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SPOTLIGHT

Non-canonical imprinting in the spotlight

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ABSTRACT

Classical genomic imprints are regulated by parent-specific DNA methylation levels inherited from the gametes in mammals. Imprints control gene expression in a parent-of-origin manner and are essential for development. A distinct class of so-called 'non-canonical' imprints was recently discovered; these are seemingly regulated by histone methylation and govern parent-specific expression of developmentally important genes, most notably in the placenta. This new class of imprinted genes expands the repertoire of asymmetric parental contributions in mammalian embryogenesis, and raises new questions about the functionality of imprinted gene regulation in mammalian development. In this Spotlight, we summarize the latest findings regarding non-canonical imprinting, mainly from the mouse model, and discuss what we know about the conservation of this phenomenon and how it impacts mammalian development.

Introduction

Unlike most vertebrates, mammalian uniparental embryos derived from two maternal (parthenotes; see Glossary, Box 1) or two paternal (androgenotes; see Glossary, Box 1) genomes fail to develop beyond the mid-gestation stage, even in carefully controlled laboratory settings (Surani et al., 1984; McGrath and Solter, 1984; Barton et al., 1985). Close examination of the failed uniparental embryos revealed gross abnormalities in placenta size: the placenta of parthenotes is drastically smaller and, conversely, the placenta of androgenotes is enlarged. Why does mammalian development, in particular placenta size homeostasis, require exactly one maternal and one paternal genome?

Subsequent studies suggested that the failure of uniparental zygotes to develop was due to the requirement of maintenance of parent-specific DNA methylation (see Glossary, Box 1) at so-called 'imprinting control regions' (ICRs; see Glossary, Box 1), of which there are roughly 21 in mouse and human (Tucci et al., 2019). ICRs are the basis of canonical imprinting (see Glossary, Box 1) and control the expression of ∼150 genes in *cis* in the mouse genome. Misexpression of canonical imprinted genes in embryonic (precursors to the adult soma) and extra-embryonic cells (precursors to the placenta) has been associated with uniparental embryo lethality (Walsh et al., 1994; Weinberg-Shukron et al., 2022). Gene dose misregulation is frequently associated with pathologies, including cancers, fragile-X syndrome, Huntington's disease and imprinting disorders, such as Angelman and Prader-Willi syndromes (Tucci et al., 2019). Therefore, deregulation of imprinted genes in uniparental and cloned embryos due to a lack of imprinting may drive the disruption of placenta homeostasis and engender developmental arrest. For more on the regulation of gene expression in the placenta, we refer the reader to recent reviews (Hanna, 2020; Pastor and Kwon, 2022).

Recent work has identified a non-canonical form of imprinting (see Glossary, Box 1) , mediated by histone methylation. In this Spotlight, we discuss how these non-canonical imprints were discovered, their importance in development and their conservation across mammalian species. Although non-canonical imprinting is found in the early morula stage embryo, this Spotlight focuses on extra-embryonic imprinting in the mouse, as it is the most well-characterized barrier to the development of uniparental and cloned embryos.

The molecular basis of imprinted gene expression

Oocyte and sperm chromatin differ drastically in DNA methylation levels, and there are thousands of so-called differentially methylated regions between gametes. However, parental DNA methylation level differences are largely harmonized by the blastocyst stage (Fig. 1A) (Wang et al., 2014; Guo et al., 2014; Peat et al., 2014; Xia and Xie, 2020). Exceptionally, ICRs maintain asymmetric parental DNA methylation levels throughout embryogenesis and into adulthood (Fig. 1B). Mechanistically, parental methylation levels are maintained through the action of sequence- and DNA methylation-specific DNA-binding proteins that recruit chromatin modifiers to the methylated allele (Li et al., 2008; Strogantsev et al., 2015; Takahashi et al., 2019). Canonical imprints exert their control over nearby genes through a variety of *cis*- acting mechanisms, including repression via DNA methylation and histone 3 lysine 9 trimethylation (H3K9me3; see Glossary, Box 1), through long non-coding RNA transcription, 3D genome organization and recruitment of other chromatin regulating complexes (reviewed by Tucci et al., 2019).

Gametic chromatin asymmetry extends beyond DNA methylation. The sperm genome is packaged with protamines (see Glossary, Box 1) that are replaced by histones soon after fertilization (Nonchev and Tsanev, 1990). Meanwhile, the oocyte exhibits a specific histone modification landscape, with broad domains of the repressive Polycomb Repressive Complex 2 (PRC2)-deposited histone 3 lysine 27 trimethylation (H3K27me3; see Glossary, Box 1) (Zheng et al., 2016). Interestingly, a subset of these parental chromatin differences persists throughout preimplantation development (Mei et al., 2021; Chen et al., 2021; Lismer et al., 2020) (Fig. 1C). Thus, like DNA methylation, histone modifications deposited in gametes and maintained during development may regulate gene expression in a parent-of-origin manner. In recent years, it has become clear that H3K27me3 confers a non-canonical form of imprinted gene expression in developing mice (Inoue et al., 2017, 2018; Chen et al., 2019; Santini et al., 2021) (Fig. 1D).

Discovery and regulation of non-canonically imprinted genes

Beyond canonical imprints, an additional layer of imprinting was hinted at, with the discovery that the paternal-specific expression of *Gab1*, *Sfmbt2* and *Slc38a4* in the placenta is independent of oocyte DNA methylation (Okae et al., 2012, 2014). Notably, *Gab1*, *Sfmbt2* and *Slc38a4* have important roles in placental development, e.g. in nutrient transport, and are highly expressed in the placenta (Itoh

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Box 1. Glossary

Androgenotes. Motherless individuals generated from two paternally inherited (i.e. from spermatozoa) genomes.

Canonical imprints. A class of imprints that are established by differential DNA methylation levels in the oocyte and sperm. Although most parent-specific DNA methylation is lost during embryogenesis, a subset of imprint control regions maintain asymmetric parental DNA methylation levels. This process involves methylation- and sequence-specific proteins (ZFP57/ZFP445) and chromatin silencing complexes (DNMT1/KAP1/SETDB1) that promote silencing via DNA methylation and H3K9me3 deposition.

DNA methylation. An epigenetic modification that occurs at position 5 on the cytosine ring typically at palindromic CpG dinucleotides in mammals. DNA methylation at promoters is generally associated with transcriptional repression and is the molecular basis of canonical genomic imprinting.

ERVK retrotransposons. A family of endogenous retroviruses. Recombination events at the long-terminal repeats (LTRs) of ERV elements result in solo-LTRs, which no longer encode viral proteins but retain promoter and enhancer elements. Such solo LTRs may act as alternative promoters to genic transcription.

Histones. Proteins around which DNA is wrapped. Histone post-translational modifications can exert myriad effects on genome regulation as well as confer epigenetic memory; this Spotlight focuses on mono-, di- and tri-methylation of lysine residues.

