to the formation of parental-origin-specific regulatory conformations (Figure 4C). Another possibility is that access to shared enhancers might be insulated through CTCF binding, enabling *Gtl2* expression and *Dlk1* repression (Figure 4D) consistent with a similar mechanism that is well established for the *Igf2*/*H19* domain, where imprinted gene expression is controlled by differentially methylated CTCF binding sites in the ICR. The H19-CTCF sites are methylated on the paternally inherited chromosome and are thus only able to bind CTCF on the maternal chromosome, where they function as an insulator, preventing *Igf2* from being expressed (Bell and Felsenfeld, 2000; Hark et al., 2000). In ESCs, the H19-ICR has been recently shown to form a maternal chromosome-specific sub-TAD boundary that splits the imprinted domain into two, which is similar to what is observed in the *Dlk1-Dio3* locus. Deletion of one of the CTCFs *in vitro* has been shown to cause upregulation of *Dlk1* (Llères et al., 2019). However, the extent to which the *Gtl2* CTCFs can regulate gene expression *in vivo* remains to be established.

The Rian-DMR: A paternal chromosome-specific regulatory element?

Rian (RNA imprinted and accumulated in the nucleus) is a lncRNA that has more than 20 predicted alternative transcripts in mice. Its expression from the maternal chromosome has not been dissociated from *Gtl2*, and an individual promoter for this gene has not been identified or a transcription unit clearly defined; hence, it can be considered as a *Gtl2*-associated transcript driven by the IG-DMR and Gtl2-DMR. Nonetheless, RNA from this region acts as the host transcript for miRNAs and two clusters of C/D snoRNAs (Cavaillé et al., 2002). In humans, the *Rian* ortholog, *MEG8* (along with *MEG3*), has been shown to be upregulated in many cancers and is thought to regulate many different pathways by acting as a molecular sponge for various miRNAs (Ghafouri-Fard et al., 2022). A Rian-DMR has been described in the second intron of the gene (Figure 1E; Figure 3A), and as opposed to other DMRs, in the *Dlk1-Dio3* domain, this region is methylated in both sperm and oocytes, and then becomes hypomethylated in the blastocyst. The paternally inherited allele remains hypomethylated throughout development, whereas the maternally inherited copy becomes hypermethylated by e6.5 (Zeng et al., 2014). This hypermethylation on the maternal allele may be due to the normal methylation accumulation on actively transcribed gene bodies. Upon maternal transmission of the IG-DMR deletion, the Rian-DMR is lost; however, unlike the Gtl2-DMR, the Rian-DMR becomes biallelically hypomethylated (Figure 3B). This once again illustrates that appropriate methylation of the germline ICR is necessary to establish the epigenotype of the entire domain.

In mice, the DMR consists of a small CpG island that contains 12 copies of a GGCG repeat. This region is conserved and G-rich in eutherian mammals; however, the GGCG repeat is only seen in mice and rats. Upstream of the repeats is a conserved CTCF binding domain. Interestingly, this motif lacks CpG dinucleotides, so binding is not affected by methylation. In agreement with this, CTCF occupancy has been shown to be biallelic at this site (Zeng et al., 2014). Until now, the role of the *Rian*-DMR has been unclear, but a recent study has thrown some light on its function. Han et al. (2022) have shown that the DMR functions as an insulator in mouse MLTC-1 cells. Intriguingly, the CTCF binding region was only able to act as an insulator in the presence of the repeat element. They further deleted the entire DMR, and the CTCF site repeats individually to assess the role of the region on gene expression. A 661bp deletion of the entire DMR led to reduced *Dlk1* and *Rtl1* expression and increased expression of *Gtl2*, *Rian*, and *Mirg*. When the CTCF binding site alone was deleted, a similar but less pronounced effect was observed. Interestingly, the tandem repeat deletion only affected the expression of the downstream gene *Mirg*. These data indicate that the Rian-DMR functions on the unmethylated paternal chromosome to ensure the correct expression of *Dlk1* and *Rtl1* and the repression of *Gtl2* and its associated transcripts (Zeng et al., 2014). However, more recent *in vivo* data from mice with a 434bp deletion of the CTCF binding site and the GGCG repeats show that the loss of this region has little phenotypic effect as maternal and paternal heterozygotes and homozygotes all survive to adulthood. Furthermore, no effect was observed at e12.5 on *Dlk1*, *Rtl1*, *Dio3*, *Gtl2*, *Rian*, or *Mirg* expression on either maternal or paternal transmission of the deletion. These mice do show increased expression of two miRNAs within the *Rian* gene, miR-118 and miR-341. Both miRNAs are significantly upregulated in paternal heterozygotes and homozygotes but not in maternal heterozygotes at e12.5. In addition, RNAseq data indicated that many other miRNAs in the region become upregulated on paternal deletion, suggesting a role of the Rian-DMR in preventing miRNA expression on the paternal chromosome (Zhang et al., 2023).

The Dlk1-DMR and *Dlk1* isoforms

Both the promoter of *Dlk1* and the last exon of *Dlk1* contain CpG islands (Takada et al., 2000). Whereas the *Dlk1* promoter does not show any parental-origin-specific methylation pattern, the smaller CpG island within the fifth exon is completely unmethylated on the maternal allele and partially methylated on the paternal allele (Takada et al., 2002). This differentially methylated region is termed the Dlk1-DMR (Figure 1B). Similar to the Gtl2-DMR, the Dlk1-DMR acquires paternal allele-specific

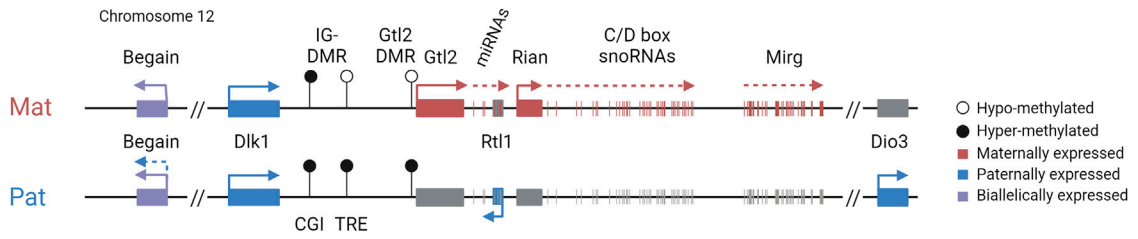

FIGURE 5
Selective absence of imprinting in the neurogenic niche; in the postnatal subventricular zone, niche astrocytes and neuronal stem cells exhibit biallelic expression of *Dlk1*, whereas *Gtl2* retains imprinting and remains exclusively maternally expressed. This selective absence of imprinting is accompanied by increased methylation at the IG-CGI but not at the IG-TRE or Gtl2-DMR.

methylation following fertilization (Gagne et al., 2014). The methylation pattern of this DMR remains dynamic in late embryonic development and into adulthood. Interestingly, the level of Dlk1-DMR methylation does not correlate with the level of *Dlk1* expression. Takada et al. (2002) reported a different methylation profile per tissue (lung, muscle, liver, kidney, and brain), suggesting that the methylation is cell-type specific and that allele-specific methylation differences at the Dlk1-DMR may not have a role to play in transcriptional control. The function of the Dlk1-DMR remains to be elucidated.

Alternative splicing at exon 5 generates a membrane-bound and secreted isoform of *Dlk1*. The secreted isoforms, produced with a longer part of exon 5, include a juxtamembrane motif for cleavage by extracellular proteases, which is absent from constitutively membrane-bound isoforms. In the neurogenic niche, secreted *Dlk1* is predominantly expressed by niche astrocytes, whereas neural stem cells (NSCs) express membrane-bound *Dlk1* (Ferrón et al., 2011). Interestingly, membrane-bound DLK1 in NSCs is stimulated by astrocyte-secreted DLK1, and communication between these cell types in the neurogenic niche regulates NSC self-renewal.

Selective absence of imprinting of *Dlk1* not *Gtl2* in the neurogenic niche

Recent evidence suggests that imprinted genes can be selectively "switched on" or "switched off" in particular cell types or at specific developmental time-points to initiate a change in gene dosage that is essential for normal development (Ferrón et al., 2011; Ferrón et al., 2015). Intriguingly, some imprinted genes show a selective absence of imprinting in the neurogenic niche (Lozano-Ureña et al., 2017). The *Igf2* gene, which is canonically expressed from the paternally inherited copy, is biallelically expressed in the choroid plexus (DeChiara et al., 1991; Giannoukakis et al., 1993; Lehtinen et al., 2011), and this selective absence of *Igf2* imprinting is required for neurogenesis.

The vertebrate-specific atypical Notch ligand gene, *Dlk1*, is dosage-sensitive with different tissue-specific sensitivities to altered expression levels (Moon et al., 2002; Da Rocha et al., 2009). DLK1 is involved in a range of processes, including non-shivering thermogenesis, metabolism, and behavior (Wallace et al., 2010; Charalambous et al., 2012; García-Gutiérrez et al., 2018; Montalbán-Loro et al., 2021). In humans, *DLK1* variants are associated with age at menarche (Day et al., 2017), type I diabetes (Wallace et al., 2010), and a range of cancers, including neural, breast, and liver cancer (Yin et al., 2006; Cai et al., 2016; Makoukji et al., 2016; Buccarelli et al., 2020). DLK1 is, therefore, a biomedically relevant key player in a diverse range of processes. Similar to *Igf2*, the *Dlk1* gene shows selective absence of imprinting in the postnatal neurogenic niche, resulting in the activation of the repressed maternal allele *via* an unknown mechanism (Figure 5). This absence of imprinting is essential for normal adult neurogenesis (Ferrón et al., 2011). Unlike *Dlk1*, the neighboring gene *Gtl2* keeps its imprinting in the neurogenic niche, suggesting a selective gene-specific regulation (Ferrón et al., 2011).

An important evolutionary question remaining to be elucidated is the time of switch in imprinted gene dosage. Although the dosage change is described as a selective absence of imprinting, it is not known whether biallelic expression of *Dlk1* is "switched on" in specific cell types, representing a recently evolved function, or whether imprinting is "switched off" during development, representing an ancestral state prior to the evolution of imprinting, where *Dlk1* is biallelically expressed (Edwards et al., 2008). Remarkably, this selective requirement for a double dose for neurogenesis is shared by the imprinted *Igf2* gene (Ferrón et al., 2015). This emphasizes the importance of exquisite dosage control of certain genes by genomic imprinting, and the adaptability and flexibility of this epigenetically regulated mechanism in particular developmental contexts.

In conclusion, selective regulation of imprinting is probably a normal mechanism for modulating gene dosage to control stem cell potential in brain development and within the neurogenic niches throughout development and adult life (Perez et al., 2016). The dosage sensitivity of functionally important imprinted genes and the finding of highly selective absence of imprinting at *Dlk1* and *Igf2* in the brain suggest tight regulation of parental-origin-specific monoallelic expression. Dissecting the molecular players that participate in regulating imprints during postnatal neurogenesis will provide insights into the wider epigenetic control of the neurogenic process and uncover the molecular mechanisms underlying normal NSC function to understand tumoral processes in the adult brain. Therefore, unmasking the mechanism that regulates this time- and tissue-specific change in gene dosage is crucial for expanding our understanding of the

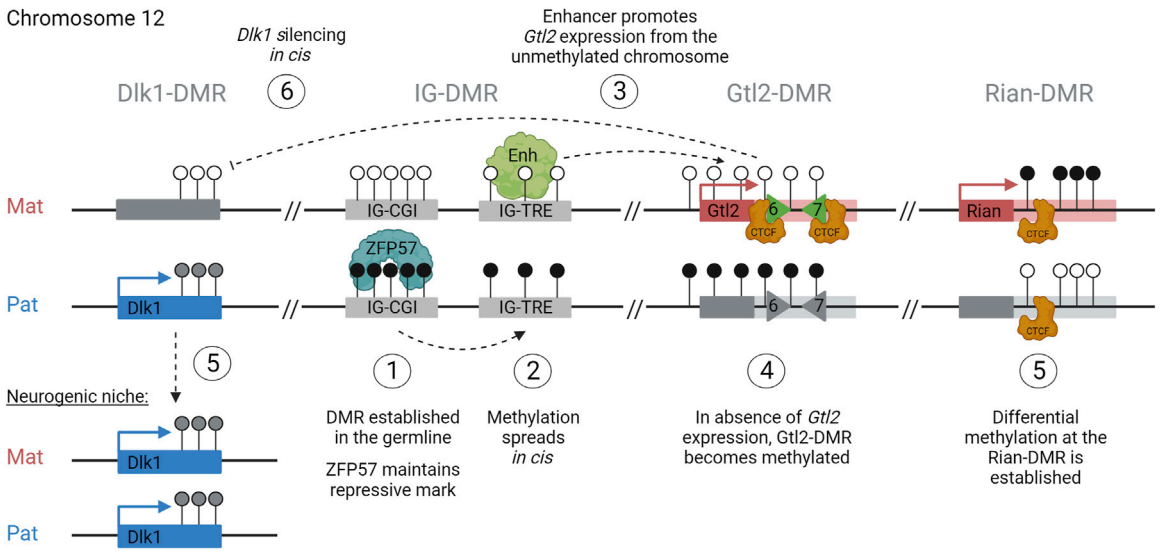

FIGURE 6
How to build an imprint: step 1: establishment of the germline-DMR (IG-CGI), methylated in sperm and unmethylated in oocytes. Step 2: maintenance of methylation across the entire paternal ICR (CGI + TRE). Step 3: monoallelic expression of *Gtl2* from the maternal unmethylated allele. Step 4: accumulation of methylation over the paternal Gtl2-DMR. Step 5: establishment of somatic DMRs at *Dlk1* and *Rian*. Step 6: regulation of gene expression profiles in *cis*, silencing *Dlk1* from the maternal copy.

physiological pathways regulated by imprinted genes in pathology and health.

Discussion

How to build an imprinted domain

Clearly, appropriate allele- and tissue-specific expression of the Dlk1-Dio3 region is necessary for normal mammalian development. Assessing different models that remove various elements in the region has allowed us to dissect the chain of events that is necessary to establish and maintain imprinted gene expression.

The first stage of the hierarchy is the establishment of the germline-DMR. In sperm, the IG-DMR becomes fully methylated before e19.5 (Hiura et al., 2007), whereas in oocytes, the region remains unmethylated (Figure 6, step 1). After fertilization, the presence of DNA methylation across the paternal IG-CGI allows the recruitment of oocyte-loaded and zygotically expressed ZFP57 to the region, which in turn recruits TRIM28 and DNMTs and ensures that the entire ICR, including the IG-TRE, remains unmethylated on the paternal chromosome (Figure 6, step 2). Meanwhile, on the maternally inherited chromosome, the unmethylated IG-CGI is unable to bind ZFP57 and the IG-TRE remains unmethylated. This allows the IG-TRE to bind transcription factors and act as an enhancer for *Gtl2*, causing it to be monoallelically expressed from the maternally inherited chromosome from e3.5 (Figure 6, step 3). DNA methylation starts to accumulate over the paternal copy of the Gtl2-DMR at e5.5 and is complete by e6.5 (Figure 6, step 4). At the same time, the other somatic DMRs are also established at *Dlk1* and *Rian* (Figure 6, step 5). Once established, the Gtl2-DMR can control imprinted gene expression on the maternal chromosome either *via* the lncRNA recruiting PRC2, direct silencing, or insulator activity (Figure 6, step 6). On the paternal chromosome, the unmethylated Rian-DMR regulates paternal miRNA expression, possibly through its insulating properties.

