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Noncanonical imprinting: intergenerational epigenetic inheritance mediated by Polycomb complexes

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Genomic imprinting is illustrative of intergenerational epigenetic inheritance. The passage of parental genomes into the embryo is accompanied by epigenetic modifications, resulting in imprinted monoallelic gene expression in mammals. Some imprinted genes are regulated by maternal inheritance of H3K27me3, which is termed noncanonical imprinting. Noncanonical imprinting is established by Polycomb repressive complexes during oogenesis and maintained in preimplantation embryos and extraembryonic tissues, including the placenta. Recent studies of noncanonical imprinting have contributed to our understanding of chromatin regulation in oocytes and early embryos, imprinted X-chromosome inactivation, secondary differentially DNA-methylated regions, and the anomalies of cloned mice. Here, I summarize the current knowledge of noncanonical imprinting and remark on analogous mechanisms in invertebrates and plants.

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Introduction

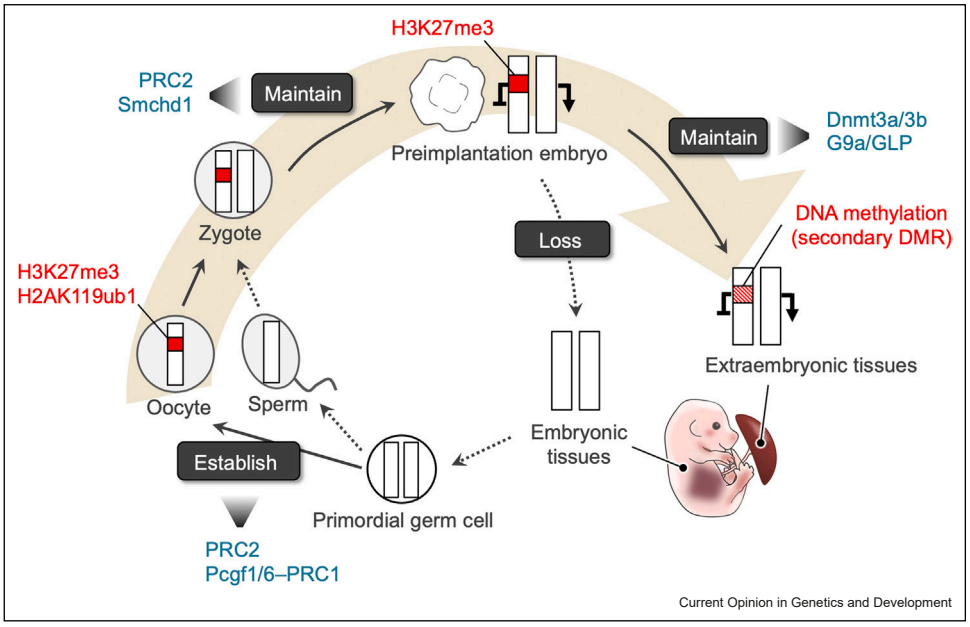
The genomic imprinting field has evolved around DNA methylation (DNAme). DNAme is deposited during male and female gametogenesis, resulting in germline differentially DNA-methylated regions (gDMRs). Parental allele-specific DNAme at some gDMRs persists throughout development and controls imprinted gene expression. DNAme-mediated 'canonical' imprinting is disrupted in DNA methyltransferase 3a (*Dnmt3a*) or 3l (*Dnmt3l*) maternal knockout (matKO) embryos [1,2]. However, some paternally expressed imprinted genes (PEGs), which do not have gDMRs, maintain their imprinting in the matKO embryos [3–5]. This observation suggests the existence of a DNAme-independent, 'noncanonical', mechanism for imprinting.

In 2017, the core of noncanonical imprinting was discovered to be a repressive histone modification, trimethylation of histone H3 at lysine 27 (H3K27me3) [6]. Integrative analyses of parental allele chromatin accessibility, DNA methylome, and H3K27me3 ChIP-seq datasets in mouse zygotes and preimplantation embryos revealed that maternal gene repression and inaccessibility at DNA hypomethylated regions are attributable to H3K27me3 that is passed down from oocytes to embryos. Maternal H3K27me3 regulated all PEGs whose imprinting was reported to be DNAme-independent. This study raised many questions about the mechanisms, functions, and evolutionary conservation of maternal H3K27me3-mediated noncanonical imprinting.