Histone 3 lysine 27 trimethylation (H3K27me3). A post-translational epigenetic modification. H3K27me3 is deposited by Polycomb Repressive Complex 2 (PRC2) and is associated with transcriptional silencing. H3K27me3 is associated with non-canonical genomic imprinting.

Histone 3 lysine 9 di-methylation (H3K9me2) and tri-methylation (H3K9me3). Post-translational epigenetic modifications. H3K9me2 and H3K9me3 are deposited by G9a/GLP and SETDB1/SUV39H1/SUV39H2, respectively, and are associated with transcriptional silencing. The DNA methylated allele of canonical imprints is therefore enriched for H3K9me3. H3K9 methylation has also recently been implicated in non-canonical genomic imprinting.

Imprinting control regions (ICRs). The basis of canonical imprinting. They are regulated by DNA methylation and govern lifelong, parent-specific gene expression in all tissues. There are ∼21 such ICRs described in mouse and human (18 maternally methylated and 3 paternally methylated imprints) that govern the expression of ∼150 genes in the mouse.

Non-canonical imprints. A class of imprints that are established by H3K27me3 in the oocyte and, through an elusive mechanism, result in maternally DNA methylated imprints that govern paternal-specific gene expression in extra-embryonic cells. The full extent of non-canonical imprinting, including which genes are affected or whether it is a general feature of eutherian gene regulation, is an active area of research.

Parthenotes. Fatherless individuals generated from two maternally inherited (i.e. from oocytes) genomes.

Protamines. Arginine-rich proteins that package DNA in sperm. They are distinct from histones and are replaced soon after fertilization.

Somatic cell nuclear transfer (SCNT). A technique used to exchange the nucleus of an unfertilized egg with a nucleus obtained from a donor somatic cell, resulting in the cloning of the donor individual. The process is notoriously inefficient, in part due to epigenetic barriers imposed by genomic imprinting.

***Xist*.** A long non-coding RNA that is expressed from one of the X chromosomes in mammalian females and subsequently leads to near-complete *cis* gene repression along the entire chromosome (X chromosome inactivation). In mice and rats, a broad domain of H3K27me3 coats the promoter of *Xist*, resulting in non-canonical paternally biased expression and preferential inactivation of the paternal X chromosome in the placenta.

et al., 2000; Miri et al., 2013; Bogutz et al., 2019; Xie et al., 2022). This suggests that previous placental phenotypes in uniparental embryos may not be entirely due to canonical imprinting.

The role of histone modifications in non-canonical imprinting

The identification of the molecular basis of non-canonical imprinting, as well as the discovery of further candidates that are subject to this form of regulation, was enabled by technical advancements in low-input, high-throughput sequencing assays that allowed for genome-wide characterization of rare cell populations such as mouse oocytes and early embryos. Indeed, genome-wide profiling of zygotes uncovered an asymmetry in chromatin accessibility between the parental genomes, which is reflected in discordant accessibility patterns between parthenogenic and androgenetic morula stage embryos (Inoue et al., 2017). Genetic ablation of *Eed*, which encodes a core subunit of PRC2, and overexpression of *Kdm6b*, which encodes a H3K27me3 demethylase, demonstrated that this asymmetric parental chromatin accessibility depends on H3K27me3 levels deposited in oocytes (Inoue et al., 2017, 2018) (Fig. 2A,B). Importantly, lack of the H3K27me3 imprint in oocytes is associated with deregulation of a few dozen genes in the early embryo and post-implantation placental precursor cells, including *Gab1*, *Jade1*, *Smoc1*, *Sfmbt2* and *Slc38a4* (Inoue et al., 2018). Subsequent studies confirmed non-canonical imprinting by maternal H3K27me3, but also showed that, interestingly, H3K27me3 is generally lost on both alleles by the post-implantation stage in rodents (Zheng et al., 2016) and even earlier in other mammals (Lu et al., 2021). Indeed, maternal-specific H3K27me3 is not maintained at non-canonical imprints in the developing placenta, where imprinted expression occurs (Fig. 1D) (Chen et al., 2019; Hanna et al., 2019). As such, although H3K27me3 may act as the primary genomic imprint, it cannot be responsible for imprinted gene expression in the placenta, per se. Rather, maternal-specific DNA methylation is observed at non-canonical imprints in the placenta (Chen et al., 2019; Hanna et al., 2019). Thus, maternal H3K27me3 is seemingly supplanted by DNA methylation via an undefined recruitment mechanism.

Further studies implicated the histone 3 lysine 9 di-methylation (H3K9me2; see Glossary, Box 1) methyltransferases G9a and GLP in the establishment and maintenance of non-canonical imprinting (Zeng et al., 2021b). Removing G9a and/or GLP in the growing oocyte results in upregulation of non-canonical imprinted genes *Gab1* (Demond et al., 2023) and *Sfmbt2* (Meng et al., 2022). Why only two non-canonically imprinted genes are upregulated in oocytes lacking G9a is unknown, but it may be related to the similar observation that oocytes lacking DNA methylation also show few changes in canonical imprinted gene expression levels (Xu et al., 2019). Instead, the failure to establish putative H3K9me2 imprints in oocytes results in misexpression later in development. For example, *G9a* maternal knockout (KO) embryos show biallelic expression of *Gab1, Smoc1* and *Jade1* in placenta precursor cells (Zeng et al., 2021b; Andergassen et al., 2021). Similarly, *G9a* zygotic mutations also result in biallelic expression of *Gab1*, *Smoc1*, *Sall1* and *Sfmbt2* in the early placenta (Zeng et al., 2021b; Andergassen et al., 2021), and of *Slc38a4* in the embryo (Auclair et al., 2016), hinting at a role for H3K9me2 in the establishment and maintenance of non-canonical imprinting. In other words, maternal knockouts of two distinct chromatin modifying pathways – PRC2 (H3K27me3) and G9a/GLP (H3K9me2) – both result in loss of non-canonical imprinting (Wagschal et al., 2008; Inoue et al., 2018; Zeng et al., 2021b; Matoba et al., 2022). Do both complexes exert the primary imprint in a non-redundant manner? A side-by-side comparison of zygotic *Eed* and *G9a/Glp* KOs demonstrated that G9a/GLP play a dominant role in non-canonical imprint maintenance (Andergassen et al., 2021). Given the established association between H3K9me3, DNA methylation and canonical