Remaining questions

Although much has been learned about how imprinting is established in this domain, many questions remain. First, it is not clear how the IG-DMR becomes methylated in the male germline yet remains unmethylated in oogenesis and how the maternal copy eludes *de novo* methylation in later development. Recently, it was shown that a mouse IG-DMR transgene acquired methylation during the post-fertilization period rather than in the sperm (Matsuzaki et al., 2023). This suggests that the transgene is lacking the sequence that initially attracts methylation to the element in the sperm. This "post-fertilization imprinted methylation" was previously reported in mouse and human *H19* ICR transgenes as well (Matsuzaki et al., 2009). However, after implantation, the YAC transgene of the IG-DMR became highly methylated from both copies, suggesting that the IG-DMR fragment tested did not protect the maternal IG-DMR from genome-wide *de novo* DNA methylation. Interestingly, the fragment did not contain most of the IG-TRE, indicating that one function of the IG-TRE may be to protect the maternal sequence from global *de novo* methylation after implantation. Although maintenance of hypomethylation at the maternal *H19* ICR is known to involve CTCF and Sox/Oct factors (Sakaguchi et al., 2013), the mechanism at the maternal IG-DMR is not fully understood; however, this region also contains Sox/Oct binding motifs.

Second, the mechanism by which the maternal IG-TRE directs monoallelic expression in the domain remains to be elucidated. It is known to contain many transcription factor binding motifs and

shows low-level expression, suggesting that it most likely functions as an enhancer for *Gtl2*. However, it is possible that the IG-TRE also contains a silencer element that is capable of directly repressing the paternally expressed genes on the maternally inherited chromosome. Experiments dissecting this region further are necessary to tease apart these options.

Evidence from mice harboring deletions of the Gtl2-DMR and *in vitro* deletions of the Rian-DMR indicate that somatic DMRs can regulate parent-of-origin-specific expression in later development, but the mechanisms through which this is achieved are not fully understood. Intriguingly, both these DMRs are known to bind CTCF, so they may influence gene expression through mechanisms such as enhancer blocking. The parental-specific sub-TAD identified *in vitro* with the Gtl2-DMR at the border indicates that it has strong insulator activity. However, whether these parental-specific conformations are a cause or a consequence of differential expression patterns between the two chromosomes is uncertain (Figure 6). The role the *Gtl2* lncRNA itself plays in the regulation of gene expression in the region also needs further exploration as it is uncertain whether it recruits PRC2 to the domain *in vivo* to bring about the epigenetic silencing of genes. Much of the research on conformation and the role of the lncRNA at the *Dlk1-Dio3* locus has been performed *in vitro*, limiting the resolution of information, and thus, findings may not be recapitulated *in vivo*. For instance, as *Dlk1* is lowly expressed in mESCs, experiments designed to look at the effect of perturbation models on paternal gene expression patterns are not informative in culture.

Finally, the Dlk1-Dio3 locus is interesting as the expression varies between tissues and cell types. We recently showed that there are two weakly biased genes at the edge of the *Dlk1-Dio3* region: *Wdr25* and *Wars*. Both genes showed a weak skew toward paternal expression, but only in brain tissues. This bias was shown to be under the control of the IG-DMR (Edwards et al., 2023), suggesting that its influence may be more extensive in neuronal tissues. Weakly biased genes were also found at the periphery of other imprinted regions, and further studies are needed to understand the functional and mechanistic implications of this observation. In addition to tissue-specific differences, unique cell types display selective absence of imprinting at the *Dlk1-Dio3* domain in a temporal and spatial-specific manner. The mechanism that switches between monoallelic and biallelic expression remains to be elucidated and may provide insights into transcriptional control with wider implications for non-imprinted domains as well (Figure 6). Together, these observations indicate that the mechanisms regulating the imprinting of the *Dlk1-Dio3* locus may vary between tissues and time-points in development.

This review highlights the importance of using *in vivo* models to tease apart the complex chain of epigenetic events that is required to establish and maintain imprinted gene expression throughout development. We also demonstrate that cell-type-specific modulation of this hierarchy is necessary to ensure the correct gene dosage in certain tissues, such as in the neurogenic niche—however, what these mechanistic steps are remains unclear. Together, this work illustrates how studying one imprinted region in detail can add a layer of sophistication to how we think about the epigenetic control of genome function and its consequences for spatial and temporal regulation more generally.

Methods

Southern blot (*Figure 3*): DNA was isolated by standard techniques (Sambrook et al., 2001). A total of 10 µg of restriction enzyme-digested DNA was separated on a 0.5% TBE gel before transferring to Hybond-N+ (GE Healthcare Life Sciences) nylon membranes. Membranes were pre-hybridized in ULTRAhyb (Ambion) for at least 1 h. The probe was a PCR fragment amplified with 5′-AGTGGCCCAACTTCTATCGG and 5′-GGAACAGAGACCTCCTAAGG, which was labeled with [α-32P]dCTP using the Megaprime DNA labeling system (GE Healthcare Life Sciences) and then purified with ProbeQuant G50 Micro-Columns (GE Healthcare Life Sciences) before being added to the hybridization solution and incubated at 42°C overnight. Filters were washed to a stringency of 0.2X SSC/0.1%SDS at 65°C and then exposed to PhosphorImager Screens (Molecular Dynamics).

Author contributions

AW-S: conceptualization, funding acquisition, visualization, writing–original draft, and writing–review and editing. NY: data curation, investigation, methodology, validation, and writing–review and editing. AF-S: conceptualization, funding acquisition, project administration, resources, supervision, writing–original draft, and writing–review and editing. CE: conceptualization, funding acquisition, project administration, supervision, writing–original draft, and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Navigating the brain and aging: exploring the impact of transposable elements from health to disease

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Transposable elements (TEs) are mobile genetic elements that constitute on average 45% of mammalian genomes. Their presence and activity in genomes represent a major source of genetic variability. While this is an important driver of genome evolution, TEs can also have deleterious effects on their hosts. A growing number of studies have focused on the role of TEs in the brain, both in physiological and pathological contexts. In the brain, their activity is believed to be important for neuronal plasticity. In neurological and age-related disorders, aberrant activity of TEs may contribute to disease etiology, although this remains unclear. After providing a comprehensive overview of transposable elements and their interactions with the host, this review summarizes the current understanding of TE activity within the brain, during the aging process, and in the context of neurological and age-related conditions.

KEYWORDS

transposable elements, epigenetics, brain, aging, neurological disorders, LINE-1, ERV

Introduction

Transposable elements (TEs) are mobile genetic elements able to move across the genome, independently of their host, either through a cut-and-paste mechanism or by a copy-and-paste mechanism (Wells and Feschotte, 2020). These sequences represent approximately 41% and 48% of the mouse and human genomes respectively, which is particularly relevant when compared to the much smaller percentage of coding sequences (1.5%) (Hermant and Torres-Padilla, 2021; Hoyt et al., 2022). Long considered as purely "junk DNA," TEs were originally identified in maize by Barbara McClintock more than 60 years ago (McClintock, 1950; McClintock, 1951). She referred to them as "controlling elements" and played a fundamental role in highlighting their capacity to influence gene expression (McClintock, 1956). Since this pioneering work, TEs have been shown to play major roles in genome evolution, structural variation, genome size expansion, spatial organization, genetic diversity and gene regulation (Cordaux and Batzer, 2009; Chuong et al., 2017; Choudhary et al., 2023). On the other hand, unchecked activity of TEs can have nefarious effects, namely inducing mutations, disrupting genes, hindering the transcriptional regulation of genes and leading to the production of extranuclear nucleic acids that can induce cellular toxicity. For that reason, the host maintains a tight control over TE activity, mainly at the transcriptional and epigenetic levels, keeping them silent to prevent deleterious changes. This control is frequently broken in disease, such as cancer and neurological disorders, and during aging. Moreover, silencing mechanisms appear also

partially released in certain developmental contexts or tissues, such as in the brain, raising the possibility of an actual functional role conferred by TE activity, in particular in neuronal lineages. However, the contribution of TEs to both physiological and pathological contexts, particularly in the brain, are poorly understood. Specifically, is the aberrant activity of TEs merely a consequence of the disease, or could it contribute to certain pathological phenotypes? In this review, we provide a comprehensive overview about transposable elements and their interactions with the host. Additionally, we summarize the current knowledge regarding TE activity in physiological contexts, with a specific emphasis on the brain and aging, as well as neurological and age-related disorders.

Insights into transposable elements

Classification

TEs can be divided into two main classes, according to their transposition mechanism. Class I TEs, or retrotransposons, mobilize their DNA via an RNA intermediate, through a "copy-and-paste" mechanism, in a process known as retrotransposition. Class II TEs, or DNA transposons, mobilize through a "cut-and-paste" mechanism, in a process referred to as transposition (Finnegan, 1989). DNA transposons, which are no longer active in most mammalian species, represent a minority of the human (3%) and mouse (1%) genome (Pace and Feschotte, 2007; Hoyt et al., 2022). In turn, class I retrotransposons constitute the vast majority of TEs in mammals and are divided into two main subclasses according to their mechanism of chromosomal integration: long terminal repeat (LTR) retrotransposons, and non-LTR retrotransposons (Figure 1) (Wells and Feschotte, 2020).

LTR retrotransposons, also called endogenous retroviruses (ERVs), are remnants of exogenous retroviruses that were incorporated in the host germline as a result of ancient viral infections (Mao et al., 2021; Hoyt et al., 2022). A full length, autonomous, ERV has an average length of 7.5 kb and consists of two identical LTRs, which are non-coding regions containing *cis*-regulatory sequences, such as promoters, enhancers, or polyadenylation signals. The LTRs usually flank a set of three ORFs that encode the viral proteins: *gag*, which encodes structural proteins that form virus-like particles (VLPs); *pro-pol*, which encodes the enzymes necessary for the viral life cycle (reverse transcriptase, integrase, and protease); and *env*, which encodes the envelope proteins (Figure 1) (Küry et al., 2018). Most ERV copies have accumulated mutations that prevent their retrotransposition. In addition, recombination events between the two LTRs of a proviral insertion often lead to ERVs being reduced to a single LTR, or solo LTR, leaving behind remnants of regulatory sequences scattered throughout the genome (Thomas et al., 2018). All ERV subfamilies are no longer active in the human genome, except the evolutionary young HERV-K subfamily HML-2 (human mouse mammary tumor virus like-2), which shows signs of transcriptional activity and intact ORFs, still capable of producing some of the proteins required for VLPs formation (Garcia-Montojo et al., 2018). In contrast, several ERV subfamilies are still active in mice, such as IAP (intracisternal A-particle) and MusD elements. IAPs are highly abundant and competent for both transcription and retrotransposition. Importantly, ERV insertions contribute to 10%–12% of spontaneous germline mutations in laboratory mice (Maksakova et al., 2006; Stocking and Kozak, 2008).

Non-LTR retrotransposons are composed of two main subtypes: the autonomous LINEs (Long Interspersed Nuclear Elements) and non-autonomous SINEs (Short Interspersed Nuclear Elements).

LINEs constitute approximately 21% of the mammalian genomes and are autonomous, meaning that they produce all the machinery necessary for their own retrotransposition (Fueyo et al., 2022; Hoyt et al., 2022). LINEs are on average 6-7 kb long and composed of a 5′ untranslated region (UTR), comprising an RNA polymerase (RNApol) II promoter with both sense and antisense activity, two open reading frames (ORFs), and a 3′UTR with a polyadenylation signal (Evans and Erwin, 2021). ORF1 encodes an RNA-binding protein that has a nucleic acid chaperone activity required for retrotransposition (Martin and Bushman, 2001; Martin, 2006), while ORF2 encodes a protein with both reverse transcriptase and endonuclease activity (Figure 1) (Mathias et al., 1991; Feng et al., 1996). During retrotransposition, the encoded proteins (ORF1p and ORF2p) bind the RNA from which they originate, in *cis*, and the resulting ribonucleoprotein (RNP) translocates into the nucleus, where reverse transcription and retrotransposition takes place (Terry and Devine, 2020). In mammalian genomes, LINEs are dominated by a single family, LINE-1, which accounts for approximately 17% and 21% of the human and mouse genome respectively, constituting the largest proportion of TE-derived sequences in mammals (Waterston et al., 2002; Terry and Devine, 2020; Hoyt et al., 2022). The majority of LINE-1 copies are no longer functional due to the accumulation of mutations or 5′ truncations. Current estimations predict that only 80–100 LINE-1 copies are intact and still retrotransposition-competent (RC-LINE-1) in humans (Brouha et al., 2003; Evans and Erwin, 2021) and around 3,000 in the mouse genome (DeBerardinis et al., 1998). In addition, a larger number of elements with disrupted ORF sequences still harbor an intact 5′UTR and are hence transcriptionally active (Penzkofer et al., 2017). These elements belong to the evolutionary youngest LINE-1 subfamilies, called L1MdA, L1MdTf and L1MdGf in mice and some of the human-specific LINE-1 (L1Hs) from PA-1 subfamily called the transcribed-active elements subset (L1Ta-subset) in humans (Richardson et al., 2015). It is worth noting that in humans, LINE-1 are the only active autonomous elements (Hoyt et al., 2022).

Unlike LINEs, SINEs are non-autonomous elements. They do not encode any proteins and rely on the machinery produced by LINE-1 elements for their retrotransposition. Although they show a strong *cis*-preference, LINE-1-derived proteins, ORF1p and ORF2p, are able to bind in *trans* SINE RNAs (Dewannieux et al., 2003; Raiz et al., 2012). SINEs are derived from tRNAs or 7SL RNAs (Daniels and Deininger, 1985). Given this ancient origin and due to extensive accumulation of mutations during evolution, current SINE elements are highly diverse. There are two main families of SINEs in the human genome: Alu elements, constituting approximately 11% of the genome and being the TE family with the highest copy number; and the evolutionary young SINE-VNTR-Alu (SVA) elements, comprising only 0.1%–0.2% of the genome (Evans and Erwin, 2021; Hoyt et al., 2022). Alu are approximately 300 bp long, composed of highly similar left and right monomers, transcribed by RNApol III and they terminate with a poly (A) tract (Richardson

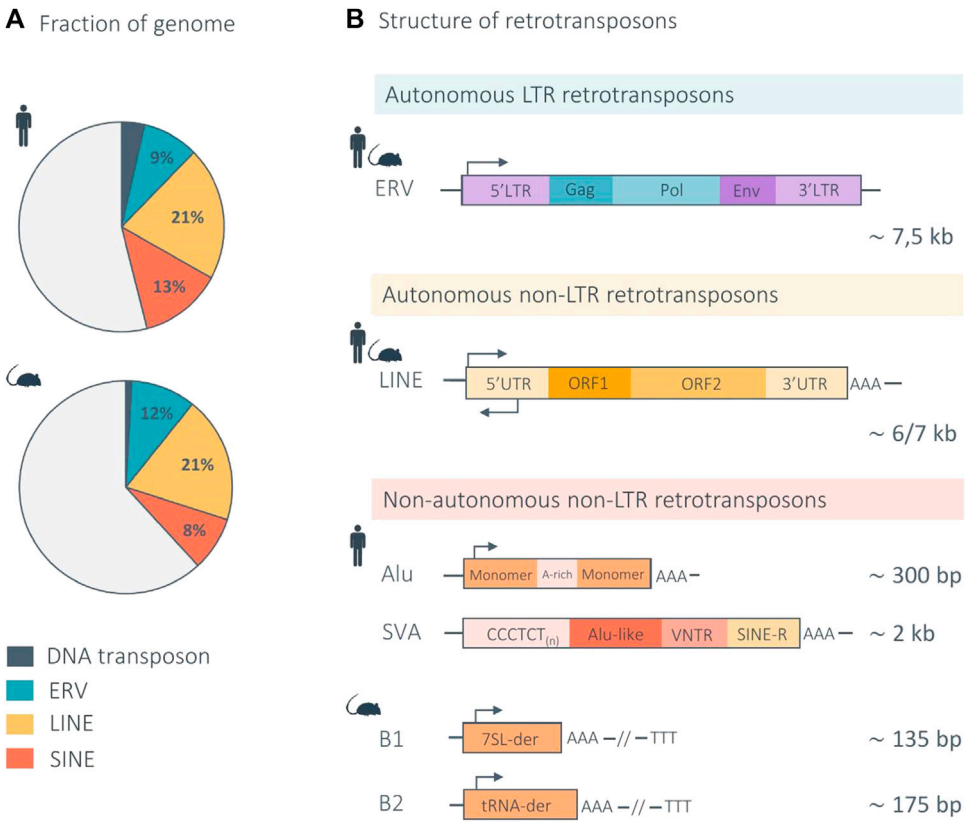

FIGURE 1
Structure of mammalian retrotransposons and genomic proportions in the human and mouse genome. **(A)** The pie charts indicate the genomic proportion of each retrotransposon class in the human and mouse genome. Light gray represents non-repeat DNA. **(B)** Retrotransposons are divided into two main subclasses according to their mechanism of retrotransposition: LTR and non-LTR retrotransposons. LTR elements, also called endogenous retroviruses (ERVs), are autonomous and share a genomic structure similar in the human and mouse genome. Non-LTR retrotransposons are further divided into two main subtypes: the autonomous LINEs (Long Interspersed Nuclear Elements) and non-autonomous SINEs (Short Interspersed Nuclear Elements). The genomic structure of LINEs is similar in the human and mouse genome. The main non-autonomous non-LTR elements are Alu and SVA in the human genome, and B1 and B2 in the mouse genome. Arrows indicate the approximate position and orientation of the promoter for each element. der, derived (Waterston et al., 2002; Richardson et al., 2015; Deniz et al., 2019; Hoyt et al., 2022).

et al., 2015). SVAs result from the fusion of an Alu sequence, a variable number of tandem repeats (VNTR) and a LTR fragment (SINE-R) (Figure 1). The youngest elements of these families are still active, comprising approximately 200,000 Alu elements from the Y, Ya5, Ya8, and Yb8 subfamilies and around 40% of the youngest SVA elements belonging to SVA-D, SVA-E, SVA-F, and SVA-F1 subfamilies (Comeaux et al., 2009; Hancks and Kazazian, 2010). The main SINE families in mice are the B1 and B2 elements, each representing 2%–3% of the genome (Waterston et al., 2002). If the presence of active SINEs B1 and B2 have been shown by cell culture-based experiments, the exact number of active elements in the mouse genome is still unknown (Dewannieux and Heidmann, 2005).