Establishment of noncanonical imprinting and Polycomb regulation in oocytes

Noncanonical imprinting is established by Polycomb-repressive complex 2 (PRC2) and PRC1 that respectively deposit H3K27me3 and monoubiquitylation of histone H2A at lysine 119 (H2AK119ub1) during oocyte growth (Figure 1) [7,8]. H3K27me3 and H2AK119ub1 are broadly colocalized at more than 10 000 non- and lowly expressed genes, including noncanonical imprinted genes, in fully grown oocytes (FGOs) [8–11]. Each Polycomb domain spans up to several megabases in FGOs. Such broad Polycomb domains are not established in sperm. The boundaries of Polycomb domains are partly determined by H3K36me3, which is deposited at highly expressed genes by Setd2 and counteracts PRCs [12]. Polycomb domains in FGOs spatially self-interact to form higher-order structures called Polycomb-associating domains (PADs) [13]. PADs are disrupted and PAD-associated genes are derepressed by depletion of Ring1a/1b, essential subunits of PRC1, but not by depletion of Eed, an essential subunit of PRC2 [13]. This indicates that PRC1-mediated H2AK119ub1, but not PRC2-mediated H3K27me3, is central to gene silencing in oocytes. Consistently, H2AK119ub1 distribution is unchanged in *Eed* KO FGOs [10]. By contrast, H3K27me3 is lost at ~20% of genes, including several noncanonical imprinted genes, in FGOs depleted of Pcgf1/6, essential components of variant PRC1,

Figure 1



Ontogeny and known players of noncanonical imprinting. Noncanonical imprinting is established by PRC2 with the help of Pcgf1/6-PRC1 during oocyte growth and is maintained by PRC2 during preimplantation development. Smchd1 is involved in the maintenance, or possibly establishment, for some genes. After implantation, noncanonical imprinting is maintained in extraembryonic tissues by acquiring secondary DMR in a manner dependent on Dnmt3a/3b and G9a/GLP. By contrast, noncanonical imprinting is lost in embryonic tissues, thereby resetting the imprinted state before primordial germ cell development.

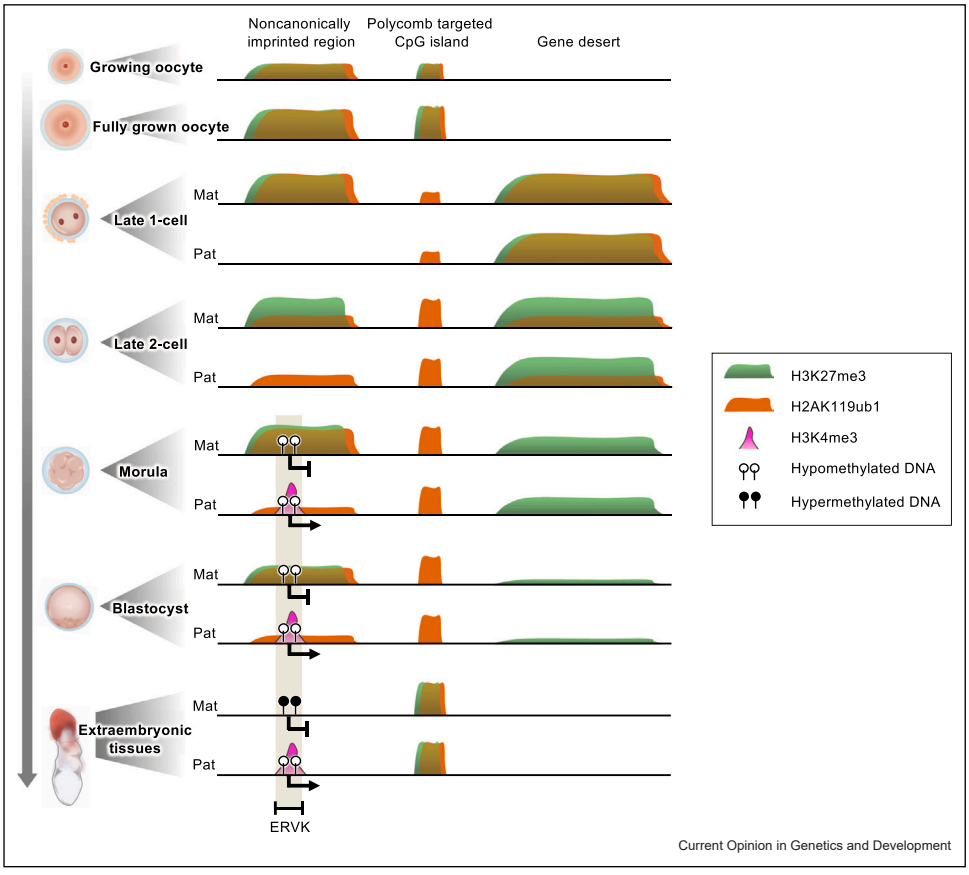
wherein H2AK119ub1 is massively reduced [8]. This indicates that PRC1 acts upstream of PRC2 and is required for the establishment of noncanonical imprinting, at least in a subset of genes. Importantly, the H3K27me3-deficient state in *Pcgf1/6* KO oocytes is irreversibly inherited by embryos even in the presence of PRC2, resulting in loss of noncanonical imprinting and placental enlargement [8]. This indicates that H3K27me3 loss in oocytes is no longer repaired after fertilization and thus impacts later development.