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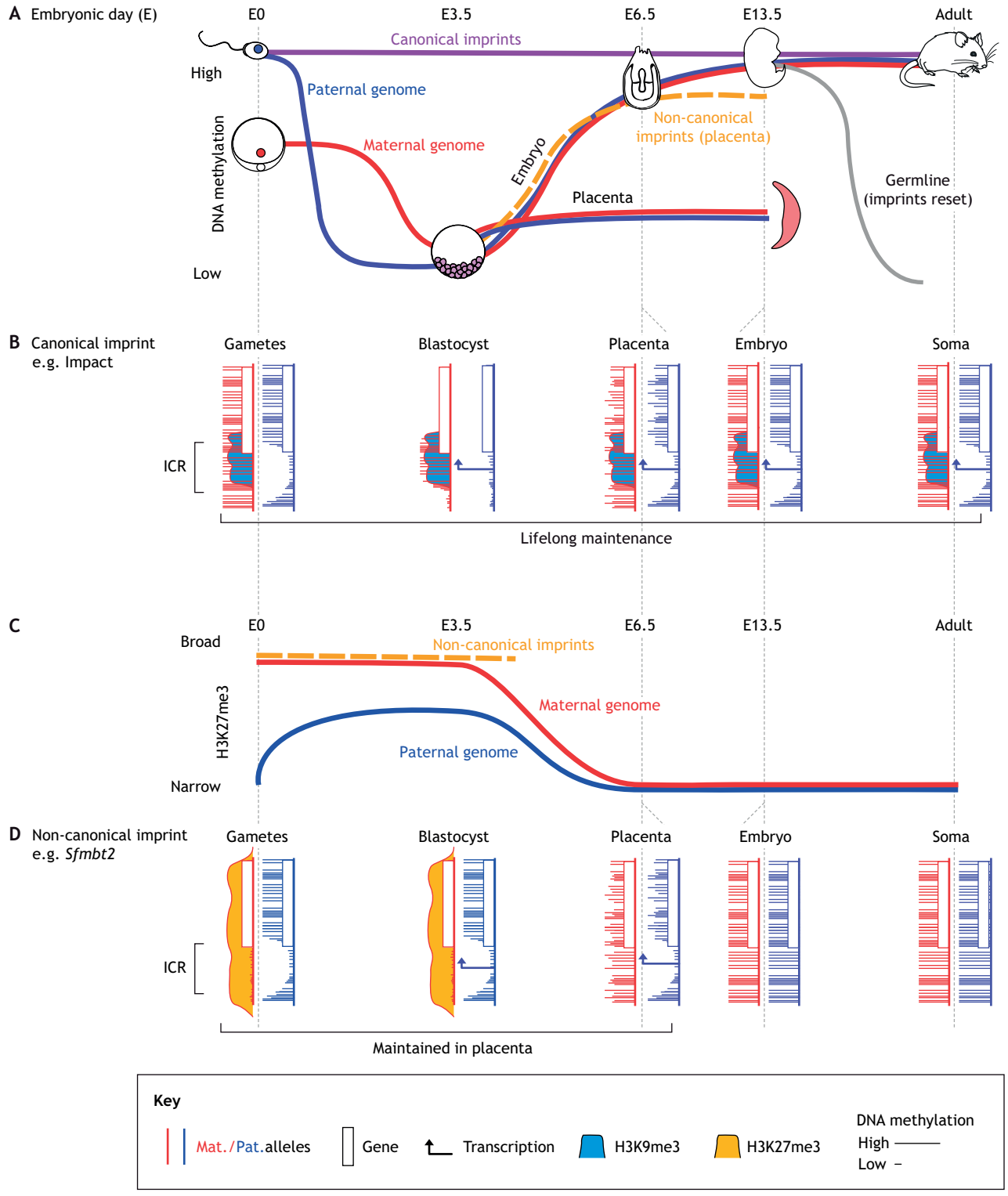

Fig. 1. Chromatin dynamics and imprinted gene expression during mouse development. (A) Global DNA methylation levels are shown during key stages of mouse embryogenesis. DNA methylation levels of the methylated allele of canonical imprints are indicated by a purple line. Maternal allele DNA methylation levels at non-canonical imprints are indicated by a dotted orange line. (B) An example of a canonically imprinted, paternally expressed, maternally methylated gene. DNA methylation levels are indicated by horizontal lines; the length of the line corresponds to the average methylation levels over individual CpG dinucleotides in a population of cells. The relevant region for imprinting is annotated as 'ICR' (the imprinting control region). Note the maintenance of H3K9me3 on the silent allele. The variable levels of DNA methylation in the placenta likely reflect incomplete re-methylation of parental genomes in this tissue. (C) Broad and narrow distribution of H3K27me3 is shown for the same developmental stages as in A. H3K27me3 is enriched over atypically broad domains in oocytes and early embryos, which are progressively returned to relatively narrow peaks by the epiblast stage. H3K27me3 levels at non-canonical imprints are indicated by a dotted orange line. (D) An example of a paternally expressed, non-canonically imprinted gene is shown. The primary imprint is H3K27me3, but this is supplanted by maternal-specific DNA methylation in the placenta. H3K9me2 has also recently been implicated in non-canonical imprinting but is not depicted here, as it is unknown whether H3K9me2, per se, is acting as the primary imprint.

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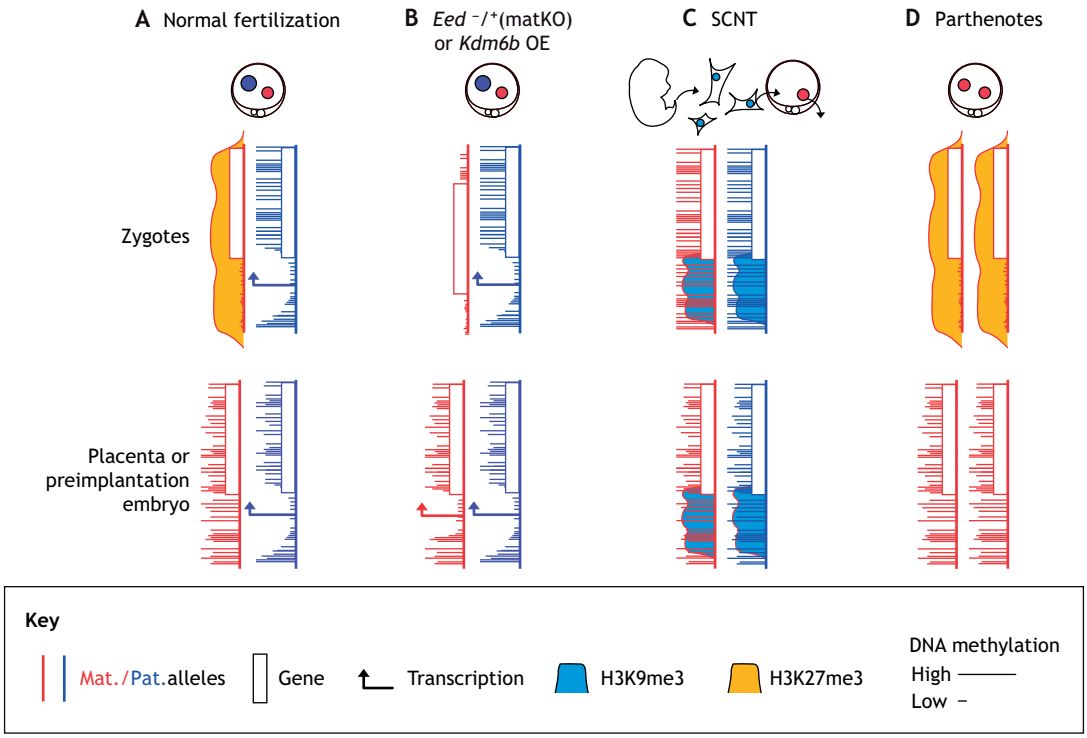