Host-transposable element interactions

Although TEs represent a large proportion of mammalian genomes, only a small fraction remains currently active. Indeed, in humans, less than 0.05% of these elements are able to mobilize (Mills et al., 2007). This is because newly inserted TEs usually do not provide an immediate fitness advantage to the host, consequently they tend to become fixed mainly through genetic drift, accumulating neutral mutations over evolutionary time. As a result, older TE insertions in genomes have accumulated mutations that render them non-functional, while more recently inserted TEs retain the capacity for activity, both in terms of transcription and, occasionally, transposition (Bourque et al., 2018). For example, using LINE-1's allele frequency and sequence divergence as a proxy for age, a study investigated the correlation between LINE-1 activity and age. They found that putative young LINE-1 with low sequence divergence are active in cultured cells and generally polymorphic in the human population. In contrast, highly diverged LINE-1 sequences are most often fixed and inactive (Brouha et al., 2003).

In order to persist throughout evolution, TEs must achieve a delicate equilibrium between their expression and repression in the genome of their hosts. This allows them to replicate and propagate within the genome while avoiding deleterious effects on the host cell functions, as this would not be favorable for their survival (Bourque et al., 2018). The intricate relationship between TEs and the host is thus very complex. A recent review proposed a model to explain

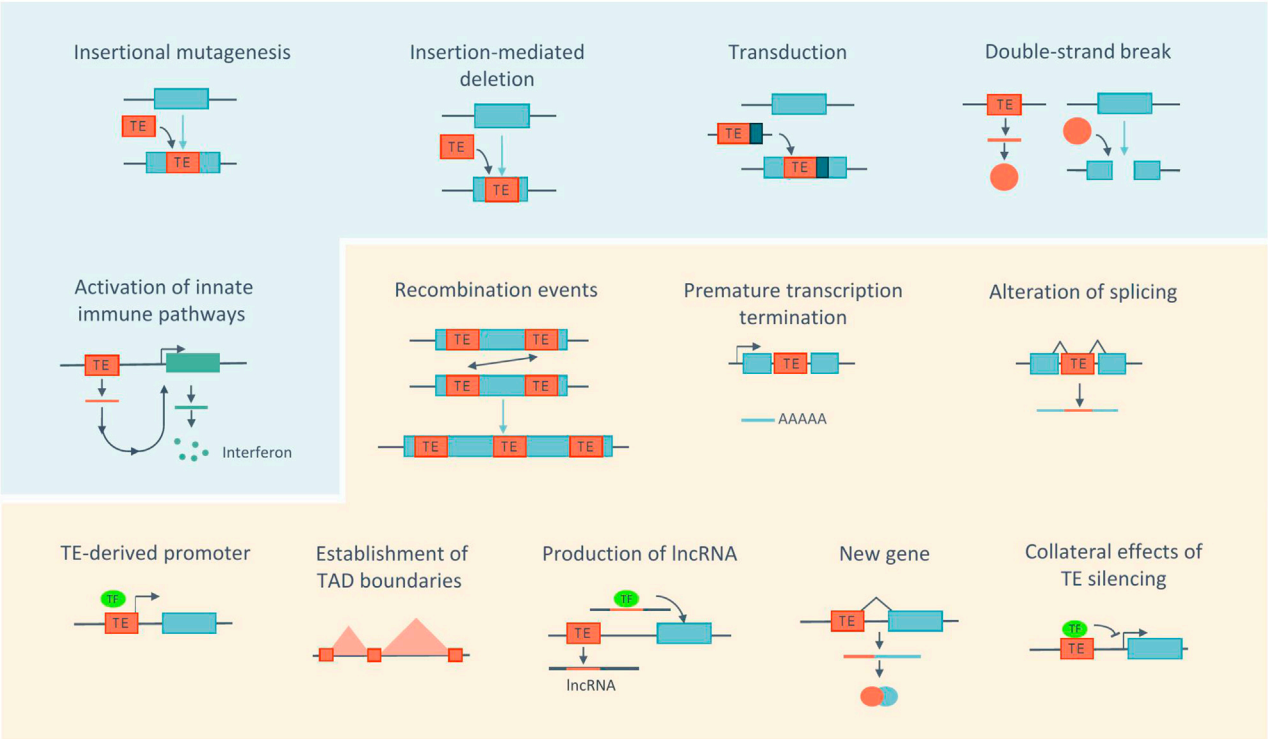

FIGURE 2
Impact of TEs at the molecular and cellular levels. Actively transposing TEs (blue panel) can be a source of genome instability by inducing mutations, deletions, transduction or DNA damage. The accumulation of products derived from TE may cause inflammation. Even without being active (orange panel), TEs have the ability to alter the host genome through recombination events, alteration of transcription, of gene expression regulation and 3D chromatin architecture. Sequences derived from TEs can also be co-opted by the host. Blue boxes represent genes. TF, transcription factor. Adapted from (Cordaux and Batzer, 2009; Sundaram and Wysocka, 2020; Fueyo et al., 2022).

host-TE interactions, suggesting that an initial period of cooperation could resolve in one of three ways: conflict or arms race, where the host develops silencing mechanisms to control TEs, which TEs may in turn counteract with anti-silencing mechanisms; cooperation and evasion, where TEs develop self-regulatory mechanisms, which can lead to the development of a mutualistic relationship between TEs and the host; and finally, co-option or domestication, when the host is able to repurpose some of the TE activity for its own benefit (Cosby et al., 2019).

Molecular and cellular impacts of transposable elements

The presence of TEs can alter the host genome or the transcriptome in numerous ways, contributing to genome evolution and diversification but also potentially affecting genome stability (Figure 2). Active TEs that have the ability to move across the genome represent a source of mutations, as insertions of TEs into protein-coding genes or regulatory regions can disrupt gene function (Cordaux and Batzer, 2009). In addition, insertions can lead to deletions at the target site (Gilbert et al., 2002). TEs may also contribute to exon shuffling through transduction, a process in which flanking sequences are moved with the element and consequently inserted into new locations (Moran et al., 1999; Richardson et al., 2015).

TEs can also contribute to genome instability via their encoded products (Hedges and Deininger, 2007). In particular, the LINE-1 ORF2p encoded-protein can create double-strand breaks (DSBs) at endonuclease target sites (Gasior et al., 2006). In addition, the accumulation of TE-derived products in the cytoplasm, including RNA, extrachromosomal DNA copies and proteins can lead to the activation of innate immune pathways and subsequently trigger inflammation (Saleh et al., 2019).

Furthermore, TEs can induce changes in the host genome even without being active. Recombination events can occur between dispersed TE sequences due to their repetitive nature and high copy number, generating large genomic rearrangements, including deletions, duplications, and inversions (Sen et al., 2006; Han et al., 2008; Lee et al., 2008; Cordaux and Batzer, 2009). In this context, a recent study analyzed the genomes of three individuals and identified 493 genomic rearrangements mediated by TEs, highlighting how this contributes to genome diversity (Balachandran et al., 2022).

In addition, TEs carry a number of regulatory motifs or sequences, which can affect host gene expression, independently of their activity. TEs, such as LINE-1, have internal polyadenylation signals, which can lead to elongation defects, by promoting premature termination of transcription (Perepelitsa-Belancio and Deininger, 2003; Han et al., 2004). TEs, such as Alu and LINE-1, possess splice site signals, which may lead to cryptic splicing, exon

skipping or incorporation of their sequence into transcripts (Saleh et al., 2019). Importantly, TEs also contain transcription factor binding sites (TFBSs) and *cis*-regulatory elements (CREs) including promoters and enhancers. The vast majority of them no longer mediate the transcription of TEs, but can be repurposed to regulate the expression of host genes (Sundaram and Wysocka, 2020; Fueyo et al., 2022). An example of this is the transcription of host genes by the LINE-1 antisense promoter in human cells (Nigumann et al., 2002). Additionally, it has been proposed that TEs contribute to the pluripotency gene regulatory network by harboring 25% of the binding sites for pluripotency factors, including OCT4 and NANOG, in both the human and mouse genomes (Kunarso et al., 2010; Sundaram and Wysocka, 2020; Fueyo et al., 2022). Furthermore, different TE families have been shown to contribute to the evolution of innate immunity in mammals by acting as interferon (IFN)-inducible enhancers. In humans, most of these co-opted regulatory elements are found within ERVs (Chuong et al., 2016). However, non-LTR elements, such as L1M2a, were also shown to act as IFN-inducible enhancers (Buttler et al., 2023). In contrast, in mice, B2 elements are the predominant source of these regulatory sequences (Horton et al., 2023).

TEs can also impact host gene expression by affecting the 3D chromatin architecture, either by acting as insulator elements or by being enriched at the boundaries of topologically associating domains (TADs). TADs are chromatin domains where enhancer-promoter interactions are favored, and their boundaries are enriched for binding sites of the zinc finger protein CTCF, many of which derive from TE sequences. This protein not only demarcates these boundaries but also mediates chromatin loop formation (Diehl et al., 2020; Sundaram and Wysocka, 2020; Fueyo et al., 2022).

Beyond regulatory sequences, TE-derived sequences can also be co-opted or exapted for host gene function. One striking example of this are long non-coding RNAs (lncRNAs). Indeed, 83% of the human and 66% of the mouse lncRNAs contain at least one TE (Kelley and Rinn, 2012). The presence of the TE sequence may play a role in regulating lncRNAs expression, processing and localization. For example, they can provide polyadenylation signals or contribute to post-transcriptional adenosine-to-inosine editing. TEs can also serve as functional domains within lncRNAs. Indeed, some studies have demonstrated that mutation or deletion of TEs from the lncRNA sequence can impact its function by altering its localization and expression (Fort et al., 2021).

In addition to lncRNA genes, the exaptation of TE sequences led to the emergence of key protein-coding genes, with both conserved and species-specific functions (Bourque et al., 2018). For example, the coding sequences from different TE families have been domesticated on multiple occasions to integrate genes involved in placental development in both humans and mice (*Syncytin* genes) (Dupressoir et al., 2012) and in brain development (*Arc* gene) (Pastuzyn et al., 2018).

Lastly, TEs can influence, indirectly, the expression of host genes at the epigenetic level as both silencing mechanisms or loss of silencing in certain contexts can spread beyond the TE itself and affect nearby host gene expression (Choi and Lee, 2020; Fueyo et al., 2022).

Silencing mechanisms

As the immediate uncontrolled activity of TEs can have negative consequences on the genome (see previous section), the host has developed various mechanisms operating at different levels to prevent their expression and transposition (Klein and O'Neill, 2018). At the transcriptional level, silencing mechanisms comprise the deposition of epigenetic modifications on chromatin, primarily involving DNA methylation as well as repressive histone modifications (Garcia-Perez et al., 2016; Deniz et al., 2019; He et al., 2019). Although DNA methylation of CpG-rich promoters is prevalent on most TE families in somatic lineages, these marks are widely erased and reprogrammed during pre-implantation development. Therefore, in embryonic stem cells (ESCs), TEs are primarily repressed through the action of several histone lysine methyltransferases. Hence, in mouse ESCs, trimethylation of lysine 9 on histone H3 (H3K9me3) is deposited by SETDB1 and SUV39H1/2 at specific ERVs and LINE-1 elements, depending on the family considered and their evolutionary age, and is necessary for their silencing and heterochromatinization through HP1 (heterochromatin protein 1) recruitment (Matsui et al., 2010; Karimi et al., 2011; Bulut-Karslioglu et al., 2014). Moreover, dimethylation of lysine 9 on histone H3 (H3K9me2), deposited by the G9a enzyme, is necessary for the silencing of a distinct family of ERVs (MERVL elements) (Maksakova et al., 2013). Repression by trimethylation of H3K27 (H3K27me3) appears limited to a specific ERV family in ESCs (Murine Leukemia Virus (MLV) elements) (Leeb et al., 2010), but can be acquired by other TE families upon genome-wide demethylation (Walter et al., 2016). In parallel, the repressor KAP1 (KRAB-associated protein 1, also called TRIM28) acts as a cofactor essential for silencing and for the recruitment of SETDB1 and other histone-modifying enzymes to specific TE families. KAP1 itself is recruited to TEs by Krüppel-associated box domain-containing zinc finger proteins (KRAB-ZFPs), the largest TF family in mouse and human, which confer the sequence specificity for binding to specific TE families/subfamilies (Schultz et al., 2002) or by the TF Yin Yang 1 (YY1) at specific ERV elements (Lee et al., 2018). KAP1-mediated repression appears particularly relevant for young families of TEs in both mouse and human ESCs. For example, in human ESCs, ancient LINE-1 families have accumulated mutations, rendering them unable to be bound by KAP1/KRAB-ZFPs and to be transcribed. Younger LINE-1 families are bound and repressed by KAP1, while the youngest and more active human-specific L1Hs elements are not yet bound by KAP1/KRAB-ZFPs, but instead repressed by DNA methylation, which may be deposited by small RNA-based mechanisms (Castro-Diaz et al., 2014). Upon implantation and ESC differentiation, permanent silencing of most TEs is ensured by DNA methylation, which is catalyzed by DNA methyltransferases (DNMTs) and then maintained throughout development by the maintenance methyltransferase DNMT1, without the necessity for continual expression of sequence-specific TE-recognizing repressors (Jansz, 2019). DNMTs are recruited to LINE-1 and ERV sequences in ESCs either by the human silencing hub (HUSH) complex, which interacts directly with H3K9me3 or by KAP1/KRAB-ZFPs (Robbez-Masson et al., 2018). Moreover, a binding site for the TF YY1 (Yin Yang 1) located in the 5′UTR and conserved among LINE-1 elements was shown to

mediate DNA methylation of young LINE-1 promoters in human ESCs and differentiated cells, possibly through the recruitment of DNMTs (Sanchez-Luque et al., 2019). Furthermore, repression of TEs through KAP1/KRAB-ZFPs, which was initially thought to be restricted to ESCs, is also active in neuronal progenitor cells (NPCs), where KAP1 is necessary for the establishment of H3K9me3 at ERVs and their repression (Fasching et al., 2015; Brattås et al., 2017).