The gene-selective loss of H3K27me3 upon massive loss of H2AK119ub1 in *Pcgf1/6* KO FGOs implicates the existence of H2AK119ub1-dependent and -independent mechanisms for H3K27me3 establishment in oocytes. The faithful establishment of H3K27me3 at the majority of genes suggests that the chromatin-targeting mechanisms of PRC1 and PRC2 are largely independent in oocytes [8]. It is possible that Kdm2b-containing PRC1.1 and PCL-containing PRC2.1 independently target CpG islands (CGIs) to deposit H2AK119ub1 and H3K27me3, respectively [14]. By contrast, the defective establishment of H3K27me3 at a subset of genes suggests that H2AK119ub1 is required for H3K27me3. Given that the H3K27me3 loss is tightly coupled with gene derepression in *Pcgf1/6* KO FGOs [8] and that transcription potently counteracts PRC2 [14], I speculate that the gene selectivity might be attributable to transcription activators, such as specific transcription factors and Trithorax complexes, in oocytes. Addressing to these questions would definitely expand our knowledge of Polycomb regulation *in vivo*.

Short-term maintenance of noncanonical imprinting

After fertilization, noncanonical imprinting is maintained through preimplantation development and during subsequent extraembryonic tissue development (Figures 1 and 2). In preimplantation embryos, maternal H3K27me3 domains are retained until the blastocyst stage and mediate maternal allele repression [6,9,15,16]. A 'read-and-write' self-sustaining property of PRC2 might underlie the stability of H3K27me3 [17]. On the paternal allele, H3K27me3 is deposited at gene deserts, but not at gene-rich regions harboring maternal H3K27me3 [9] (Figure 2). The avoidance of paternal H3K27me3 deposition can be partly explained by paternal allele-specific enrichment of H3K36me3, which antagonizes PRC2 [12]. However, other mechanisms are likely involved, because paternal allele-specific H3K36me3 is restricted to gene bodies of noncanonical

Figure 2



Epigenome dynamics at noncanonically imprinted regions, CGIs, and gene deserts. Broad H3K27me3 domains coating noncanonical imprinted genes are formed, along with H2AK119ub1, during oocyte growth. The H3K27me3 domains are retained during preimplantation development. Accompanied by paternal allele gene expression in embryos, H3K4me3 is deposited on the ERVKs nearby the promoters at the paternal allele. DNAme is deposited on the ERVKs at the repressive maternal allele after the blastocyst stage. H2AK119ub1 is globally remodeled after the 1-cell stage. The parental asymmetry of H2AK119ub1 at noncanonical imprinted genes is temporarily resolved at the 2-cell stage and is then restored by the morula stage. H2AK119ub1, but not H3K27me3, is deposited at Polycomb-targeted CGIs during preimplantation development. At gene deserts, both H2AK119ub1 and H3K27me3 are enriched at the late 1-cell stage, but then H2AK119ub1 is displaced in a manner preceding H3K27me3. The H2AK119ub1 distribution in extraembryonic cells is illustrated based on our unpublished data.