Fig. 2. Loss of non-canonical imprinting in parthenogenic and SCNT embryos. (A) H3K27me3, DNA methylation and transcription levels at non-canonically imprinted loci in normal zygotes and post-implantation placenta, as in Fig. 1D. (B) Embryos generated without non-canonical imprints by genetic ablation of *Eed* in oocytes (matKO) or overexpression (OE) of *Kdm6b* in zygotes result in overexpression of non-canonically imprinted genes. This analysis was performed in morula-stage embryos, not in the developing placenta. (C) Embryos generated by somatic nuclear cell transfer (SCNT) often maintain somatic chromatin patterns and fail to express non-canonically imprinted genes in the placenta. The failure to reactivate gene expression is associated with the aberrant maintenance of somatic H3K9me3 levels. (D) Embryos generated by two maternally derived genomes carry two copies of each non-canonical imprint and fail to express key developmental genes in the placenta.

imprinting (Fig. 1B) (Wang et al., 2018; Yang et al., 2022), it is plausible that H3K9me2 is required for the maintenance, and perhaps establishment, of non-canonical imprinting. To formally address the role of H3K27me3 and H3K9me2 in non-canonical imprinting establishment and maintenance, a higher order mutant for *Eed* and *G9a/Glp* would potentially reveal the epistatic or synergistic relationship. Additionally, genome-wide maps of H3K9me2 levels in the developing embryo and placenta are required to determine whether the histone modification is maintained beyond implantation and in the placenta.

Finally, non-canonically imprinted genes *Jade1*, *Sfmbt2* and *Smoc1* are transcriptionally upregulated in *Smchd1* maternal KO placentas (Wanigasuriya et al., 2020). *Smchd1* encodes a protein that mediates repressive chromatin over long ranges; initially discovered as a regulator of X-chromosome inactivation (Blewitt et al., 2008), subsequent studies have implicated SMCHD1 in repressing Hox genes (Benetti et al., 2022) and the silent allele of non-canonical imprinted genes, including *Xist* (see Glossary, Box 1) (Wanigasuriya et al., 2020). How SMCHD1 controls expression of non-canonical imprints without altering levels of DNA methylation (Wanigasuriya et al., 2020) or H3K27me3 (Chen et al., 2015) remains an active area of research.

An ERVK-mediated basis of non-canonical imprinting

Careful examination of non-canonical gene expression revealed that the residual solo long terminal repeats (LTRs) of the ERVK family of retrotransposons (see Glossary, Box 1) often serve as alternate promoters when genes are paternally expressed in the placenta (Hanna et al., 2019). Indeed, the genetic basis of non-canonical imprints was confirmed by excising the non-canonically imprinted ERVK alternate promoter of the *Gab1* gene, which resulted in a moderate loss of imprinted expression of *Gab1* in extra-embryonic cells (Hanna et al., 2019).

It was subsequently shown that non-canonically imprinted ERVK elements are regulated by G9a/H3K9me2 (Zeng et al., 2021b; Andergassen et al., 2021), which appears to be a general mode of ERVK control during mouse embryogenesis (Zeng et al., 2021a). Taken together, it is tempting to speculate that G9a/H3K9me2 may regulate ERVKs and non-canonical imprints, akin to of SETDB1/H3K9me3 repressing other families of LTR retrotransposons (Matsui et al., 2010) and canonical imprints (Wang et al., 2018; Yang et al., 2022). Although imprinted ERVK alternate promoters do not have any obvious shared motifs (Hanna et al., 2019), future studies will hopefully address the mechanism that targets G9a/H3K9me2 to these regions.

Notably, species-specific ERV insertions drive species-specific DNA methylation establishment over canonical imprints in the female germline (Brind'Amour et al., 2018; Bogutz et al., 2019). Perhaps functional non-canonical imprints can be identified through species-specific ERVK element insertions near genes that show species-specific imprinted expression. Indeed, a rat-specific ERVK insertion in the promoter of *Slc38a1* coincides with rat-specific non-canonical imprinting (Richard Albert et al., 2023). Whether by establishing canonical imprints in oocytes or by promoting non-canonical paternal expression in extraembryonic tissues, both cases

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represent fascinating examples of transposable elements as vectors of potentially adaptive gene regulation.

Developmental importance of non-canonical imprinted genes

While elucidating the mechanism of non-canonical imprinting has deepened our understanding of the extent of intergenerational epigenetic inheritance and of gene regulation in general, the findings raise a key question: the precise expression levels of which non-canonically imprinted genes are crucial for normal placenta homeostasis and, consequently, for normal mammalian *in utero* development?

Several recent studies have explored the developmental role of non-canonical imprints using complementary and overlapping genetic approaches. Using a candidate-based approach of previously defined non-canonical imprinted genes (Inoue et al., 2017, 2018), four of these studies tested whether restoring dose expression of deregulated non-canonically imprinted genes in somatic cell nuclear transfer (SCNT, see Glossary, Box 1) embryos – where this mode of imprinting is normally lost (Fig. 2C) – is sufficient to facilitate normal development (Wang et al., 2020; Xie et al., 2022; Matoba et al., 2018; Inoue et al., 2020).

Modulating expression of the master regulator of X-chromosome inactivation, the long noncoding RNA gene *Xist*, resulted in moderate increase in developmental success, yet placental defects remained in SCNT embryos (Matoba et al., 2018). *Slc38a4* encodes an amino acid carrier expressed from the paternal genome in the placenta (Bogutz et al., 2019). Paternal KO of *Slc38a4* results in a diminished placenta size and intrauterine growth restriction in biparental embryos (Matoba et al., 2019). Conversely, loss of imprinted expression of *Slc38a4*, as occurs in SCNT embryos, results in placenta overgrowth (Xie et al., 2022). Interestingly, restoring *Slc38a4* expression levels in SCNT embryos partially rescues the placenta overgrowth phenotype but does not result in an increase in live births (Xie et al., 2022). The fact that a partially rescued placenta phenotype did not coincide with an increase in live births was unexpected given the clear link between placenta homeostasis and embryonic outcomes as discussed above. Finally, an independent group showed that correcting expression levels of either *Sfmbt2*, *Jade1*, *Gab1* or *Smoc1* in SCNT embryos partially rescues the placenta overgrowth phenotype and results in a notable increase in live birth rates (17/162 compared with 0/565 in control SCNT), with *Sfmbt2* having the largest effect (Wang et al., 2020).