In addition, repression of TEs can also occur at the post-transcriptional level, via RNA silencing-based mechanisms (Heras et al., 2014; Garcia-Perez et al., 2016; Goodier, 2016). A study in human cells showed that the bidirectional transcription of LINE-1 promoters can be processed into small interfering RNAs (siRNAs), which reduce the stability of the LINE-1 RNA (Yang and Kazazian, 2006). In addition, the microRNA miR-128 was shown to inhibit LINE-1 retrotransposition in human induced pluripotent stem cells (iPSCs) and cancer cells, by binding either directly to LINE-1 RNA or to the 3′UTR of nuclear import factor transportin 1 (TNPO1) mRNA, which encodes a protein necessary for the nuclear import of LINE-1 RNP complexes (Hamdorf et al., 2015; Idica et al., 2017). Furthermore, a distinct and conserved pathway active predominantly in germ cells exists, wherein a set of small RNAs called *Piwi*-interacting RNAs (piRNAs) can target complementary TE transcripts for degradation in the cytoplasm and direct DNA methylation to genomic TE sequences (Wang et al., 2023).

Finally, post-translational mediated repression commonly targets the LINE-1 RNP complex for destabilization and degradation (Saleh et al., 2019). It has been proposed that the zinc-finger antiviral protein ZAP colocalizes with LINE-1 RNA and ORF1p in cytoplasmic stress granules to promote RNP degradation, and prevents LINE-1 and Alu retrotransposition (Moldovan and Moran, 2015). Furthermore, uridine residues can be transferred to LINE-1 mRNA in the cytoplasm by TUT7 (terminal uridyl transferase 7) and the MOV10 RNA helicase, which may prevent ORF2p-mediated reverse transcription initiation in the nucleus (Warkocki et al., 2018).

Transposable elements activity in the healthy brain and during aging

Healthy brain

Whereas TEs are kept silenced in most somatic tissues, one organ escaping this rule is the brain. Indeed, somatic retrotranspositions have been shown to occur in the healthy human and rodent brain, which could contribute to the establishment of neuronal somatic mosaicism. The first study demonstrating somatic retrotransposition in the neuronal lineage reported mobilization of an engineered human LINE-1 *in vitro,* in NPCs derived from rat hippocampus neural stem cells, and also *in vivo*, in the brain of transgenic mice bearing a similar transgene (Muotri et al., 2005). This was then further shown to occur in NPCs derived from human ESCs or from human fetal brain stem cells (Coufal et al., 2009). In addition, a qPCR assay demonstrated increased endogenous LINE-1 copy number in various brain regions, in particular the hippocampus, compared to the heart and liver of the same donor (Coufal et al., 2009). These observations were confirmed by DNA sequencing approaches, however to different frequencies. Bulk DNA sequencing of various human brain regions identified an extensive number of somatic insertions of LINE-1, as well as Alu and SVA, with widespread events mapping to protein coding genes expressed in the brain (Baillie et al., 2011). Sequencing of single human neuronal nuclei reported frequency of somatic LINE-1 insertions ranging from <0.6 to 13.7 unique insertions per neuron (Evrony et al., 2012; Upton et al., 2015). While the exact rate remains uncertain, collectively, these studies provide evidence of somatic retrotransposition, predominantly impacting LINE-1 elements in the neural lineage, including NPCs and non-dividing neuronal cells (Macia et al., 2017). These observations have important implications for neuronal plasticity and diversity. However, the actual functional significance of these events in brain function remains an open question. Moreover, as recent sequencing studies have focused mainly on somatic retrotransposition events and their frequency, the exact number of individual TE insertions whose expression is perturbed, and the resulting impact on gene regulation through *cis*-regulatory mechanisms, remain unknown.

Aging

Physiological aging is another process linked with disrupted TE activity. At the molecular level, aging is associated with extensive epigenetic alterations, including changes in histone modifications and DNA methylation patterns, as well as global heterochromatin loss and redistribution (López-Otín et al., 2013). These epigenetic alterations may, in turn, impact the expression and mobilization of TEs in aged cells and tissues (Cardelli, 2018). Changes in chromatin architecture were reported in senescent human fibroblasts, revealing a general compaction of euchromatic gene-rich regions, contrasting with an overall opening of constitutive heterochromatin in gene-poor regions. This was associated with increased expression of evolutionary young subfamilies of Alu, SVA and LINE-1 elements, along with indications of LINE-1 retrotransposition (Cecco et al., 2013a). Similar observations were reported in aged mouse somatic tissues, such as liver and muscle, for various retrotransposon subfamilies (Cecco et al., 2013b). In addition, a progressive increase in TE expression with age was observed in a study that examined total RNA-seq dataset from cell lines derived from healthy individuals from 1 to 94 years-old (LaRocca et al., 2020). At the mechanistic level, besides global epigenetic alterations, it was shown that the binding of sirtuin 6 (SIRT6), a strong repressor of LINE-1 elements, is reduced upon aging. SIRT6 coordinates the packaging of the LINE-1 5′UTR into repressive heterochromatin, through mono-ADP ribosylation of the corepressor KAP1 (Meter et al., 2014).

This raises the question of whether increased transcription and transposition is merely a consequence of aging, or whether it could also actively contribute to it (Maxwell, 2016). Increased transposition could contribute to the elevated DNA damage and related genomic instability associated with aging (Driver and McKechnie, 1992; Laurent et al., 2010; Sedivy et al., 2013). Increased TE expression has also been proposed to actively contribute to aging by promoting sterile inflammation, an aging-associated hallmark (López-Otín et al., 2013). Indeed, WT aged and SIRT6 knockout mice, along with senescent human fibroblasts,

present increased expression of LINE-1, as well as elevated cytoplasmic LINE-1 cDNAs. Although the origin of LINE-1 cDNAs in the cytoplasm is still unclear, this can trigger a strong type-I interferon (IFN-I) response, via activation of c-GAS (Cecco et al., 2019; Simon et al., 2019). IFN-I response was mitigated after knockdown of LINE-1 expression using siRNAs, as well as after treatment with nucleoside reverse transcriptase inhibitors (NRTIs), which inhibit the LINE-1 reverse transcriptase (Simon et al., 2019). In addition, in aged mice, NRTI treatment appears to improve age-associated inflammation observed in multiple tissues (Cecco et al., 2019; Simon et al., 2019). Interestingly, healthspan- and lifespan-increasing interventions, such as calorie restriction or pharmacological interventions, were shown to reduce TE expression and LINE-1 transposition in aged mice supporting a potential causal effect of increased TE expression in aging (Cecco et al., 2013b; Wahl et al., 2021). However, diminished TE expression could also be simply a consequence of the reduced aging.

Recently, the HERV-K subfamily HML-2 was also implicated as a potential contributor to cellular senescence through the activation of innate immune pathways. Indeed, it was shown that not only the expression of HML-2 elements is augmented in senescent human mesenchymal stem cells (hMSCs), but also that these elements were able to produce VLPs, which could be released extracellularly and induce senescent phenotypes in young cells (Liu et al., 2023). In addition, increased levels of ERVs were observed in different model organisms, including IAPs and MusD in aged mice (Barbot, 2002; Cecco et al., 2013b), ERV-K and ERV-W in aged cynomolgus monkeys (Liu et al., 2023) and HERV-K, HERV-W and HERV-H in human tissues and serum derived from old individuals (Balestrieri et al., 2015; Nevalainen et al., 2018), as well as in senescent hematopoietic stem cells (Capone et al., 2018). Strikingly, the repression of ERV expression upon treatment with Abacavir, an NRTI, led to an alleviation of cellular senescence and tissue aging in mice (Liu et al., 2023).

Collectively, these findings show that increased expression of TEs is a hallmark of aging. In addition, they pose TEs as key drivers of cellular senescence, primarily through the activation of innate immune pathways, either in a cell-autonomous way or in a paracrine manner in the case of HERV elements. However, questions about the consequences of TEs expression remain to be investigated such as their possible impact on gene expression through *cis*-regulatory mechanism or chromatin architecture modifications. Eventually, TE expression might also be implicated in the development of aging-associated disorders reviewed in the next section.

Transposable elements activity in neurological and age-related disorders

Active TEs capable of mobilizing in the genome represent a source of genomic variability, which may be harmful to the host. In fact, germline insertions of TEs have been widely linked with genetic diseases (Hancks and Kazazian, 2016). Moreover, somatic *de novo* insertions of TEs have also been reported in various cancers (Burns, 2017). In addition, TEs are able to impact the host even without mobilizing since they bear important regulatory elements and encode proteins with multiple biochemical activities (Wells and Feschotte, 2020). For instance, upon loss of DNA methylation in human NPCs, young LINE-1 elements were shown to function as alternative promoters for various genes with neuronal-related functions or linked to neurological disorders, suggesting that the misregulation of LINE-1 expression during brain development could contribute to the onset of neurological diseases later in life (Jönsson et al., 2019). Accordingly, the misregulation of both the expression and mobilization of TEs has been implicated in several pathological contexts, including in neurological and age-related disorders (Table 1) (Saleh et al., 2019; Ahmadi et al., 2020; Burns, 2020; Terry and Devine, 2020; Evans and Erwin, 2021; Gorbunova et al., 2021). However, in most cases, the contribution of TEs to pathology remains unclear. Therefore, in the following sections, some of the most important findings implicating TEs in different neurological and age-related disease contexts will be discussed.

Rett syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder that affects predominantly young females, with a frequency ranging from 1/10,000 to 1/15,000 live female births (Marano et al., 2021). Clinical features of RTT include regression of spoken language, gait abnormalities and stereotypical hand movements (Neul et al., 2010). RTT symptoms start to manifest in early childhood and develop progressively over stages (Kyle et al., 2018). Approximately 95% of typical RTT cases are caused by mutations in the X-linked methyl-CpG binding protein 2 (*MECP2*) gene (Amir et al., 1999; Neul et al., 2010). *MECP2* encodes an epigenetic regulatory protein, which binds methylated cytosines in CG and CA contexts and interacts with transcriptional co-repressor complexes. As such, one of the main functions of MECP2 is to repress gene expression in a DNA methylation-dependent manner. Moreover, MECP2 is also believed to play a role in transcriptional activation, modulation of alternative splicing and microRNA (miRNA) processing, and chromatin remodeling (Lyst and Bird, 2015; Marano et al., 2021). *MECP2* is ubiquitously expressed, but was shown to be expressed at ~10-fold higher levels in neurons compared to other cell types, making it one of the most abundant proteins in neuronal nuclei (Skene et al., 2010). Consistent with its high abundance, MECP2 binds methylated DNA broadly throughout the genome and its absence causes global alterations of the neuronal epigenome, leading to transcriptional changes affecting many genes and suggesting that MECP2 fine-tunes neuronal gene expression (Marano et al., 2021).

Besides genes, MECP2 was also shown to bind and repress the expression of methylated TE sequences, such as LINE-1 and IAP retrotransposons in mouse brain (Muotri et al., 2010; Skene et al., 2010). Moreover, increased LINE-1 retrotransposition was observed in neuroepithelial cells of *MECP2*-null mice, in human NPC derived from RTT iPSCs, and in postmortem brains of RTT patients (Muotri et al., 2010). More recently, whole genome sequencing of postmortem brain samples from RTT patients and healthy controls confirmed a higher number of somatic insertions of the human-specific LINE-1 subfamily, L1Hs, in RTT brains (Jacob-Hirsch et al., 2018). In addition, a targeted

TABLE 1 List of neurological and age-related disorders associated with perturbed TE activity.

Disease	Cause	Implicated TE families	Impact on TE activity and potential TE-driven mechanisms	References
Rett syndrome	Mutation in *MECP2* gene	LINE-1 ERV	Increased LINE-1 and IAP expression Increased LINE-1 retrotransposition	Muotri et al. (2010), Skene et al. (2010), Jacob-Hirsch et al. (2018), Zhao et al. (2019)
Aicardi-Goutières syndrome	Mutations in *TREX1*, *RNASEH2*, *SAMHD1*, *ADAR1* and *IFIH1* genes	LINE-1 SINE	Accumulation of DNA and RNA derived from LINE-1 and Alu in the cytosol leading to IFN-1-induced immune response	Crow et al. (2006a), Crow et al. (2006b), Stetson et al. (2008), Zhao et al. (2013), Rice et al. (2014), Hu et al. (2015), Li et al. (2017), Thomas et al. (2017), Benitez-Guijarro et al. (2018), Chung et al. (2018), Herrmann et al. (2018)
Ataxia-telangiectasia	Mutation in *ATM* gene	LINE-1	Increased LINE-1 expression and retrotransposition inducing expression of interferon stimulated genes	Coufal et al. (2011), Jacob-Hirsch et al. (2018), Takahashi et al. (2022)
Amyotrophic lateral sclerosis	TDP-43 cytoplasmic accumulation	ERV LINE-1 SINE	Increased LINE-1, SINE and ERV expression Increased LINE-1 retrotransposition Expression of HERV-K or of its *env* gene leading to neuronal toxicity and cell death	Li et al. (2015), Liu et al. (2019), Tam et al. (2019)
Frontotemporal dementia	TDP-43 cytoplasmic accumulation	LINE-1 SINE ERV	Increased LINE-1, SINE and ERV expression Increased LINE-1 retrotransposition	Li et al. (2012), Liu et al. (2019)
Alzheimer's disease	Hyperphosphorylation of Tau protein	LINE-1 SVA ERV	Increased expression of LINE-1, SVA, HERV HERV-K(HML-2) RNA activating Toll-like receptors (TLRs) HERV-K transcripts leading to neurodegeneration and microglia accumulation	Guo et al. (2018), Sun et al. (2018), Dembny et al. (2020), Ramirez et al. (2022), Evering et al. (2023)
Hutchinson-Gilford Progeria syndrome	Mutation in *LMNA* gene	LINE-1 ERV	Increased expression of LINE-1 inhibiting expression of SUV39H1 and inducing heterochromatin loss Increased HERV-K expression and accumulation of VLPs activating innate immune pathways	Vazquez et al. (2019), LaRocca et al. (2020), Valle et al. (2022), Liu et al. (2023)
Werner syndrome	Mutation in *WRN* gene	LINE-1 ERV	Increased expression of LINE-1 inhibiting expression of SUV39H1 and inducing heterochromatin loss Increased HERV-K expression and accumulation of VLPs activating innate immune pathways	Valle et al. (2022), Liu et al. (2023)

bulk sequencing approach using PCR revealed that the lack of MECP2 leads to changes in the genomic pattern of L1Hs somatic insertions in cortical neurons of RTT patients. These insertions were found to be enriched in introns and in the sense orientation, which could potentially impact gene expression (Zhao et al., 2019). All together, these studies demonstrated that MECP2 plays a role in the silencing of TE sequences, mainly from the LINE-1 family. However, the extent to which the expression of other TE families is affected in RTT and whether this could play a role in transcriptome changes and in the etiology or progression of RTT remains unknown.

Aicardi-Goutières syndrome

Aicardi-Goutières syndrome (AGS) is a progressive inflammatory encephalopathy characterized by spasticity, psychomotor retardation, intracranial calcification, white matter changes and cerebrospinal fluid lymphocytosis (Aicardi and Goutières, 1984; Crow et al., 2020; 2013). This syndrome is phenotypically and genotypically heterogeneous, as it can manifest itself with different degrees of severity and results from mutations in various genes involved in nucleic acid metabolism and signaling, including *TREX1*, *RNASEH2*, *SAMHD1*, *ADAR1*, and

IFIH1/MDA5 (Crow et al., 2006a; 2006b; Stephenson, 2008; Rice et al., 2012; 2009; Oda et al., 2014).