imprinted genes, while maternal H3K27me3 domains are much broader [12]. I speculate that *de novo* H3K27me3 deposition activity of PRC2 might be very limited during preimplantation development, possibly because PRC2 is trapped by a large amount of pre-existing H3K27me3 for its maintenance. Future investigations are warranted to understand the mechanism of the paternal allele protection from PRC2 in preimplantation embryos.

Whereas the maternal allele bias of H3K27me3 is maintained during preimplantation development, H2AK119ub1 is more dynamic (Figure 2). H2AK119ub1 is colocalized with H3K27me3 in late 1-cell zygotes where it somehow contributes to maintenance of H3K27me3 [8]. Then it becomes progressively redistributed to typical Polycomb-targeted CGIs of developmental genes [8,10,11]. Whereas H3K27me3 disappears from CGIs shortly after fertilization, H2AK119ub1 represses developmental genes in preimplantation embryos [10]. Consequently, H2AK119ub1 does not show global parental asymmetry at the late 2-cell stage and beyond. Exceptionally, noncanonical imprinted genes show maternally biased H2AK119ub1 enrichment in morula embryos. This parental asymmetry is likely a consequence, but not the determinant, of imprinted gene expression because H2AK119ub1 removal by transient overexpression of H2A deubiquitylases did not induce expression of the maternal allele [10].

How H3K27me3 mediates gene repression is an important open question. H3K27me3 reader proteins, such as CBX subunit-containing forms of PRC1, might

execute H3K27me3-dependent gene repression [18]. Alternatively, H3K9me3 might be involved, as it is seemingly associated with maternal H3K27me3 from the 2- to 8-cell stages [19]. Recent studies demonstrated that several noncanonical imprinted genes, including *Xist*, are partially derepressed in *Smc hinge domain-containing 1* (*Smchd1*) matKO embryos, suggesting that Smchd1 plays a role in establishment or maintenance of noncanonical imprinting [20,21]. Since Smchd1 acts downstream of PRC1 on the inactive X (Xi) chromosome [22], a PRC1–Smchd1 pathway might be involved in noncanonical imprinting. Future studies, including rigorous epigenomic analyses of *Smchd1* mutants, are needed to understand the mechanisms of H3K27me3-dependent gene repression in early embryos.

Long-term maintenance of noncanonical imprinting

Long-term maintenance of noncanonical imprinting involves a Polycomb-to-DNAme relay (Figures 1 and 2). While maternal H3K27me3 domains are largely lost before implantation, DNAme is deposited on endogenous retrovirus-K (ERVK) long terminal repeats (LTRs), which are located nearby promoters of noncanonical imprinted genes and also act as the imprinted promoters in extraembryonic tissues [23–26]. Maternal allele-specific acquisition of DNAme results in secondary differential DNA-methylated regions (sDMRs). Genetic deletion of the LTR at *Gab1* results in partial loss of imprinting, demonstrating that sDMRs indeed contribute to imprinting maintenance [23]. The establishment of sDMRs and the maintenance of noncanonical imprinting require *Dnmt3a/3b* and the H3K9 methyltransferases, *G9a/Glp* [24–26], yet it remains elusive how G9a/GLP are required and whether H3K9 methylation itself is required. The LTRs at the paternal allele do not acquire DNAme, possibly because H3K4me3, which is known to repel Dnmt3, is enriched on the paternal allele at peri-implantation [23,24]. In epiblasts, the LTRs acquire DNAme at both alleles, accompanied by gene silencing [23].