Using a complementary approach to SCNT, Matoba et al. used naturally fertilized *Eed* KO oocytes to generate embryos devoid of non-canonical imprints with much greater efficiency. *Xist* imprinting was again shown to be crucial for proper development, especially in male embryos with only one X chromosome (Matoba et al., 2022). However, even though correcting for *Xist* dose enabled more embryos to develop to term, developmental delays and placenta overgrowth were observed (as noted above), likely due to loss of imprinted expression of other non-canonically imprinted genes. Indeed, paternal deletion of *Slc38a4* partially rescued the placenta overgrowth phenotype in maternal *Eed*+*Xist* KO embryos. Furthermore, careful genetic manipulation to correct expression levels of the imprinted intronic miRNA cluster of *Sfmbt2* showed the greatest rescue of placenta overgrowth. Taken together, these six recent studies suggest that the correct dose of non-canonically imprinted genes *Xist, Slc38a4*, *Sfmbt2* and *Sfmbt2* miRNAs helps orchestrate placenta size homeostasis. However, it is possible that this is only a partial list of functional non-canonical imprints; more research is required to elucidate the number and function of this class of genes during development.

Conservation of imprinting

A striking aspect of canonical imprints is their general conservation in eutherian mammals (Tucci et al., 2019; Kobayashi, 2021), although it should be stressed that canonical imprinting has generally been investigated based on homology with known imprinted genes identified in the mouse: the chief mammalian model organism. Thus, a comprehensive analysis of imprinted gene expression – canonical and non-canonical – in embryonic and extra-embryonic tissues in a range of mammals is required to determine the relative conservation of imprinting.

What is clear is that therian (eutherians and marsupials) genomes exhibit canonical imprinting, whereas there is no evidence of imprinting in monotremes, which are egg-laying mammals (Renfree et al., 2009). Even less is known about the conservation of non-canonically imprinted gene expression (Kobayashi, 2021). For example, analysis of H3K27me3 level dynamics in early embryos suggests that rodents may be unique in their retention of the mark, at least compared with cow, pig and human (Lu et al., 2021). Indeed, H3K27me3 profiling in uniparental macaque embryos found only sparse associations between paternally expressed genes and maternally biased H3K27me3 (Chu et al., 2021). Primates, including crab eating macaques and humans, clearly show extensive placenta-specific imprinting, albeit at non-overlapping sets of genes (Chu et al., 2021; Xu et al., 2021; Monteagudo-Sánchez et al., 2019), and many of these imprints are associated with a germline DMR (Xu et al., 2021), suggesting that non-canonical imprinting could be less important in the primate lineage. However, it should be noted that in macaque placenta, over 200 paternally expressed genes were not obviously associated with maternal DNA methylation (Chu et al., 2021). This at least opens up the possibility that non-canonical imprinting is occurring, if largely by a different mechanism than that reported in mice and rats.

Intriguingly, a recent screen in the rat model identified non-canonical imprinting of *Sfmbt2* and *Slc38a4*, which are demonstrably important for mouse placenta development (as discussed above) (Richard Albert et al., 2023). Furthermore, eight rat-specific imprints were identified, all of which were transcribed from the paternal allele exclusively in extra-embryonic cells, suggesting a non-canonical mode of imprinted regulation. Compared with canonical imprints identified in rat, all of which were previously characterized in mouse or human, these results suggest non-canonical imprinting is rapidly evolving.

Thus, although the data were limited at the time of writing, it appears that non-canonical imprinting controls expression of a subset of genes in rodents, although the mechanisms dictating the phenomenon as well as the sets of genes implicated may vary between species. Transposable elements are known to be drivers of evolution, including of the placenta (Chuong, 2013; Chuong et al., 2013; Frost et al., 2023), and may have helped accelerate the appearance of imprinted genes. Determining whether non-canonical imprinting occurs in all mammals, and whether ERVKs or different ERV family members may contribute, will provide insights into the convergent evolution of such a mode of gene regulation.

Perspectives

Non-canonical imprinting represents an emerging and fascinating subject for epigenetics and developmental research (Raas et al., 2021; Inoue, 2022). As discussed here, many questions still remain regarding the underlying mechanisms, the scope and the

evolutionary conservation of this mode of gene regulation. Although mechanistic studies outside the mouse model remain a general challenge, agnostic exploratory studies, such as those recently described in rats (Richard Albert et al., 2023), will hopefully begin to shed light on the other aspects.

A striking recent study provided strong evidence that genomic imprints remain a barrier for uniparental offspring in mammals. Modification of the methylation status of seven canonical imprints [paternally methylated *H19* and *Gtl2* (*Meg3*)/*Dlk1* and maternally methylated *Igf2r*, *Snrpn*, *Kcnq1ot1*, *Nespas* (*Gnasas1*) and *Peg10* ICRs] using epigenome editing in artificially activated diploid oocytes resulted in the generation of an adult mouse (Wei et al., 2022). However, this was an extremely rare outcome, as it represented the only survivor out of 192 implanted embryos. This low efficiency may suggest inefficient DNA methylation editing or that an insufficient number of canonical ICRs are modified. It is also possible that the biallelic expression of non-canonical imprints may have contributed to the very low rate of developmental success (Fig. 2D). Epigenome editing can be used to modify chromatin marks besides DNA methylation (Policarpi et al., 2022 preprint). An interesting avenue to pursue will be the modification of non-canonical imprints, perhaps in combination with canonical imprints, in order to assess the importance of parental asymmetries for proper developmental progression. Although the technology is still in its infancy, epigenome editing could be conducted *in vitro* using recently developed *ex utero* development systems (Aguilera-Castrejon et al., 2021; Amadei et al., 2022), which may mitigate both the ethical considerations and labour intensiveness of generating *in vivo* transgenic material. Hopefully, continued research will continue to unravel the epigenetic regulatory mechanisms, as well as the evolutionary underpinnings, of the fascinating biology of imprinted gene control.

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References
Aguilera-Castrejon, A., Oldak, B., Shani, T., Ghanem, N., Itzkovich, C., Slomovich, S., Tarazi, S., Bayerl, J., Chugaeva, V., Ayyash, M. et al. (2021). Ex utero mouse embryogenesis from pre-gastrulation to late organogenesis. *Nature* **593**, 119-124. doi:10.1038/s41586-021-03416-3

Amadei, G., Handford, C. E., Qiu, C., De Jonghe, J., Greenfeld, H., Tran, M., Martin, B. K., Chen, D.-Y., Aguilera-Castrejon, A., Hanna, J. H. et al. (2022). Embryo model completes gastrulation to neurulation and organogenesis. *Nature* **610**, 143-153. doi:10.1038/s41586-022-05246-3

Andergassen, D., Smith, Z. D., Kretzmer, H., Rinn, J. L. and Meissner, A. (2021). Diverse epigenetic mechanisms maintain parental imprints within the embryonic and extraembryonic lineages. *Dev. Cell* **56**, 2995-3005.e4. doi:10.1016/j.devcel.2021.10.010

Auclair, G., Borgel, J., Sanz, L. A., Vallet, J., Guibert, S., Dumas, M., Cavelier, P., Girardot, M., Forné, T., Feil, R. et al. (2016). EHMT2 directs DNA methylation for efficient gene silencing in mouse embryos. *Genome Res.* **26**, 192-202. doi:10.1101/gr.198291.115