The *TREX1* gene encodes the three-prime repair exonuclease 1, an exonuclease involved in the degradation of cytosolic DNA. It was shown that depletion of TREX1 in the mouse leads to the accumulation of single-stranded DNA (ssDNA) derived from TEs, highlighting retroelement-derived DNA as a substrate of TREX1 (Crow et al., 2006a; Stetson et al., 2008). Moreover, an increase in TE derived-extrachromosomal DNA, of which LINE-1 are a major source, was reported in *TREX1*-deficient NPCs obtained after differentiation of human pluripotent stem cells. Further differentiation showed increased apoptosis in neurons and astrocytes exhibiting increased IFN-I secretion, thus contributing to greater neurotoxicity compared to control cells. Knockdown of LINE-1 RNA using shRNAs or inhibition of reverse transcription using NRTIs reduced the levels of extranuclear ssDNA and IFN-1 secretion in *TREX1*-deficient cells (Thomas et al., 2017). It was further shown that TREX1-mediated LINE-1 suppression could also occur independently of its nuclease activity, through ORF1p degradation (Li et al., 2017).

The *RNASEH2A, RNASEH2B* and *RNASEH2C* genes encode the three proteins composing the human ribonuclease H2 enzyme complex. Mutations in any of the three units is the most frequent cause of AGS (Crow et al., 2006b). It has been suggested that RNASEH2 degrades LINE-1 RNA after reverse transcription, being thus required for efficient completion of the retrotransposition cycle. Mutations in the *RNASEH2* genes therefore result in decreased LINE-1 retrotransposition and may lead to the accumulation of cytoplasmic LINE-1 RNA (Benitez-Guijarro et al., 2018).

Mutations in the *SAMHD1* gene also cause AGS (Rice et al., 2009). This gene encodes the SAM domain and HD domain containing protein 1 (SAMHD1), a nucleocytoplasmic shuttling protein with dNTP triphosphohydrolase activity (Du et al., 2019). SAMHD1 is known to inhibit LINE-1 retrotransposition activity in dividing cells by a mechanism still not fully understood (Zhao et al., 2013; Hu et al., 2015; Herrmann et al., 2018). On the one hand, it was suggested that SAMHD1 reduces ORF2p expression (Zhao et al., 2013). On the other hand, SAMHD1 is known to promote the formation of stress granules in the cytoplasm, which may induce the sequestration of LINE-1 RNP and prevent retrotransposition (Hu et al., 2015). The inhibition of LINE-1 retrotransposition by SAMHD1 could restrain TE-derived DNA accumulation in the cytoplasm, preventing the aberrant synthesis of interferon and inflammatory cytokines explaining, at least in part, this characterized feature of AGS associated with SAMHD1 mutations (Hu et al., 2015).

Finally, the *ADAR1* gene encodes an adenosine deaminase acting on double-stranded RNA (dsRNA) (Rice et al., 2012). This protein was shown to bind transcripts derived from Alu elements and to prevent activation of dsRNA sensors such as MDA5, a cytoplasmic viral RNA receptor involved in IFN production and response. *ADAR1* KO in NPCs results in non-edited Alu sequences that tend to form dsRNAs, which trigger IFN-1 production via the activation of MDA5 (Chung et al., 2018). A recent study demonstrated the activation of IFN-1 in the brain of mice carrying *ADAR1* mutation (Guo et al., 2021). In addition, gain-of-function mutations in the *IFIH1* gene, which encodes the MDA5 receptor, were identified in AGS patients. These mutations might lower the recognition threshold of MDA5, enabling not only the recognition of exogenous dsRNA, but also of dsRNA derived from TEs. Eventually, the constitutive activation of the receptor triggers an innate immune response (Rice et al., 2014).

All the mutations described above are associated with the accumulation of cytoplasmic DNA or RNA species derived from TEs. In addition, IFN-1-induced immune response triggering the expression of interferon stimulated genes is observed in almost all AGS patients, except the ones with *RNASEH2B* mutations (Crow and Manel, 2015). However, the mechanistic link between TE product accumulation and the inflammatory phenotype observed in AGS is still poorly understood.

Ataxia-Telangiectasia

Ataxia-Telangiectasia (AT) is an autosomal recessive disorder characterized by progressive cerebellar degeneration, immunodeficiency, predisposition to develop cancer, radiation sensitivity and premature aging (Rothblum-Oviatt et al., 2016). It is caused by a loss-of-function mutation in the Ataxia-Telangiectasia mutated (*ATM*) gene, which encodes a serine/threonine kinase that activates the DNA repair machinery in response to DNA damage (Savitsky et al., 1995; Shiloh, 2001), highlighting a possible link between genomic instability and neurodegeneration (McKinnon, 2017).

An increase in the retrotransposition efficiency of an engineered human LINE-1 was detected in NPCs derived from *ATM*-deficient hESCs and in *ATM* KO transgenic mice. In addition, an increase in human-specific LINE-1 (L1Hs) copy number was observed in postmortem human brain tissue from AT patients compared to healthy controls (Jacob-Hirsch et al., 2018). This led to the hypothesis that the ATM protein recognizes intermediates created during LINE-1 integration as sites of DNA damage, and consequently increases the number and the length of the resulting retrotransposition events (Coufal et al., 2011).

In a more recent study, an increased expression of evolutionary younger LINE-1 subfamilies and a concomitant decreased expression of TE epigenetic silencers, including MECP2 and KAP1, was observed in cerebellar samples from AT patients. Interestingly, targeted upregulation of the young mouse L1MdTf subfamily using a CRISPR activation (CRISPRa) strategy in the cerebellum of transgenic mice was sufficient to induce progressive ataxia and expression of interferon stimulated genes. In addition, treatment with NRTIs led to the attenuation of disease progression. This study thus delineates a causal link between increased LINE-1 activity and neurodegeneration, using a mouse model (Takahashi et al., 2022). However, whether LINE-1 enhanced activity triggers neurodegeneration directly remains to be functionally demonstrated, as the observed neurodegeneration could be a consequence of increased DNA damage as previously shown (Blaudin de Thé et al., 2018) or of CRISPRa off-target effects.

Neurodegenerative disorders

In addition to the aforementioned neurological disorders, perturbed TE activity has been linked to various

neurodegenerative disorders, some examples of which will be developed in the following section. Amyotrophic lateral sclerosis (ALS), a disease marked by loss of motor neuron function and frontotemporal dementia (FTD), which is associated with loss of frontal and temporal cortexes, are two neurodegenerative diseases associated with disrupted TE activity (Ling et al., 2013). One of the major hallmarks of these pathologies is the loss of nuclear TAR DNA-binding protein 43 (TDP-43) and its subsequent cytoplasmic accumulation (Neumann et al., 2006). This protein was shown to bind to TE-derived transcripts from all the main classes including LINEs, SINEs, and ERVs. The association between TDP-43 and TE-derived transcripts was shown to be reduced in FTD patients and mouse models exhibiting TDP-43 dysfunction show an increase of TE-derived transcripts, which match the ones identified as TDP-43 targets (Li et al., 2012). In agreement with these findings, another study analyzed the transcriptomes of 148 ALS postmortem cortexes and identified a subset of ALS patients with TDP-43 dysfunction and increased expression of TEs, especially young LINE-1 and SVA elements (Tam et al., 2019). Moreover, the effect of nuclear TDP-43 loss and its cytoplasmic aggregation were investigated in postmortem brain samples of FTD and ALS-FTD patients. Chromatin decondensation around LINE-1 insertions was reported, as well as increased LINE-1 DNA content, indicative of increased retrotransposition. Accordingly, it was hypothesized that TDP-43 may regulate the expression of TEs in the brain under physiological conditions through unknown mechanisms, further suggesting that TE derepression may be implicated in ALS and FTD (Liu et al., 2019). TDP-43 was also shown to regulate the expression of HERV-K elements. Indeed, postmortem brain tissue from ALS patients display increased expression of HERV-K. In addition, the expression of HERV-K in human neurons *in vitro* resulted in retraction and beading of neurites, neuronal toxicity and cell death. Moreover, the expression of the HERV-K *env* gene in the neurons of transgenic animals led to the development of several pathological features reminiscent of ALS, including motor dysfunction (Li et al., 2015). All these observations point towards a contribution of HERV-K to neurodegeneration.

Another neurodegenerative disease that has been associated with the activation of TEs is Alzheimer's disease (AD). One of the neuropathological signatures of AD is the hyperphosphorylation of Tau protein, which leads to the subsequent formation of intracellular neurofibrillary tangles (NFTs) (Iqbal et al., 2005; Klein et al., 2019). Expression of a pathogenic form of Tau was shown to induce heterochromatin loss in motor neurons in mice and in hippocampal neurons from AD patients. The heterochromatin relaxation was shown to be triggered by oxidative stress-induced DNA damage and to be associated with aberrant expression of genes linked with pluripotency and developmental processes, which are normally silent in the brain (Frost et al., 2014). Heterochromatin loss, as well as a reduction of Piwi protein and piRNAs levels, could lead to the increased expression of TEs, including specific subfamilies of LINE-1, SVAs, and HERVs, observed in postmortem brain samples of AD patients. Increased TEs expression could contribute to neurodegeneration by innate immune response activation and/or by promoting genome instability (Guo et al., 2018; Sun et al., 2018).

A recent study investigating TE expression in the brain of three different tauopathy mouse models reported an increase in retrotransposon transcript levels, especially from the ERV class, including IAP, IAP-E, MULV, MERVL, ERV-β4 subfamily members, but also LINE-1 and B1/B2 elements. Moreover, an increase in IAP encoded-gag protein levels and a higher copy number of LINE-1, IAP, ETn and specific ERV-K elements was detected, suggesting that these elements are actively retrotransposing in the context of tauopathy (Ramirez et al., 2022). Moreover, RNA from HERV-K (HML-2) was shown to bind to and activate the murine TLR7 and human TLR8 (Toll-like receptor) expressed in neurons and microglia, resulting in neurodegeneration and microglia accumulation, an important hallmark of AD (Dembny et al., 2020). Recently, a model was proposed to explain the impact of ERV transcripts in neurodegeneration. Innate immune sensors are activated by cytoplasmic HERV-derived nucleic acids, which lead to the secretion of IFN-1 and other inflammatory signals. In response to these signals, microglia release cytokines that can be sensed by astrocytes. These reactive astrocytes produce neurotoxins and are unable to maintain synaptic connections, which could ultimately lead to neuronal death and neurodegeneration (Evering et al., 2023).

Premature aging progeria syndromes

Another group of age-related disorders where TEs have been implicated are premature aging disorders, including Hutchinson-Gilford Progeria syndrome (HGPS) and Werner syndrome (WS). Both HGPS and WS recapitulate many of the phenotypes associated with normal aging (Ghosh and Zhou, 2014). HGPS is a genetic disorder classified as a laminopathy, caused by single-base substitutions in the *LMNA* encoding lamin A/C, which results in the activation of a cryptic splice site leading to the production of a protein truncated of 50 amino acids, called progerin (Eriksson et al., 2003; Sandre-Giovannoli et al., 2003; Worman and Bonne, 2007; Noda et al., 2015). WS, on the other hand, is caused by mutations in the *WRN* gene, which encodes a RecQ helicase known as the WRN protein that has both exonuclease and helicase activities (Yu et al., 1996; Kudlow et al., 2007). These two premature aging disorders are associated with epigenetic changes, including loss of heterochromatin. Indeed, the lamin A/C proteins, structural components of the nuclear lamina, promote the anchoring of heterochromatin to the nuclear periphery (Goldman et al., 2004; Scaffidi and Misteli, 2005; Shumaker et al., 2006). The WRN protein, known to be involved in DNA repair, plays a role in heterochromatin stability through interactions with heterochromatin proteins, including the histone methyltransferase SUV39H1 and HP1α (Zhang et al., 2015).

Since heterochromatin loss is associated with loss of silencing of TEs, there has been a growing interest in exploring whether TEs could contribute to premature aging disorders. In one study, it was demonstrated that SIRT7-mediated deacetylation of H3K18 plays a role in silencing LINE-1 by facilitating its association with lamin A/C in mouse fibroblasts. Consequently, absence of SIRT7 or depletion of lamin A/C results in transcriptional upregulation of LINE-1 elements in mouse and human cells, consistent with observations from RNA-seq data from fibroblasts of HGPS patients (Vazquez et al., 2019; LaRocca et al., 2020).

Recently, LINE-1 RNA was implicated as a causal agent of heterochromatin erosion in premature aging syndromes. Increased expression of L1Hs elements was observed in hMSCs differentiated from iPSCs derived from HGPS and WS patients. The accumulation of LINE-1 RNA in the nucleus led to an increased interaction with SUV39H1, resulting in the inhibition of its enzymatic activity, loss of heterochromatin and increased expression of senescence-associated secretory phenotype (SASP) genes. Interestingly, an improvement of the senescent phenotype in dermal fibroblasts of progeria patients and HGPS mice was reported following LINE-1 RNA depletion using antisense oligonucleotides (ASOs), but not using NRTIs. In addition, LINE-1 RNA depletion led to an upregulation of pathways associated with nuclear organization, cell proliferation and transcription regulation, together with downregulation of pathways associated with aging, inflammatory response and DNA damage. Together, these results point to an important role of LINE-1 RNAs in the progression of premature aging disorders through the negative regulation of SUV39H1 enzymatic activity (Valle et al., 2022). However, the mechanism by which LINE-1 RNA inhibits SUV39H1 activity remains an open question.

A recent study showed that the HERV-K (HML-2) retrotransposon family also contributes to the senescence phenotype of premature aging syndromes. HERV-K expression was found to be upregulated in HGPS and WS cellular models, where the accumulation of viral proteins and VLPs could trigger innate immune responses thereby contributing to senescence. Importantly, as indicated in the aging section, HERV-K VLPs could be released in a paracrine manner and trigger senescence in non-senescent cells. Consistent with these results, tissues from HGPS cynomolgus monkeys exhibited an increase in ERV-W-Env protein levels. Moreover, this study showed that CRISPRa-mediated activation of HERV-K induced premature senescence, and that repression of HERV-K using shRNA, CRISPR interference or NRTI treatment reduced cellular senescence phenotypes and tissue aging in mice (Liu et al., 2023).

All together, these studies suggest a causal relationship between increased TEs expression and aging-associated phenotypes, which can be alleviated by repressing TEs. This opens up new possibilities for premature aging treatment and offers a strategy to be applied to other aging-associated disorders.

Concluding remarks

Once considered as purely "junk DNA," TEs are now recognized as major drivers of genome evolution and genetic diversity. As their immediate impact may be deleterious, the host has developed silencing mechanisms to restrict their expression and retrotransposition, in particular in somatic lineages. It is now accepted that the brain stands out as an exception, exhibiting increased activity of TEs from specific families or subfamilies. It is still unclear whether this is linked to the relaxation of epigenetic mechanisms in neuronal lineages or the presence of specific factors promoting TE expression, or most likely a combination of both. Furthermore, while the biological importance of these observations for neuronal plasticity and diversity is intriguing, it remains unknown and challenging to investigate experimentally.

The aging process, as well as the neurological and age-related disorders described in this review and showing perturbed TE activity, share significant common hallmarks, such as increased DNA damage from retrotransposition, the cytoplasmic accumulation of nucleic acid species from TEs, and the induction of IFN-1 immune response, which can trigger inflammation. Although a causal link between TE expression and neurodegeneration or aging-associated phenotypes is observed in models of AT and progeroid syndromes, the relative contribution of these different features to pathological phenotypes and the sequence of events are unclear. In addition, the potential *cis*-regulatory roles of TE promoters and their influence on transcriptional networks in the various disease contexts remain poorly explored. Regardless, products encoded by TEs, including transcripts and proteins, merit further investigation, in particular as potential candidates for the development of biomarkers of biological age or neurological disorders (LaRocca et al., 2020).

Genome editing technologies such as CRISPR-Cas9 will be essential tools to further unravel the contribution of TEs in physiological or disease contexts (Fueyo et al., 2022). For example, these methods could be used to induce transcriptional silencing of TEs families or subfamilies known to be aberrantly expressed in disease. This would enable determining whether some of the common transcriptome changes or pathological phenotypes are reversed following TE silencing. This could also be used to address whether interfering with TE expression could impact brain development or function.