While noncanonical imprinting is maintained exclusively in the extraembryonic cells, imprinting of *Slc38a4* is well-maintained in both extraembryonic and embryonic cell lineages. This is likely because *Slc38a4*, but not the other noncanonical imprinted genes, has a gDMR at the promoter [27,28]: the promoter and gene body of *Slc38a4* are marked by heritable DNAme and H3K27me3, repspectively, in oocytes. The combinatorial behavior of canonical and noncanonical imprinting of *Slc38a4* seems to involve alternative promoter usage in a developmental stage- and tissue-specific fashion [28]. The case study of *Slc38a4* implies that the lack of gDMRs might be a key to accomplish extraembryonic cell lineage-specific maintenance of noncanonical imprinting, although this hypothesis awaits experimental validation.

Functions of noncanonical imprinting

Noncanonical imprinting controls at least 30 PEGs, the majority of which are transiently imprinted before implantation and become bi-allelically expressed afterward [6–8,15,16,23,24,29]. In the extraembryonic tissues of postimplantation embryos, noncanonical imprinting regulates at least nine PEGs and the chromosome-2 microRNA cluster (C2MC), a rodent-specific miRNA cluster containing 72 miRNA precursors (Table 1).

Imprinting functions can be studied by a loss-of-imprinting (LOI) approach. Thus far, *Eed*, *Ezh1/2*, and *Pcgf1/6* matKO mutants have been generated as LOI models for noncanonical imprinting [7,8,30,31]. *Eed* and *Ezh1/2* encode essential subunits of PRC2, while *Pcgf1/6* encode essential components of variant PRC1, which deposits H2AK119ub1. All of these matKO mutants consistently show partial lethality (sublethality) after implantation and the survivors exhibit placental enlargement at term [7,8,29–31]. These phenotypes are truly caused by LOI, because restoration of monoallelic gene expression of *Xist* and autosomal PEGs (e.g. C2MC and *Slc38a4*) suppresses embryonic lethality and placental enlargement in *Eed* matKO embryos (Figure 3a) [29].

Xist encodes a long noncoding RNA that induces X-chromosome inactivation (XCI). In mouse preimplantation embryos, *Xist* is expressed from the paternal X chromosome (Xp). Consequently, Xp is selectively inactivated, and this persists through extraembryonic development, a process called imprinted XCI [32]. The Xp selectivity is attributable to H3K27me3-mediated repression of *Xist* on the maternal X chromosome (Xm) during preimplantation development (Figure 3b) [15]. Loss of maternal H3K27me3 results in ectopic Xm-*Xist* expression, leading to downregulation of Xm-linked genes (XmCI) in LOI embryos [7,8,15,30,31]. The XmCI in these embryos is resolved by the late blastocyst stage via unknown mechanisms (Figure 3b). Possibly, XmCI-mediated downregulation of *Rnf12/Rlim*, a positive regulator of *Xist*, might contribute to the downregulation of *Xist* and X-reactivation [7]. Despite being transient, XmCI before implantation does have detrimental ripple effects on later development, because prevention of XmCI by maternal *Xist* KO greatly suppresses the postimplantation lethality of *Eed* matKO embryos (Figure 3b) [29]. These studies indicate that noncanonical imprinting of *Xist* is important for mouse development by preventing XmCI before implantation. Nonetheless, given that *Eed/Xist* double matKO embryos still show partial prenatal lethality by E13.5 [29], noncanonical imprinting may have developmentally relevant functions other than the regulation of XCI, a possibility that awaits further investigation.

Table 1

Noncanonical imprinted genes in the mouse.