Barton, S. C., Adams, C. A., Norris, M. L. and Surani, M. A. H. (1985). Development of gynogenetic and parthenogenetic inner cell mass and trophectoderm tissues in reconstituted blastocysts in the mouse. *J. Embryol. Exp. Morphol.* **90**, 267-285. doi:10.1242/dev.90.1.267

Benetti, N., Gouil, Q., del Fierro, A. T., Beck, T., Breslin, K., Keniry, A., McGlin, E. and Blewitt, M. E. (2022). Maternal SMCHD1 regulates Hox gene expression and patterning in the mouse embryo. *Nat. Commun.* **13**, 4295. doi:10.1038/s41467-022-32057-x

Blewitt, M. E., Gendrel, A.-V., Pang, Z., Sparrow, D. B., Whitelaw, N., Craig, J. M., Apedaile, A., Hilton, D. J., Dunwoodie, S. L., Brockdorff, N. et al. (2008). SmcHD1, containing a structural-maintenance-of-chromosomes hinge domain, has a critical role in X inactivation. *Nat. Genet.* **40**, 663-669. doi:10.1038/ng.142

Bogutz, A. B., Brind'Amour, J., Kobayashi, H., Jensen, K. N., Nakabayashi, K., Imai, H., Lorincz, M. C. and Lefebvre, L. (2019). Evolution of imprinting via lineage-specific insertion of retroviral promoters. *Nat. Commun.* **10**, 1-14. doi:10.1038/s41467-019-13662-9

Brind'Amour, J., Kobayashi, H., Richard Albert, J., Shirane, K., Sakashita, A., Kamio, A., Bogutz, A., Koike, T., Karimi, M. M., Lefebvre, L. et al. (2018). LTR retrotransposons transcribed in oocytes drive species-specific and heritable changes in DNA methylation. *Nat. Commun.* **9**, 3331. doi:10.1038/s41467-018-05841-x

Chen, K., Hu, J., Moore, D. L., Liu, R., Kessans, S. A., Breslin, K., Lucet, I. S., Keniry, A., Leong, H. S., Parish, C. L. et al. (2015). Genome-wide binding and mechanistic analyses of Smchd1-mediated epigenetic regulation. *Proc. Natl. Acad. Sci. USA* **112**, E3535-E3544. doi:10.1073/pnas.1413625112

Chen, Z., Yin, Q., Inoue, A., Zhang, C. and Zhang, Y. (2019). Allelic H3K27me3 to allelic DNA methylation switch maintains noncanonical imprinting in extraembryonic cells. *Sci. Adv.* **5**, eaay7246. doi:10.1126/sciadv.aay7246

Chen, Z., Djekidel, M. N. and Zhang, Y. (2021). Distinct dynamics and functions of H2AK119ub1 and H3K27me3 in mouse preimplantation embryos. *Nat. Genet.* **53**, 551-563. doi:10.1038/s41588-021-00821-2

Chu, C., Zhang, W., Kang, Y., Si, C., Ji, W., Niu, Y. and Zhang, Y. (2021). Analysis of developmental imprinting dynamics in primates using SNP-free methods to identify imprinting defects in cloned placenta. *Dev. Cell* **56**, 2826-2840.e7. doi:10.1016/j.devcel.2021.09.012

Chuong, E. B. (2013). Retroviruses facilitate the rapid evolution of the mammalian placenta. *BioEssays* **35**, 853-861. doi:10.1002/bies.201300059

Chuong, E. B., Rumi, M. A. K., Soares, M. J. and Baker, J. C. (2013). Endogenous retroviruses function as species-specific enhancer elements in the placenta. *Nat. Genet.* **45**, 325-329. doi:10.1038/ng.2553

Demond, H., Hanna, C. W., Castillo-Fernandez, J., Santos, F., Papachristou, E. K., Segonds-Pichon, A., Kishore, K., Andrews, S., D'Santos, C. S. and Kelsey, G. (2023). Multi-omics analyses demonstrate a critical role for EHMT1 methyltransferase in transcriptional repression during oogenesis. *Genome Res.* **33**, 18-31. doi:10.1101/gr.277046.122

Frost, J. M., Amante, S. M., Okae, H., Jones, E. M., Ashley, B., Lewis, R. M., Cleal, J. K., Caley, M. P., Arima, T., Maffucci, T. et al. (2023). Regulation of human trophoblast gene expression by endogenous retroviruses. *Nat. Struct. Mol. Biol.* **30**, 527-538. doi:10.1038/s41594-023-00960-6

Guo, F., Li, X., Liang, D., Li, T., Zhu, P., Guo, H., Wu, X., Wen, L., Gu, T.-P., Hu, B. et al. (2014). Active and passive demethylation of male and female pronuclear DNA in the mammalian zygote. *Cell Stem Cell* **15**, 447-458. doi:10.1016/j.stem.2014.08.003

Hanna, C. W. (2020). Placental imprinting: Emerging mechanisms and functions. *PLoS Genet.* **16**, e1008709-e16. doi:10.1371/journal.pgen.1008709

Hanna, C. W., Pérez-Palacios, R., Gahurova, L., Schubert, M., Krueger, F., Biggins, L., Andrews, S., Colomé-Tatché, M., Bourc'his, D., Dean, W. et al. (2019). Endogenous retroviral insertions drive non- canonical imprinting in extra-embryonic tissues. *Genome Biol.* **20**, 1-17. doi:10.1186/s13059-019-1833-x

Inoue, A. (2022). Noncanonical imprinting: intergenerational epigenetic inheritance mediated by Polycomb complexes. *Curr. Opin. Genet. Dev.* **78**, 102015. doi:10.1016/j.gde.2022.102015

Inoue, A., Jiang, L., Lu, F., Suzuki, T. and Zhang, Y. (2017). Maternal H3K27me3 controls DNA methylation-independent imprinting. *Nature* **547**, 419-424. doi:10.1038/nature23262

Inoue, A., Chen, Z., Yin, Q. and Zhang, Y. (2018). Maternal Eed knockout causes loss of H3K27me3 imprinting and random X inactivation in the extraembryonic cells. *Genes Dev.* **32**, 1525-1536. doi:10.1101/gad.318675.118

Inoue, K., Ogonuki, N., Kamimura, S., Inoue, H., Matoba, S., Hirose, M., Honda, A., Miura, K., Hada, M., Hasegawa, A. et al. (2020). Loss of H3K27me3 imprinting in the Sfmbt2 miRNA cluster causes enlargement of cloned mouse placentas. *Nat. Commun.* **11**, 2150. doi:10.1038/s41467-020-16044-8

Itoh, M., Yoshida, Y., Nishida, K., Narimatsu, M., Hibi, M. and Hirano, T. (2000). Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. *Mol. Cell. Biol.* **20**, 3695-3704. doi:10.1128/MCB.20.10.3695-3704.2000