Furthermore, the development of tools for TE annotation, the more systematic inclusion of TE sequences in next-generation sequencing analysis and the improvement of dedicated computational pipelines will undoubtedly help to understand further the extent to which TE expression and their chromatin state is perturbed in a specific context, as well as the impact on the transcriptome (Lanciano and Cristofari, 2020). In particular, it will be important to distinguish expression of TEs embedded in introns of genes from autonomous expression of TE from their own promoter. In addition, determining whether most elements or only a small subset of insertions from a given family/subfamily are impacted will be essential for the design of downstream functional analysis. Finally, mapping reads coming from the youngest and more active elements, usually overrepresented among the classes showing increased expression in disease (such as L1Hs or HERV-K in the human genome), is very challenging. In that regard, recent pipelines, such as CELLO-seq or SoloTE (Berrens et al., 2022; Rodríguez-Quiroz and Valdebenito-Maturana, 2022), exploit long read and/or single-cell RNA sequencing technologies to tackle many of the issues associated with the mapping of young TEs and allow to analyze more unambiguously TE copies at the individual and locus-specific level.

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Conflict of interest

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Roles of endogenous retroviral elements in the establishment and maintenance of imprinted gene expression

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DNA methylation (DNAme) has long been recognized as a host defense mechanism, both in the restriction modification systems of prokaryotes as well as in the transcriptional silencing of repetitive elements in mammals. When DNAme was shown to be implicated as a key epigenetic mechanism in the regulation of imprinted genes in mammals, a parallel with host defense mechanisms was drawn, suggesting perhaps a common evolutionary origin. Here we review recent work related to this hypothesis on two different aspects of the developmental imprinting cycle in mammals that has revealed unexpected roles for long terminal repeat (LTR) retroelements in imprinting, both canonical and noncanonical. These two different forms of genomic imprinting depend on different epigenetic marks inherited from the mature gametes, DNAme and histone H3 lysine 27 trimethylation (H3K27me3), respectively. DNAme establishment in the maternal germline is guided by transcription during oocyte growth. Specific families of LTRs, evading silencing mechanisms, have been implicated in this process for specific imprinted genes. In noncanonical imprinting, maternally inherited histone marks play transient roles in transcriptional silencing during preimplantation development. These marks are ultimately translated into DNAme, notably over LTR elements, for the maintenance of silencing of the maternal alleles in the extraembryonic trophoblast lineage. Therefore, LTR retroelements play important roles in both establishment and maintenance of different epigenetic pathways leading to imprinted expression during development. Because such elements are mobile and highly polymorphic among different species, they can be coopted for the evolution of new species-specific imprinted genes.

KEYWORDS

genomic imprinting, DNA methylation, H3K27me3, endogenous retroviral elements, developmental epigenetics

1 Genomic imprinting and host defense mechanisms

The first mouse imprinted genes, *H19*, *Igf2*, and *Igf2r*, were identified in 1991 (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991). The imprinting of *Snrpn* was demonstrated the following year (Cattanach et al., 1992; Leff et al., 1992), and in 1993 the first reports presenting evidence supporting a role for DNAme in the imprinting mechanism were published (Bartolomei et al., 1993; Brandeis et al., 1993; Ferguson-Smith et al., 1993; Li et al., 1993). It was already recognized at the time that the DNAme machinery exploited in mammals was derived from bacterial immune systems

that had been adapted for the transcriptional repression of repetitive sequences (Bestor, 1990). This led the late Denise Barlow to propose that genomic imprinting had evolved from host defense mechanisms, by co-opting DNAme-based functions in the parent-of-origin-specific silencing of imprinted genes (Barlow, 1993). Several different predictions of the model proposed have been confirmed, as recently reviewed (Ondičová et al., 2020). A related aspect of this model, which is the focus of this review, addresses the roles played by endogenous repetitive elements themselves in the regulation of imprinted gene expression. Here, we review recent evidence suggesting that LTR elements have been co-opted for both germline establishment and somatic maintenance of imprinted gene expression in early development.

2 Retrotransposons and canonical imprint establishment

2.1 DNA methylation imprints

Early studies on diploid biparental gynogenetic and androgenetic embryos suggested that genomic imprinting is established during gametogenesis (McGrath and Solter, 1984; Surani et al., 1984). Although the epigenetic mechanisms involved were not known at the time, DNAme was later shown to represent an important epigenetic mark, directly inherited from the mature gametes, and regulating imprinted gene expression. The monoallelic expression of canonical imprinted genes in somatic cells is maintained by differential DNAme marks (Tucci et al., 2019) established *de novo* during male or female gametogenesis by the sex-specific action of the DNA methyltransferase DNMT3A (Kaneda et al., 2004), and its co-factor DNMT3L (Bourc'his et al., 2001; Hata et al., 2002; Arima et al., 2006). Large fractions of the genome are differentially methylated between eggs and sperm, but unlike most of the differences, the gametic DNAme marks at imprinted genes survive the wave of demethylation occurring during pre-implantation stages. This survival of imprints requires the maintenance DNA methyltransferase DNMT1 (Li et al., 1993; Hirasawa et al., 2008), its partner UHRF1 (Sharif et al., 2007), and the DNAme-dependent DNA-binding factors ZFP57 and ZFP445. These KRAB zinc-finger proteins specifically bind the methylated allele of imprinted genes and protect it from demethylation during preimplantation stages via their recruitment of KAP1/TRIM28 and SETDB1. This histone methyltransferase establishes a H3K9me3 mark over the DNAme-marked region for preferential recruitment of DNMT1 via UHRF1, which recognizes H3K9me3 via its tandem Tudor domain and plant homeodomain (Li et al., 2008; Strogantsev and Ferguson-Smith, 2012; Takahashi et al., 2019; Janssen and Lorincz, 2021). In somatic cells, sequences carrying these DNAme imprints are detected as Differentially Methylated Regions (methylated on a single allele) of gametic origin (gDMR). Imprinted gDMRs are thought to be responsible for all canonical imprinted gene expression observed in embryonic and adult cells. Only 24 gDMRs have been identified in the mouse, 21 methylated in the oocyte and 3 in sperm (Proudhon et al., 2012; Bogutz et al., 2019). Both oocyte and sperm DNAme play essential roles in imprinting, but whereas most of the paternal DNAme is lost after fertilization, a portion of oocyte-derived 5-methylcytosine (5mC) survives the passive demethylation occurring during preimplantation (Smallwood et al., 2011). Although the function of most of this inherited maternal DNAme is still unknown, some of these maternal marks were shown to be required for silencing genes detrimental for placental development, the first demonstration of a role for maternal DNAme unrelated to genomic imprinting (Branco et al., 2016). Recent surveys suggest that the human genome contains more gDMRs (Zink et al., 2018; Akbari et al., 2022), with several maternally-inherited marks maintained only in the placenta (Court et al., 2014; Hamada et al., 2016; Hanna et al., 2016; Sanchez-Delgado et al., 2016).

2.2 Imprint establishment during oogenesis: a transcription-guided process

The analysis of the DNAme profile of gametes at single-base resolution using whole-genome bisulphite sequencing (WGBS) revealed that there is nothing fundamentally unique about *de novo* establishment of imprinted gDMR. Rather, these sequences acquire DNAme as part of global mechanisms methylating the mouse sperm and oocyte genomes at >80% and ~40% levels, respectively (Kobayashi et al., 2012). Mature gametes are also methylated at very different levels in human, with average levels of DNAme of ~75% and ~54% for sperm and egg, respectively (Okae et al., 2014a). In the mouse, whereas paternal gDMRs are DNA methylated in prospermatogonia from E14.5 to birth (Davis et al., 2000; Li et al., 2004), elegant embryological experiments showed that the establishment of functional maternal imprints occurs during the phase of oocyte growth taking place in postnatal ovaries (Kono et al., 1996; Obata et al., 1998; Obata and Kono, 2002). Accordingly, the genome of primary non-growing oocytes (NGO, from P1-P5 females), and of fully-grown, germinal vesicle stage oocytes (FGO or GVO, from mature females), show a drastic difference in average genomic DNAme levels, from 2% to 40%, including at several CpG islands (CGIs) (Shirane et al., 2013). The process of *de novo* DNAme therefore occurs postnatally in females, in non-dividing oocytes, and was shown to require DNMT3A and its cofactor DNMT3L, but not DNMT3B or the maintenance DNMT1 enzyme (Smallwood et al., 2011; Kobayashi et al., 2012; Shirane et al., 2013).

By comparing the DNA methylome and transcriptome of oocytes, as determined by RNA-seq, a direct correlation was observed between gene transcription and gene body DNAme (Smallwood et al., 2011; Kobayashi et al., 2012; Veselovska et al., 2015). Whereas promoter regions of active genes are hypomethylated (<15% 5 mC), their transcribed regions acquire 60%–90% DNAme, starting ~2 kb downstream of their oocyte-specific transcription start site (TSS). Strikingly, transcribed regions account for 85%–90% of the methylome of FGOs, including DNAme at all imprinted maternal gDMRs (Veselovska et al., 2015). Pioneering work from the group of Gavin Kelsey showed that oocyte transcription across the gDMR region was required for DNAme establishment at the maternal *Gnasxl/Nespas* gDMR and that most imprinted maternal gDMRs are indeed covered by an oocyte transcript initiating at an upstream promoter (Chotalia et al., 2009). Following this work, a role for oocyte transcription in *de novo* DNAme at the gDMRs of the

paternally expressed genes *Snrpn, Plagl1,* and *Kcnq1ot1* was demonstrated directly in mouse mutants in which inserted transcription termination sequences prevent oocyte transcripts from extending across the DMR region (Smith et al., 2011; Veselovska et al., 2015; Singh et al., 2017). Although DNAme blocks are not as well defined in human oocytes, a strong correlation was also noted between methylated and transcribed regions, suggesting that the link between *de novo* methylation and transcription is conserved (Okae et al., 2014a). Interestingly, other examples of transcription-coupled acquisition of DNAme at imprinted promoters emerged from the analysis of retrogenes, inserted within a host gene expressed in oocytes (Cowley and Oakey, 2010). Because of their location, the promoter of these inserted retrogenes is covered by a transcript in oocytes and acquires a maternal gDMR, leading to silencing of the maternal allele and expression from the paternal allele of the retrogene in the progeny (Wood et al., 2007).

The mechanism whereby transcribed regions acquire DNAme in oocytes was shown to be guided by both negative and positive cross-talks with specific histone post-translational modifications. Unmethylated promoter CGIs are usually marked by H3K4me3 (Mikkelsen et al., 2007) and this mark has an inhibitory effect on the action of the DNMT3A-DNMT3L complex (Guo et al., 2015), protecting these CpG-rich sequences from *de novo* DNA methylation (Ooi et al., 2007). This implies that intragenic CpG-rich regions covered by an oocyte transcript would be refractory to *de novo* DNA methylation unless methylation marks at H3K4 are previously removed from those regions. Consistent with this prediction, CGIs acquiring DNAme during oocyte growth are devoid of or lose H3K4me2/3 marks in preparation for *de novo* DNA methylation, and the H3K4 lysine demethylase KDM1B plays a dominant role in the removal of these refractory marks (Ciccone et al., 2009; Stewart et al., 2015). Interestingly, a mutant version of DNMT3A carrying two point mutations within the ADD domain, which interfere with the binding of DNMT3A to H3K4me0 *in vitro*, was recently shown to lead to dwarfism and female infertility. The global DNAme of oocytes from homozygous females is severely affected (at only 17.6%, compared to 35.9% for wild-type oocytes), leading to stochastic loss of maternal imprinted methylation, abnormal expression of different imprinted genes in the progeny, with variations between individual embryos, several of which die before mid-gestation (Uehara et al., 2023).

The recruitment of the *de novo* DNA methyltransferases DNMT3A and 3B to transcribed regions is mediated by their PWWP domain, a reader for H3K36me2/3 marks (Dhayalan et al., 2010). As originally demonstrated in somatic cells, transcribed regions acquire an H3K36me3 domain via the recruitment of the histone methyltransferase SETD2, which is in a complex with the elongating RNA polymerase II (Yoh et al., 2008). The SETD2-deposited H3K36me3 marks then recruit DNMT3B to those regions via its PWWP domain, leading to the establishment of a DNAme block over transcribed regions in ESCs (Baubec et al., 2015; Neri et al., 2017). A similar recruitment mechanism is conserved during *de novo* DNA methylation in the germline, although different approaches are exploited to establish the H3K36me-marked domains in male and female gametes. In oocytes, both SETD2-deposited H3K36me3 marks over transcribed regions as well as H3K36me2, presumably deposited by the nuclear receptor-binding SET-domain proteins NSD1 or NSD2, are implicated in establishing the maternal methylome via recruitment of the DNMT3A-DNMT3L complex (Xu et al., 2019; Yano et al., 2022). In the male germline, where the genome is more than 80% methylated (Kobayashi et al., 2012), the recruitment of DNMT3A is mediated by NSD1-deposited H3K36me2 marks, which cover broad regions of the genome (Shirane et al., 2020).

Despite this simple model implicating direct DNMT3A-H3K36me2/3 interactions, some results on mouse mutants carrying specific mutations in the DNMT3A PWWP reader domain may suggest that additional mechanisms are also at play during *de novo* DNA methylation. Two point mutations within the PWWP domain abrogating the binding of DNMT3A to H3K36me2/3 *in vitro* have been modeled in mouse. Both of these alleles, D239A and W236R, lead to dominant growth retardation phenotypes characterized by abnormal gain of DNAme at H3K27me3-marked regions. Surprisingly, gene bodies where H3K36me3 is deposited were unaffected (Heyn et al., 2019; Sendžikaitė et al., 2019). Similar observations were also made in mutant oocytes expressing only the D239A variant, in which H3K36me2/3-marked regions still acquired DNAme (Kibe et al., 2021). Although those results may suggest the existence of an alternative recruitment mechanism for the DNMT3A/3L complex, the authors also raise the possibility of residual binding of the D239A mutant PWWP domain to H3K36me2/3 *in vivo*, or a compensation via interactions between DNMT3A and DNMT3B, which also features an H3K36me2/3-binding PWWP domain (Kibe et al., 2021). The resolution of these alternative scenarios will require the direct analysis of DNMT3A D239A binding specificity *in vivo* by ChIP-seq and studies involving the simultaneous deletion of *Dnmt3b* in oocytes.