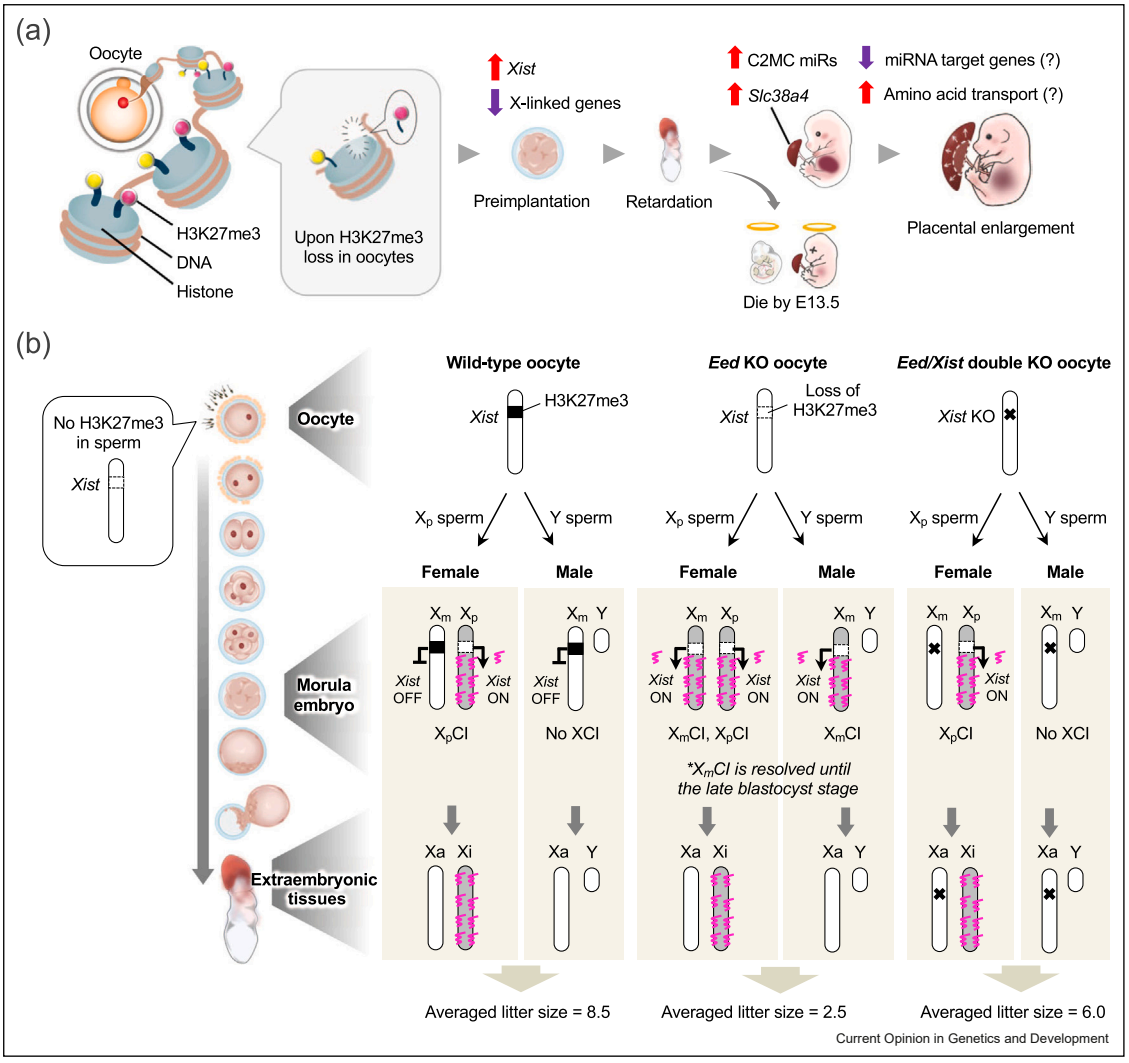
	C2MC	*Gab1*	*Gm32885*	*Jade1/Phf17*	*Platr20*	*Sall1*	*Sfmbt2*	*Slc38a4*	*Smoc1*	*Xist*
Formal gene name	Chromosome- 2 miRNA cluster	Growth factor receptor-bound protein 2-associated protein 1	Predicted lncRNA	Jade family PHD finger 1	Pluripotency-associated transcript 20, lncRNA	Spalt-like transcription factor 1	Scm-like with four mbt domains 2	Solute carrier family 38, member 4	SPARC- related modular calcium binding 1	Xi-specific transcript
Location	Chr2, embedded in an intron of *Sfmbt2*	Chr8	Chr15, located at ~100 kb upstream of *Slc38a4*	Chr3	Chr11	Chr8	Chr2	Chr15	Chr12	ChrX
Phenotypes of loss-of-function mouse mutants (KO or gene trap)	Prenatally sublethal, placental defects	Prentally lethal, placental defects	Unknown	Neonatally sublethal	Unknown	Postnatally lethal	Prenatally lethal, placental defects	Postnatally sublethal, placental defects	Postnatally lethal	Neonatally lethal in females, placental defects
Reference	[57]	[58]	N.A.	[59]	N.A.	[60]	[61]	[62]	[63]	[64]