Kobayashi, H. (2021). Canonical and non-canonical genomic imprinting in rodents. *Front. Cell Dev. Biol.* **9**, 713878. doi:10.3389/fcell.2021.713878

Li, X., Ito, M., Zhou, F., Youngson, N., Zuo, X., Leder, P. and Ferguson-Smith, A. C. (2008). A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Dev. Cell* **15**, 547-557. doi:10.1016/j.devcel.2008.08.014

Lismer, A., Siklenka, K., Lafleur, C., Dumeaux, V. and Kimmins, S. (2020). Sperm histone H3 lysine 4 trimethylation is altered in a genetic mouse model of transgenerational epigenetic inheritance. *Nucleic Acids Res.* **48**, 11380-11393. doi:10.1093/nar/gkaa712

Lu, X., Zhang, Y., Wang, L., Wang, L., Wang, H., Xu, Q., Xiang, Y., Chen, C., Kong, F., Xia, W. et al. (2021). Evolutionary epigenomic analyses in mammalian

early embryos reveal species-specific innovations and conserved principles of imprinting. *Sci. Adv.* **7**, eabi6178. doi:10.1126/sciadv.abi6178

Matoba, S., Wang, H., Jiang, L., Lu, F., Iwabuchi, K. A., Wu, X., Inoue, K., Yang, L., Press, W., Lee, J. T. et al. (2018). Loss of H3K27me3 imprinting in somatic cell nuclear transfer embryos disrupts post-implantation development. *Cell Stem Cell* **23**, 343-354.e5. doi:10.1016/j.stem.2018.06.008

Matoba, S., Nakamuta, S., Miura, K., Hirose, M., Shiura, H., Kohda, T., Nakamuta, N. and Ogura, A. (2019). Paternal knockout of Slc38a4/SNAT4 causes placental hypoplasia associated with intrauterine growth restriction in mice. *Proc. Natl. Acad. Sci. USA* **116**, 21047-21053. doi:10.1073/pnas.1907884116

Matoba, S., Kozuka, C., Miura, K., Inoue, K., Kumon, M., Hayashi, R., Ohhata, T., Ogura, A. and Inoue, A. (2022). Noncanonical imprinting sustains embryonic development and restrains placental overgrowth. *Genes Dev.* **36**, 483-494. doi:10.1101/gad.349390.122

Matsui, T., Leung, D., Miyashita, H., Maksakova, I. A., Miyachi, H., Kimura, H., Tachibana, M., Lorincz, M. C. and Shinkai, Y. (2010). Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* **464**, 927-931. doi:10.1038/nature08858

Mcgrath, J. and Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179-183. doi:10.1016/0092-8674(84)90313-1

Mei, H., Kozuka, C., Hayashi, R., Kumon, M., Koseki, H. and Inoue, A. (2021). H2AK119ub1 guides maternal inheritance and zygotic deposition of H3K27me3 in mouse embryos. *Nat. Genet.* **53**, 539-550. doi:10.1038/s41588-021-00820-3

Meng, T.-G., Lei, W.-L., Lu, X., Liu, X.-Y., Ma, X.-S., Nie, X.-Q., Zhao, Z.-H., Li, Q.-N., Huang, L., Hou, Y. et al. (2022). Maternal EHMT2 is essential for homologous chromosome segregation by regulating Cyclin B3 transcription in oocyte meiosis. *Int. J. Biol. Sci.* **18**, 4513-4531. doi:10.7150/ijbs.75298

Miri, K., Latham, K., Panning, B., Zhong, Z., Andersen, A. and Varmuza, S. (2013). The imprinted polycomb group gene Sfmbt2 is required for trophoblast maintenance and placenta development. *Development* **140**, 4480-4489. doi:10.1242/dev.096511

Monteagudo-Sánchez, A., Sánchez-Delgado, M., Hernandez Mora, J. R., Santamaría, N. T., Garatacos, E., Esteller, M., de Heredia, M., Nunes, V., Choux, C., Fauque, P. et al. (2019). Differences in expression rather than methylation at placenta-specific imprinted loci is associated with intrauterine growth restriction. *Clin. Epigenet.* **11**, 35. doi:10.1186/s13148-019-0630-4

Nonchev, S. and Tsanev, R. (1990). Protamine-histone replacement and DNA replication in the male mouse pronucleus. *Mol. Reprod. Dev.* **25**, 72-76. doi:10.1002/mrd.1080250113

Okae, H., Hiura, H., Nishida, Y., Funayama, R., Tanaka, S., Chiba, H., Yaegashi, N., Nakayama, K., Sasaki, H. and Arima, T. (2012). Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression. *Hum. Mol. Genet.* **21**, 548-558. doi:10.1093/hmg/ddr488

Okae, H., Matoba, S., Nagashima, T., Mizutani, E., Inoue, K., Ogonuki, N., Chiba, H., Funayama, R., Tanaka, S., Yaegashi, N. et al. (2014). RNA sequencing-based identification of aberrant imprinting in cloned mice. *Hum. Mol. Genet.* **23**, 992-1001. doi:10.1093/hmg/ddt495

Pastor, W. A. and Kwon, S. Y. (2022). Distinctive aspects of the placental epigenome and theories as to how they arise. *Cell. Mol. Life Sci.* **79**, 569. doi:10.1007/s00018-022-04568-9

Peat, J. R., Dean, W., Clark, S. J., Krueger, F., Smallwood, S. A., Ficz, G., Kim, J. K., Marioni, J. C., Hore, T. A. and Reik, W. (2014). Genome-wide bisulfite sequencing in zygotes identifies demethylation targets and maps the contribution of TET3 oxidation. *Cell Rep.* **9**, 1990-2000. doi:10.1016/j.celrep.2014.11.034

Policarpi, C., Munafò, M., Tsagkris, S., Carlini, V. and Hackett, J. A. (2022). Systematic epigenome editing captures the context-dependent instructive function of chromatin modifications. *bioRxiv* doi:10.1101/2022.09.04.506519

Raas, M. W. D., Zijlmans, D. W., Vermeulen, M. and Marks, H. (2021). There is another: H3K27me3-mediated genomic imprinting. *Trends Genet.* **38**, 82-96. doi:10.1016/j.tig.2021.06.017

Renfree, M. B., Papenfuss, A. T., Shaw, G. and Pask, A. J. (2009). Eggs, embryos and the evolution of imprinting: insights from the platypus genome. *Reprod. Fertil. Dev.* **21**, 935-942. doi:10.1071/RD09092

Richard Albert, J., Kobayashi, T., Inoue, A., Monteagudo-Sánchez, A., Kumamoto, S., Takashima, T., Miura, A., Oikawa, M., Miura, F., Takada, S. et al. (2023). Conservation and divergence of canonical and non-canonical imprinting in murids. *Genome Biol.* **24**, 48. doi:10.1186/s13059-023-02869-1