2.3 LTR elements expression in oocytes

Long-terminal-repeat retrotransposons (LTRs), also known as endogenous retroviruses (ERVs), are highly variable in mammalian genomes and constitute ~10% and ~9% of the mouse and human genomes, respectively (Chinwalla et al., 2002). Several families of transposable elements, mostly young LTRs, can promote transcription initiation and act as TSS during oocyte growth in both mouse and human (Peaston et al., 2004; Veselovska et al., 2015; Franke et al., 2017; Hendrickson et al., 2017). Therefore, although LTRs are usually silenced by epigenetic mechanisms implicating DNAme or repressive histone marks such as H3K9me3 (Liu et al., 2014), some of these elements, notably younger LTRs, evade these mechanisms and are active as promoter elements in growing oocytes. Some of these LTR-initiated transcripts are intergenic or antisense to known genes, but others act as oocyte-specific alternative promoters for annotated genes, forming chimeric transcripts with annotated downstream exons. This enormous potential of LTR elements to shape the oocyte transcriptome is conserved in mammals and has been documented by oocyte RNA-seq in several species, such as mouse, rat, hamster, human and cow (Franke et al., 2017; Hendrickson et al., 2017; Brind'Amour et al., 2018). Interestingly, oocytes utilize a paralogue of the general transcription factor TATA binding protein (TBP), called TBPL2 (also known as TRF3 or TBP2), for transcription initiation during

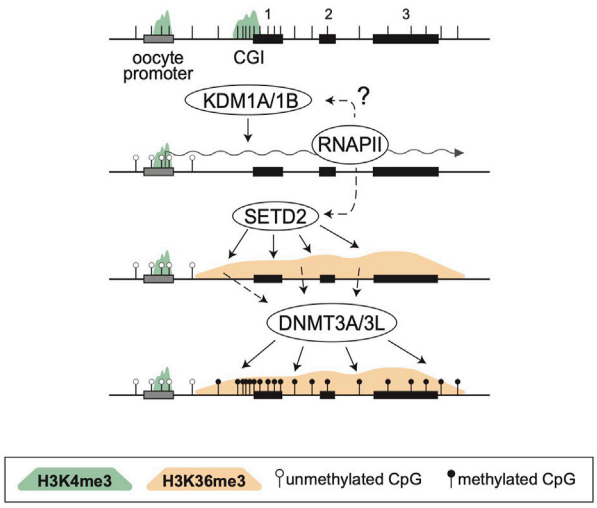

FIGURE 1
De novo DNA methylation during oocyte growth. Structure of a 3-exon gene is presented at the top, showing exons (black rectangles), a CGI promoter, the positions of CpG dinucleotides (vertical bars) and an upstream oocyte promoter (grey). In oocytes both promoters are marked with H3K4me3 (green shade) but KDM1A/1B, perhaps in association with RNAPII, remove this mark at the somatic CGI promoter. Simultaneously, SETD2 deposits H3K36me3 (orange shade) over the entire transcribed region. This mark is read by the PWWP domain of DNMT3A, which together with DNMT3L, methylates the transcribed region, including the CGI promoter.

oocyte growth (Gazdag et al., 2009). TBPL2 was shown to play an important role in oocyte transcription, including at LTR promoters, notably at those featuring a TATA-like motif (Yu et al., 2020). It will be interesting to document how the DNA methylome and imprinting are affected in *Tbpl2*$^{-/-}$ oocytes.

Given the high level of expression from specific LTR promoters in oocytes and the observation that transcribed regions are *de novo* methylated, a significant fraction of the oocyte methylome originates from transcription initiating in active LTR promoters (Brind'Amour et al., 2018). A comparative description of such an impact of LTR-initiated transcripts on the DNA methylome of mouse, rat, and human oocytes showed that transcriptionally active LTRs are responsible for wide differences in DNAme patterns in oocytes of different species (Brind'Amour et al., 2018). Note that as for non-repetitive oocyte promoters, the active LTRs themselves are not DNA methylated in oocytes and overlap with a peak of H3K4me3 active promoter mark. As for single-copy promoters, they also lead to the deposition of an H3K36me3 domain over the transcribed region and the subsequent formation of a block of DNAme starting ~2 kb downstream of the TSS provided by the LTR element (Brind'Amour et al., 2018).

2.4 Evidence for LTR-guided imprint establishment in oocytes

Since DNAme blocks acquired in oocytes and maintained during preimplantation stages play a critical role in imprinting, the work on LTR-driven transcription and DNAme in oocytes raised the following questions: Do some of the DNAme marks acquired in oocytes as a consequence of transcription from LTRs act as imprinted gDMRs allowing only paternal allele-specific expression of the downstream gene in the progeny? Has this mechanism contributed to the evolution of species-specific imprinted genes?

By analyzing known imprinted gDMRs established in mouse (21 gDMRs) and human (125 gDMRs) oocytes, 21 examples of methylated regions covered by oocyte transcripts initiated within an LTR element were identified, 4 in the mouse, and 17 in human (Bogutz et al., 2019). Based on a 2018 survey, the mouse and human genomes were found to contain approximately 260 and 228 imprinted genes, respectively, with 63 shared in both species (Tucci et al., 2019). From these figures, it follows that ~1.5% and ~7.5% of imprinted genes are regulated by oocyte promoters in mouse and human, respectively. Data from mouse oocytes show that transcription initiation from these LTRs, marked by H3K4me3, lead to downstream blocks of H3K36me3 and DNAme deposition over the transcribed region, covering the site of the associated gDMR (Figure 1). Interestingly, none of these are the 15 maternal gDMRs shared between those two species. Moreover, for the 4 mouse gDMRs, the oocyte transcripts all initiate within LTR families specific to rodents, and 12 of the 17 human gDMRs are covered by transcripts initiating from LTRs of primate-specific families, 9 of which appear conserved in chimpanzee. Whereas most of this data is correlational, CRISPR-Cas9 mediated deletions of the LTR elements acting as upstream oocyte promoters at the mouse *Impact* and *Slc38a4* imprinted genes confirmed the importance of these elements in species-specific maternal imprints. For both LTR knockouts, DNAme is lost at the gDMR of those genes in oocytes of homozygous knockout females and imprinting is lost in the progeny, with biallelic transcription of each gene (Bogutz et al., 2019). Together, the analysis presented in this study highlights a previously unappreciated role for LTR elements of endogenous retroviruses: by acting as promoters in oocytes, some of these elements can induce DNAme at a downstream CpG-rich promoter that is otherwise kept unmethylated in sperm, and can therefore lead to the formation of a new paternally-expressed imprinted gene, assuming maintenance of this maternal DNAme mark post-fertilization (Figure 2). As mentioned above, the survival of DNAme marks at gDMRs during preimplantation development relies on the binding of ZFP57 and ZFP445 to methylated TGCCGC motifs. Most of the canonical imprinted genes regulated by oocyte-specific LTR promoters contain at least one such binding site (Table 1). For the 17 human genes, ZFP57 binding has been observed by ChIP-seq at the *HTR5A* and *CLDN23* CGI promoters, which maintain their imprinted DNAme mark in many epiblast-derived tissues (Bogutz et al., 2019). For 14 of these genes, imprinted DNAme at the CGI has only been observed in the placenta, so ZFP57/445 binding would be expected to only be observed during preimplantation development and in extra-embryonic cells.

3 Retrotransposons and noncanonical imprint maintenance

3.1 Evidence for DNAme-independent imprinting

Although DNAme-based canonical imprinting provided an elegant mechanism to explain most imprinting effects, some observations suggested the existence of a parallel epigenetic

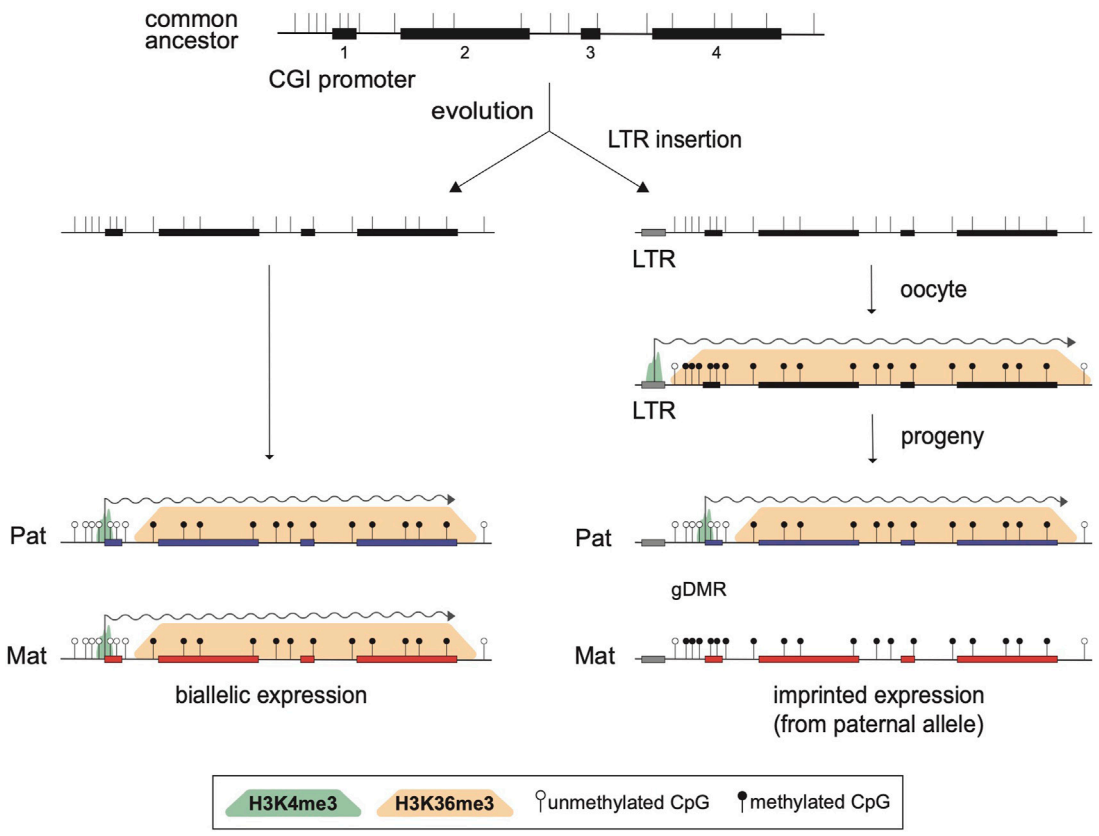

FIGURE 2
Model for the acquisition of imprinted expression via the insertion of an LTR element. Structure of a biallelically expressed ancestral gene is presented at the top, showing four exons (black rectangles), the positions of CpG dinucleotides (vertical bars), and a CpG island (CGI) promoter overlapping exon 1. Following evolution, two scenarios are considered. On the left, the locus is unchanged and the gene is expressed from both alleles in the progeny, as shown by biallelic active H3K4me3 marks (green shade) at the promoter and active transcription (wiggly arrow). One the right, a *de novo* retrotransposition event leading to the insertion of a solo LTR upstream of exon 1 is represented. The inserted LTR remains transcriptionally active in oocytes and induces the formation of blocks of H3K36me3 (orange shade) and DNAme (black lollipops) over the transcribed region. Consequently, the CGI promoter remains DNA methylated on the maternal allele in the progeny and the gene becomes a paternally expressed imprinted gene.

pathway leading to parent-of-origin effects of gametic origin. For instance, a few cases of isolated imprinted genes lacking a gDMR were reported, such as the paternally expressed genes *Sfmbt2* and *Gab1* (Wang et al., 2011; Okae et al., 2012). In the placentae of cloned mice obtained by somatic cell nuclear transfer, using cumulus or Sertoli cells as nuclear donors, those same two genes, together with *Slc38a4*, were also shown to be consistently expressed from both alleles (Okae et al., 2014b). Furthermore, these three imprinted genes maintain at least some imprinted expression in the embryonic progeny of *Dnmt3l* or conditional *Dnmt3a/3b* null females, which fail to *de novo* methylate their oocyte genome (Okae et al., 2012; 2014b). Since the imprinted expression of canonical gDMR-regulated genes was faithfully maintained in most cloned mice, the authors concluded their study of *Sfmbt2, Gab1,* and *Slc38a4* with this insightful prediction: "It is likely that an imprinting mark[s] other than DNA methylation may be required for the establishment of imprinting of these genes" (Okae et al., 2014b).

Similarly, research in the field of imprinted X chromosome inactivation (XCI) has hinted at a DNAme-independent mechanism responsible for the preferential inactivation of the paternal X in the extra-embryonic lineages of female mouse embryos (Takagi and Sasaki, 1975). Although earlier studies suggested that the maternally inherited allele of *Xist,* the lncRNA required for the initiation of XCI, is kept silent by DNAme directly inherited from oocytes (Ariel et al., 1995; Zuccotti and Monk, 1995), subsequent work with targeted or genome-wide bisulfite sequencing failed to confirm those results or reveal such a preemptive DNAme mark on the *Xist* promoter in eggs (McDonald et al., 1998; Shirane et al., 2013). Although the epigenetic imprint preventing silencing of the maternal X was shown to be established during oocyte growth, when DNAme marks are laid down (Tada et al., 2000), imprinted XCI was not perturbed in the progeny of *Dnmt3a/3b* mutant oocytes (Chiba et al., 2008), which fail to acquire DNAme (Hata et al., 2002; Kaneda et al., 2010; Shirane et al., 2013).

Together, these lines of evidence suggested that DNAme might not be the only epigenetic mark directly inherited from gametes that can lead to imprinted expression in the progeny. Note that all the evidence summarized above (for *Xist* and autosomal paternally expressed genes) pointed to a silencing mark inherited from the oocyte. The discovery of such a DNAme-independent mechanism, which has been called "noncanonical imprinting" (Inoue et al., 2017a), heralded new avenues of studies in genomic imprinting research in mammals. Features unique to noncanonical imprinting have been covered extensively by excellent recent reviews (Chen and

TABLE 1 Canonical imprinted genes regulated by an oocyte LTR promoter.

Imprinted gene		Oocyte LTR promoter	CGI coodinates return (mm10/hg19)	DNAme maintenance	ZFP57 peak[a]	TGCCGC motif[b]
Mouse	*AK008011/ Gm5790*	RMER19B	chr13:47010854-47011096	tissue-specific	yes[c]	4
	Cdh15	MTD	chr8:122864938-122865178	tissue-specific	yes[c]	2
	Slc38a4	MT2A	chr15:97054449-97054857	ubiquitous	N	2
	Impact	MTC	chr18:12973304-12973796	ubiquitous	yes	5
Human	*DNAH7*	MLT1A0	chr2:196933311-196933665	placental	no	0
	MCCC1	LTR12C	chr3:182816772-182817455	placental	no	1
	BANK1	LTR12E	chr4:102711830-102712199	placental	no	1
	RHOBTB3	LTR12C	chr5:95066877-95067812	placental	no	5
	COL26A1	HERVH	chr7:101005900-101007443	placental	no	4
	SCIN	THE1 C	chr7:12610166-12610834	placental	no	1
	AGBL3	MER51E	chr7:134671120-134671750	placental	no	0
	SVOPL	THE1D	chr7:138348963-138349444	tissue-specific	no	1
	HTR5A	MSTA	chr7:154862681-154863245	tissue-specific	yes	3
	HECW1	LTR12C	chr7:43152021-43153340	placental	no	2
	CLDN23	LTR12C	chr8:8559132-8560867	tissue-specific	yes	3
	GL/S3	MER50	chr9:4297818-4300182	placental	no	2
	ZC3H12C	MLT1A1	chr11:109963241-109964677	placental	no	2
	ST8SIA1	LTR53	chr12:22486836-22488666	placental	no	0
	SORD	LTR12F	chr15:45315202-45315543	placental	no	1
	ZFP90	MER50	chr16:68572892-68573740	placental	no	1
	ZNF396	MSTA	chr18:32956765-32957406	placental	no	2

[a]ChIP-seq ZFP57 peak from ReMap Atlas of Regulatory Regions in UCSC, genome browser.
[b]Includes motifs within and close to the CGI.
[c]Not detected in two studies in mouse ESCs (Strogantsev et al., 2015; Anvar et al., 2016).

Zhang, 2020; Hanna and Kelsey, 2021; Kobayashi, 2021; Albert and Greenberg, 2023; Inoue, 2023).