Besides the LOI models, cloned mouse embryos generated by somatic cell nuclear transfer have also provided critical insight into noncanonical imprinting functions. Since the original generation of cloned mice two decades ago, it has been known that they suffer from a high incidence of embryonic lethality and placental enlargement [33]. Analyses of imprinted gene expression in the placenta revealed that cloned embryos lose noncanonical imprinting [5,34]. This LOI is caused by the lack of broad H3K27me3 domains in donor somatic cells [34]. Use of *Xist* KO donor cells improves development of cloned embryos, indicating that *Xist* LOI is a cause of the embryonic lethality [35]. Furthermore, restoration of monoallelic expression of autosomal noncanonical imprinted genes, including C2MC, *Slc38a4*, and *Sfmbt2*, ameliorates developmental lethality and placental enlargement in cloned mice [36–38]. These findings are largely consistent with the aforementioned rescue experiment using *Eed* matKO embryos [29]. Mechanistically, LOI-induced upregulation of C2MC-harboring miRNAs causes downregulation of the putative miRNA targets, including tumor growth repression-related genes, which in turn might accelerate cell proliferation in cloned placentae [36]. Additionally, LOI-induced upregulation of *Slc38a4* increases amino acid transport and overactivates the mechanistic target of rapamycin complex 1 signaling pathway, which might also contribute to cell proliferation in cloned placentae (Figure 3a) [37]. Although the precise mechanisms and physiological meaning of placental enlargement remain elusive, correction of the noncanonical imprinting defect offers a way to normalize embryonic and placental development in cloned mice.

Conservation of noncanonical imprinting and its analogs

Analogous mechanisms of noncanonical imprinting involving intergenerational inheritance of H3K27me3 have been observed in various organisms. In *C. elegans*, H3K27me3 is transmitted from both oocytes and sperm and contributes to repression of X chromosomes during early development [39], and lack of paternally inherited H3K27me3 causes gene derepression from the paternal allele [40]. In *D. melanogaster*, oocyte-derived H3K27me3 restricts enhancer activity at zygotic genome activation [41]. In the liverwort *Marchantia polymorpha*, H3K27me3 is pervasively deposited on the paternal chromatin in zygotes and the parental asymmetry is maintained throughout embryogenesis to inactivate the entire set of paternal chromosomes [42]. In *Arabidopsis*, cold exposure induces Polycomb repression at *flowing locus C (FLC)*, and the Polycomb-dependent repressive state is maternally inherited by early embryos [43]. Moreover, maternal PRC2 regulates DNAme-independent imprinted genes in the endosperm, the extraembryonic tissue that supports embryonic growth [44]. Remarkably, PRC2 matKO *Arabidopsis* mutants exhibit enlargement of the endosperm [45,46], reminiscent of the placental

Figure 3



Functions of noncanonical imprinting. **(a)** Phenotypes of loss of noncanonical imprinting (LOI). LOI at *Xist* results in downregulation of X-linked genes in preimplantation embryos, which causes later developmental retardation and prenatal lethality. LOI at C2MC and the *Slc38a4* gene causes placental enlargement at term, possibly through downregulation of miRNA-targeted negative regulators of cell proliferation and upregulation of amino acid transport. **(b)** Dynamics of *Xist* expression and XCI. Sperm do not have *Xist* imprinting. In female morula embryos derived from wild-type oocytes, *Xist* on the Xm is silenced by maternally inherited H3K27me3, allowing Xp-specific expression of *Xist* RNA (pink). In male embryos, Xm-*Xist* is similarly silenced by H3K27me3. In extraembryonic tissues in postimplantation embryos, Xm and Xp become active (Xa) and Xi, respectively. In both female and male morula embryos derived from *Eed* KO oocytes, Xm-*Xist* is ectopically expressed, resulting in downregulation of XmCI. This aberrant XCI state is resolved by the late blastocyst stage. In both female and male morula embryos derived from *Eed*/*Xist* double-KO oocytes, ectopic expression of Xm-*Xist* is prevented, normalizing the XCI state. The averaged litter sizes of the WT and mutant female mice are indicated at the bottom [29].