Santini, L., Halbritter, F., Titz-Teixeira, F., Suzuki, T., Asami, M., Ma, X., Ramesmayer, J., Lackner, A., Warr, N., Pauler, F. et al. (2021). Genomic imprinting in mouse blastocysts is predominantly associated with H3K27me3. *Nat. Commun.* **12**, 3804. doi:10.1038/s41467-021-23510-4

Strogantsev, R., Krueger, F., Yamazawa, K., Shi, H., Gould, P., Goldman-Roberts, M., McEwen, K., Sun, B., Pedersen, R. and Ferguson-Smith, A. C. (2015). Allele-specific binding of ZFP57 in the epigenetic regulation of imprinted and non-imprinted monoallelic expression. *Genome Biol.* **16**, 112. doi:10.1186/s13059-015-0672-7

Surani, M. A. H., Barton, S. C. and Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548-550. doi:10.1038/308548a0

Takahashi, N., Coluccio, A., Thorball, C. W., Planet, E., Shi, H., Offner, S., Turelli, P., Imbeault, M., Ferguson-Smith, A. C. and Trono, D. (2019). ZNF445 is a primary regulator of genomic imprinting. *Genes Dev.* **33**, 49-54. doi:10.1101/gad.320069.118

Tucci, V., Isles, A. R., Kelsey, G., Ferguson-Smith, A. C., Tucci, V., Bartolomei, M. S., Benvenisty, N., Bourc'his, D., Charalambous, M., Dulac, C. et al. (2019). Genomic imprinting and physiological processes in mammals. *Cell* **176**, 952-965. doi:10.1016/j.cell.2019.01.043

Wagschal, A., Sutherland, H. G., Woodfine, K., Henckel, A., Chebli, K., Schulz, R., Oakey, R. J., Bickmore, W. A. and Feil, R. (2008). G9a histone methyltransferase contributes to imprinting in the mouse placenta. *Mol. Cell. Biol.* **28**, 1104-1113. doi:10.1128/MCB.01111-07

Walsh, C., Glaser, A., Fundele, R., Ferguson-Smith, A., Barton, S., Azim Surani, M. and Ohlsson, R. (1994). The non-viability of uniparental mouse conceptuses correlates with the loss of the products of imprinted genes. *Mech. Dev.* **46**, 55-62. doi:10.1016/0925-4773(94)90037-X

Wang, L., Zhang, J., Duan, J., Gao, X., Zhu, W., Lu, X., Yang, L., Zhang, J., Li, G., Ci, W. et al. (2014). Programming and inheritance of parental DNA methylomes in mammals. *Cell* **15**, 979-991. doi:10.1016/j.cell.2014.04.017

Wang, C., Liu, X., Gao, Y., Yang, L., Li, C., Liu, W., Chen, C., Kou, X., Zhao, Y., Chen, J. et al. (2018). Reprogramming of H3K9me3-dependent heterochromatin during mammalian embryo development. *Nat. Cell Biol.* **20**, 620-631. doi:10.1038/s41556-018-0093-4

Wang, L.-Y., Li, Z.-K., Wang, L.-B., Liu, C., Sun, X.-H., Feng, G.-H., Wang, J.-Q., Li, Y.-F., Qiao, L.-Y., Nie, H. et al. (2020). Overcoming intrinsic H3K27me3 imprinting barriers improves post-implantation development after somatic cell nuclear transfer. *Cell Stem Cell* **27**, 315-325.e5. doi:10.1016/j.stem.2020.05.014

Wanigasuriya, I., Gouil, Q., Kinkel, S. A., del Fierro, A. T., Beck, T., Roper, E. A., Breslin, K., Stringer, J. and Hutt, K. (2020). Smchd1 is a maternal effect gene required for genomic imprinting. *eLife* **9**, e55529. doi:10.7554/eLife.55529

Wei, Y., Yang, C.-R. and Zhao, Z.-A. (2022). Viable offspring derived from single unfertilized mammalian oocytes. *Proc. Natl. Acad. Sci. USA* **119**, e2115248119. doi:10.1073/pnas.2115248119

Weinberg-Shukron, A., Ben-Yair, R., Takahashi, N., Dujnić, M., Shtrikman, A., Edwards, C. A., Ferguson-Smith, A. C. and Stelzer, Y. (2022). Balanced gene dosage control rather than parental origin underpins genomic imprinting. *Nat. Commun.* **13**, 4391. doi:10.1038/s41467-022-32144-z

Xia, W. and Xie, W. (2020). Rebooting the epigenomes during mammalian early embryogenesis. *Stem Cell Rep.* **15**, 1158-1175. doi:10.1016/j.stemcr.2020.09.005

Xie, Z., Zhang, W. and Zhang, Y. (2022). Loss of Slc38a4 imprinting is a major cause of mouse placenta hyperplasia in somatic cell nuclear transferred embryos at late gestation. *Cell Rep.* **38**, 110407. doi:10.1016/j.celrep.2022.110407

Xu, Q., Xiang, Y., Wang, Q., Wang, L., Brind'Amour, J., Bogutz, A. B., Zhang, Y., Zhang, B., Yu, G., Xia, W. et al. (2019). SETD2 regulates the maternal epigenome, genomic imprinting and embryonic development. *Nat. Genet.* **51**, 844-856. doi:10.1038/s41588-019-0398-7

Xu, J., Shu, Y., Yao, G., Zhang, Y., Niu, W., Zhang, Y., Ma, X., Jin, H., Zhang, F., Shi, S. et al. (2021). Parental methylome reprogramming in human uniparental blastocysts reveals germline memory transition. *Genome Res.* **31**, 1519-1530. doi:10.1101/gr.273318.120

Yang, H., Bai, D., Li, Y., Yu, Z., Wang, C., Sheng, Y., Liu, W., Gao, S. and Zhang, Y. (2022). Allele-specific H3K9me3 and DNA methylation co-marked CpG-rich regions serve as potential imprinting control regions in pre-implantation embryo. *Nat. Cell Biol.* **24**, 783-792. doi:10.1038/s41556-022-00900-4

Zeng, T.-B., Pierce, N., Liao, J., Singh, P., Lau, K., Zhou, W. and Szabó, P. E. (2021a). EHMT2 suppresses the variation of transcriptional switches in the mouse embryo. *PLoS Genet.* **17**, e1009908. doi:10.1371/journal.pgen.1009908

Zeng, T.-B., Pierce, N. and Szabó, P. E. (2021b). H3K9 methyltransferase EHMT2/G9a controls ERVK-driven noncanonical imprinted genes. *Epigenomics* **13**, 1299-1314. doi:10.2217/epi-2021-0168

Zheng, H., Huang, B., Zhang, B., Xiang, Y., Du, Z., Xu, Q., Li, Y., Wang, Q., Ma, J., Peng, X. et al. (2016). Resetting epigenetic memory by reprogramming of histone modifications in mammals. *Mol. Cell* **63**, 1066-1079. doi:10.1016/j.molcel.2016.08.032