3.2 Noncanonical imprinting

The discovery of noncanonical imprinting emerged from elegant studies mapping allele-specific DNase I hypersensitive sites (DHSs) in zygotes and morulae. For these experiments, the group of Yi Zhang first established a low-input protocol for the genome-wide mapping of DHSs, liDNase-seq, suitable for preimplantation work (Lu et al., 2016). By individually analyzing the profiles of DHSs in the paternal and maternal pronuclei, they identified parental allele-specific DHSs priming allele-specific expression at the 2-cell stage (Inoue et al., 2017a). Allele-specific regions of open chromatin in early mouse embryos have also been independently mapped by ATAC-seq (Wu et al., 2016). Most of these open chromatin regions were of paternal origin and since the protection of the maternal allele at 48% of these sites did not overlap with DNA methylated regions in oocytes, the results provided support for a DNAme-independent mechanism silencing the maternal alleles (Inoue et al., 2017a). By mining ChIP-seq data for the Polycomb Repressive Complex 2 (PRC2)-mediated H3K27me3 marks in oocytes and by injection of the mRNA for the H3K27me3-specific demethylase KDM6B, Inoue et al. further showed that maternally inherited H3K27me3 was responsible for the observed protection of the maternal allele and for imprinted expression of those genes from the paternal allele in morulae (Inoue et al., 2017a). Subsequent similar studies from this group showed that the imprinted expression of the lncRNA *Xist*, responsible for paternal X chromosome inactivation in extraembryonic tissues, is also controlled via a similar noncanonical imprinting mechanism via maternal H3K27me3 marks (Inoue et al., 2017b). The genetic requirement for a functional PRC2 in the establishment of oocyte H3K27me3 imprints was shown in two independent studies documenting the loss of non-canonical imprinting at autosomal genes and *Xist* in the progeny of embryonic ectoderm development (*Eed*)- deficient oocytes (Inoue et al., 2018; Harris et al., 2019). The

observation that imprinted expression is maintained at DNAme-dependent canonical imprinted genes in those *Eed* maternal KO progeny highlights the functional independence of both imprinting mechanisms (Inoue et al., 2018). This conclusion is also supported by the maintenance of noncanonical imprinted expression in the progeny of *Dnmt3l*-deficient females, confirming that noncanonical imprinting is independent of oocyte DNAme (Chen et al., 2019). In addition to the protection of maternal alleles from assuming an open chromatin state, the maternal H3K27me3 imprints also prevent the acquisition of activating H3K4me3 marks on the maternal allele in preimplantation embryos (Chen et al., 2019). Although the available data are consistent with H3K27me3 being the epigenetic mark directly inherited from oocyte, an interplay with the Polycomb Repressive Complex 1 (PRC1)-mediated H2AK119ub1 mark has also been described: whereas H2AK119ub1 coexists with and might precede H3K27me3 establishment during oocyte growth, its depletion in zygotes does not disrupts noncanonical imprinting, unlike what was seen for the H3K27me3 marks (Chen et al., 2021; Mei et al., 2021). Nevertheless, deletions of the PRC1.6 subunits PCGF1/6 in oocytes lead to partial loss of noncanonical imprinting genes in morulae (at 9/16 genes) (Mei et al., 2021).

The work summarised above revealed a DNAme-independent mechanism of imprinting, called noncanonical imprinting, however important differences with DNAme-dependent canonical imprinting were noted. Although more than 70 genes have been detected as noncanonically imprinted and paternally expressed in preimplantation embryos, all of these genes lose their imprinted expression in epiblast-derived post-implantation tissues (Inoue et al., 2017a; Santini et al., 2021). Nevertheless, maintenance of noncanonical imprinted expression has been observed in extra-embryonic tissues, including visceral endoderm at E6.5, extra-embryonic ectoderm (EXE) at E6.5 and E7.5, ectoplacental cone at 6 somite stage (~E8.5), as well as in E9.5 and E12.5 placentae (Inoue et al., 2017a; Hanna et al., 2019; Andergassen et al., 2021; Zeng et al., 2021). Similar tissue-specific maintenance of noncanonical imprinting only in extra-embryonic lineages was observed via the mapping of allelic H3K4me3 promoter marks (Hanna et al., 2019). One exception is *Slc38a4*, which shows imprinted expression from the paternal allele in E13 fetus as well as tissue-specifically imprinted in adult adrenals, heart, and skeletal muscle (Smith et al., 2003). This is explained by the fact that *Slc38a4* is at least partially regulated by a gDMR, suggesting that both canonical and noncanonical mechanisms may regulate the expression of this gene in different tissues, perhaps via different isoforms (Smith et al., 2003; Inoue et al., 2017a; Bogutz et al., 2019; Chen and Zhang, 2020).

The expression data therefore suggest that noncanonical imprinting is mostly a transient mechanism, leading to paternal allele-specific imprinted expression of several genes (>70) in preimplantation embryos, but only maintained at some of these loci in extra-embryonic lineages (notably 7 genes: *Gab1, Phf17, Platr20, Sall1, Sfmbt2, Slc38a4,* and *Smoc1*). This would represent approximately 2.7% of mouse imprinted genes. The transient nature of this imprinting mechanism is consistent with the observation that the broad H3K27me3-marked regions inherited from the oocyte and required for noncanonical imprinting are largely maintained to the blastocyst stage, but are erased in E6.5 epiblast (Zheng et al., 2016), and are absent in embryonic stem cells, mouse embryonic fibroblasts, and adult somatic cells (Matoba et al., 2018). These observations provide an explanation for the biallelic expression seen for noncanonical imprinted genes in embryos generated by somatic cell nuclear transfer (cloning), since the maternal H3K27me3 marks responsible for noncanonical imprinting are absent in the somatic donor cells (Okae et al., 2014b; Matoba et al., 2018; Xie et al., 2022).

3.3 Evidence for LTR-guided imprint maintenance

Although noncanonical imprinting has been detected in post-implantation extra-embryonic lineages via expression and H3K4me3 data, the maternal H3K27me3 marks do not survive past the blastocyst stage (Chen et al., 2019; 2021). This raises the question of what are the mechanisms guiding and maintaining this maternal allele-specific silencing in extra-embryonic lineages of post-implantation embryos. By comparing the genomic localisations of paternal H3K4me3 peaks associated with imprinted expression, a key difference was noted between the two families of imprinted genes: at canonical imprinted genes, those H3K4me3 peaks are mostly associated with promoter CpG islands, while at noncanonical genes, the active promoter marks map to endogenous retroviral elements, notably of the ERVK family (Hanna et al., 2019). While these noncanonical imprinted ERVK promoters are not marked by H3K27me3 in E6.5 EXE, they are in fact marked by DNAme on the silent maternal allele. Since these DNAme marks at imprinted ERVKs are not present in preimplantation embryos, they constitute classical somatic DMRs (sDMRs) (John and Lefebvre, 2011), acquired in post-implantation embryos (Chen et al., 2019; Hanna et al., 2019). An essential role for both DNMT3A and DNMT3B in this postimplantation *de novo* methylation pathway was confirmed by ablating both genes in zygotes using CRISPR-Cas9. However, *Sfmbt2* appears to be an exception here, with its imprinted expression being maintained despite loss of DNAme, at least at E6.5 (Chen et al., 2019). Surprisingly, this effect at *Sfmbt2* was not observed in zygotic euchromatic histone lysine N-methyltransferase 2 (*Ehmt2*)-null embryos (also known as G9a), in which the establishment of the sDMRs at noncanonical imprinted genes does not occur and biallelic expression is observed (Auclair et al., 2016; Zeng et al., 2021).

As expected, the sDMRs at imprinted ERVKs are also lost in the progeny of *Eed*-null oocytes, confirming the importance of the maternal H3K27me3 imprints in the initiation of this imprinting process. The observations that these ERVK elements become biallelically DNA methylated in the epiblast is consistent with the maintenance of noncanonical imprinting only in extra-embryonic lineages. Although some of these ERVKs, which are mostly solo LTR elements, were shown to act as alternative promoters for noncanonical imprinted genes, it remains to be seen whether some of these elements act as extra-embryonic enhancer elements, as has been previously reported for some LTR families (Chuong et al., 2013; Hanna et al., 2019; Figure 3). How the sDMRs are established specifically in extra-embryonic lineages but not in the epiblast-derived tissues is also currently unknown.

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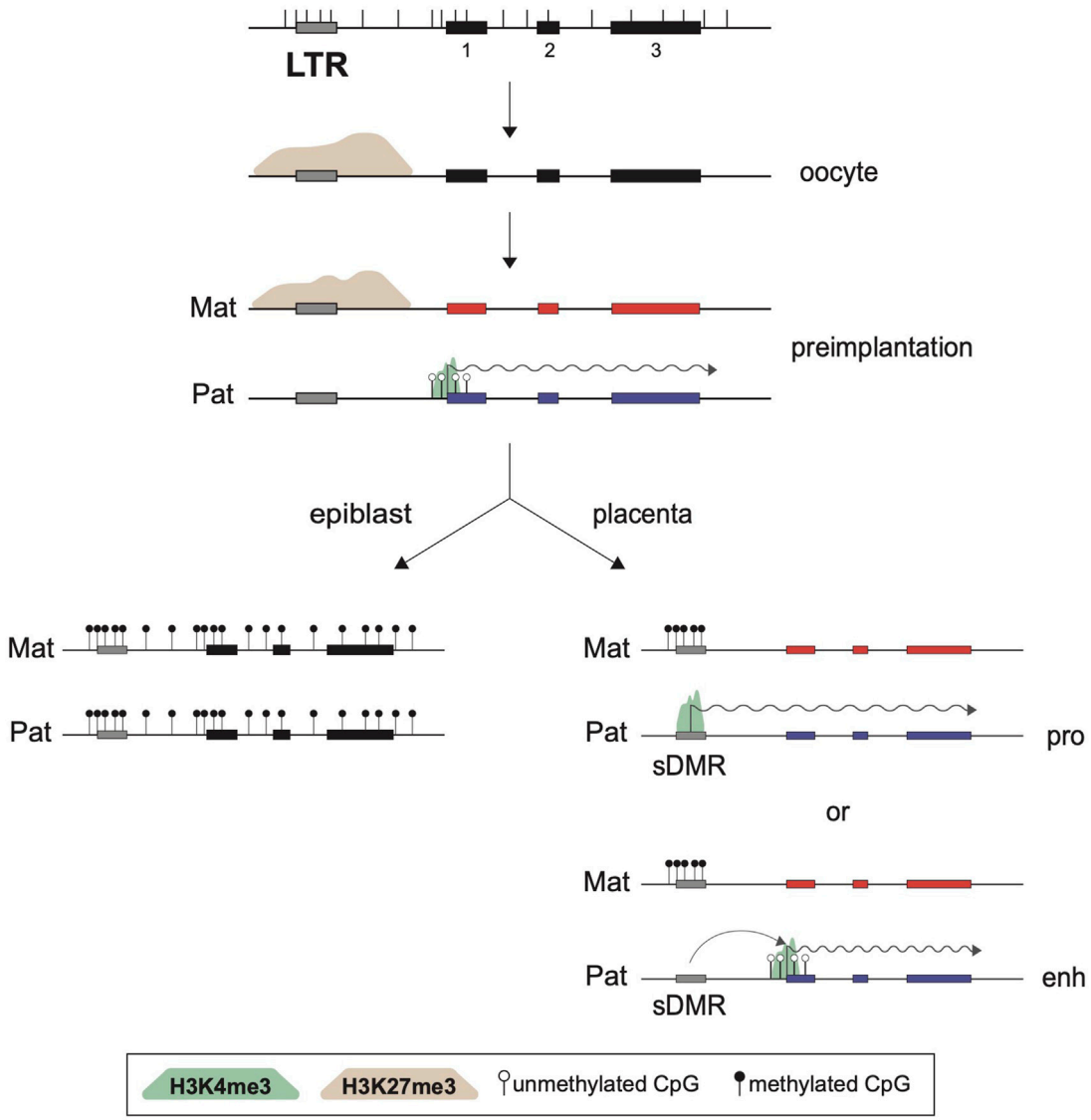

FIGURE 3
Role of LTRs in noncanonical imprinting maintenance. Structure of a noncanonical imprinted gene is presented at the top, showing three exons (black rectangles), the positions of CpG dinucleotides (vertical bars), and an upstream LTR element. In oocytes, part of the region is marked by a broad PRC2-deposited H3K27me3 domain (brown shade). This silencing epigenetic mark is inherited on the maternal allele such that only the paternal allele of the affected gene can be transcribed in preimplantation stages, as shown by the active H3K4me3 promoter mark (green shade) and transcription elongation (wiggly arrow). However, this histone imprint is only transient and is lost in all postimplantation cell lineages. In the epiblast, *de novo* DNA methylation leads to biallelic silencing, while in the extra-embryonic lineages a somatic DMR (sDMR) is generated over a nearby ERVK LTR element, with DNAme acquired exclusively on the previously H3K27me3-marked maternal allele. The LTR can then act as an alternative promoter (pro) or an enhancer (enh) to guide imprinted expression of the paternal allele. Not shown are the roles of PRC1 and its associated H2AK119ub mark in the establishment of the H3K27me3 domain in oocytes, or the implications of DNMT3A/3B, SMC hinge domain containing 1 (SMCHD1) and G9A/EHMT2 in formation of the extra-embryonic sDMRs themselves.

Together, this body of work has revealed that noncanonical imprinted genes, which so far have only been observed to be paternally expressed in extra-embryonic tissues, may rely on alternating allelic epigenetic marks for their allelic expression. The oocyte-derived H3K27me3 imprint, itself dependent on H2AK119ub1 at certain genes, must be converted into a DNAme somatic mark only on the maternal allele to achieve noncanonical imprinting in extra-embryonic tissues. This switch may be guided by the paternal H3K4me3 promoter marks over ERVK elements in preimplantation embryos, which would protect the paternal alleles from the action of the DNMT3A/3B *de novo* enzymes (Zhang et al., 2010). The observation that both the paternal H3K4me3 peaks and the sDMRs implicated in noncanonical imprinting map to endogenous retroviral promoters, notably of the ERVK family, suggests that these elements play critical roles in the maintenance of this unique tissue-specific imprinting pathway. In the cases where the imprinted ERVK element act as an alternative promoter for paternal allele-specific expression, a parallel can be drawn with the role of LTRs in the establishment of canonical imprinting, for which their activity as an oocyte promoter is critical.

4 Concluding remarks

Canonical imprints are essential for embryonic development, as shown by the early midgestational lethality of offspring obtained from oocytes deficient in *de novo* DNAme (Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004). Although much remains to be determined regarding the biological functions of noncanonical imprinted genes in extraembryonic lineages, the global loss of maternal H3K27me3 marks in conditional *Eed* or *Ezh2* mutant oocytes is compatible with development to term in the progeny, although with embryonic growth defects (Erhardt et al., 2003; Prokopuk et al., 2018). The shared placental overgrowth phenotypes observed in cloned mice and in *Eed* maternal KO conceptuses have been linked to the abnormal expression of noncanonical imprinted genes such as *Slc38a4, Gm32885* (a transcript upstream of *Slc38a4* on Chr 15), as well as a cluster of microRNAs coded within an intron of *Sfmbt2,* C2MC (Matoba et al., 2018; 2019; 2022). Together, these observations support key developmental roles for imprinted genes, both canonical and noncanonical, with an emphasis on the regulation of extra-embryonic lineages.

The fact that LTR elements, which are highly polymorphic in different mammalian species, are implicated in different aspects of both imprinting pathways suggests that they may play important roles in the emergence of new imprinted genes in different species. In their function as oocyte promoters for the establishment of maternal DNAme marks, LTRs were shown to be involved in the imprinting of non-overlapping sets of canonical imprinting genes in mouse and human (Bogutz et al., 2019). However, the three protein-coding genes imprinted by an oocyte LTR promoter in mouse (*Slc38a4, Impact,* and *Chd15*) are also imprinted in rat (Albert et al., 2023). A different picture emerges for noncanonical imprinted genes: although several noncanonical imprinted genes identified in mouse appear conserved in rat, profiling of allelic usage in this species also identified 8 rat-specific putative noncanonical imprinted genes, consistent with a rapid evolution of this imprinting mechanism in rodents (Albert et al., 2023). Nevertheless, whether the noncanonical pathway also operates in human embryos is unclear, since human *XIST* expression is not imprinted in preimplantation embryos nor in extra-embryonic membranes (Migeon and Do, 1979; Petropoulos et al., 2016), and most H3K27me3 marks are rapidly erased in human preimplantation embryo (Zheng et al., 2016; Xia et al., 2019; Lu et al., 2021). On the other hand, other studies have reported maternal-biased H3K27me3 marks and associated paternal allele-specific expression in human morulae (Zhang et al., 2019), as well as the presence of placental sDMRs corresponding to regions marked by H3K27me3 and hypomethylated in eggs, which are consistent with putative noncanonical imprinting (Hanna and Kelsey, 2021). Future work on other mammalian species will be important to establish the conservation and importance of LTR-based mechanisms of imprinting in the evolution of new imprinted genes.

Author contributions

SF: Visualization, Writing–original draft, Writing–review and editing. K-WC: Writing–original draft, Writing–review and editing. LL: Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

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