enlargement seen in PRC2 matKO mouse mutants. It seems that both mice and flowing plants share a maternal PRC2-mediated mechanism of extraembryonic tissue-size regulation. This harks back to the genetic conflict theory positing that maternal imprints suppress placental (and fetal) overgrowth to save maternal resources [47]. Why organisms adopted H3K27me3 as a means of intergenerational epigenetic inheritance is an interesting question. I envision that the high sustainability of H3K27me3 is beneficial to continuously retain a repressive memory throughout the drastic epigenetic (re-) programming period of gametogenesis and subsequent early embryogenesis. Furthermore, PRCs might have been more advantageous than DNAme machineries, not only because PRCs are more widely conserved among organisms [18], but also because DNAme is mutagenic in an evolutionary timescale and therefore PRCs are safer tools for gene silencing.

Conservation of noncanonical imprinting among mammals is under debate. Comprehensive identification of imprinted genes and epigenomic analyses in rat embryos demonstrated that some PEGs are associated with oocyte H3K27me3, but not DNAme, and acquire sDMRs in the extraembryonic cells of postimplantation embryos in which they are imprinted [48]. Furthermore, maternal H3K27me3 domains are retained throughout preimplantation development in rats [49]. These data strongly suggest that noncanonical imprinting is conserved in rats. However, noncanonical imprinted genes identified in the mouse or rat are not imprinted in human [48,50]. Therefore, the question remains whether H3K27me3 inheritance *per se* is conserved. H3K27me3 CUT&RUN analyses in human embryos demonstrated that H3K27me3 is briefly inherited until the 2-cell stage, but then largely erased before zygotic genome activation [49]. Erasure of H3K27me3 was also observed in bovine and porcine preimplantation embryos [51]. These data argue against the conservation of H3K27me3 inheritance in mammals other than rodents. However, it has also been reported that some of sDMRs in human placentae are associated with oocyte H3K27me3, but not DNAme [50], raising the possibility that a remnant H3K27me3 or an unknown epigenetic cascade beginning with briefly inherited H3K27me3 might lead to establishment of sDMR in the human placenta. Epigenetic perturbation experiments and profiling of more histone modifications in nonrodent embryos are warranted in the future.

Future perspectives

Genomic imprinting is an excellent model to understand epigenetic mechanisms for gene regulation. Studies of canonical imprinting have indeed uncovered fundamental mechanisms, such as chromatin insulation, looping, and noncoding RNA-mediated gene silencing [52]. Studies of noncanonical imprinting have also begun to reveal how PRC1 and PRC2 interplay *in vivo*, how sDMRs are established, how imprinted XCI is executed, and why cloned mice are developmentally defective. Yet, many important questions, as mentioned above, remain to be elucidated and await further investigations. Moreover, given that PRCs are more widely conserved than DNAme among organisms [18], it raises the possibility that analogous mechanisms of noncanonical imprinting, such as transient or cell-type-specific imprinting, might be present in nonmammalian animals that are thought to lack imprinting. Future studies are awaited to understand the bandwidth of biological events that involve noncanonical imprinting.

Genomic imprinting proposes the concept that epigenetic changes in the parental generation can have lifelong impacts on the offspring. Based on this, the issue of whether and how DNAme in gametes, early embryos, and the offspring is modulated by the parental environment or by *in vitro* culture/manipulation of gametes and embryos has been intensively studied [53,54]. Now that we know that defects in H3K27me3-mediated noncanonical imprinting also affect later embryonic and placental development, and possibly even postnatal growth [55], it has emerged that changes of histone modifications in oocytes and early embryos might also have lifelong impacts on the offspring. From this perspective, it is noteworthy that certain inter-/trans-generational phenomena in mice do not appear to rely on DNAme [54,56]. In the future, it will be important to address whether histone modifications in oocytes and early embryos are susceptible to any environmental cues and how they might have physiological/pathological effects on the offspring.

Data Availability

No data were used for the research described in the article.

Conflict of interest statement

Nothing declared.

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