Maternal H3K27me3-dependent autosomal and X chromosome imprinting

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Abstract | Genomic imprinting and X-chromosome inactivation (XCI) are classic epigenetic phenomena that involve transcriptional silencing of one parental allele. Germline-derived differential DNA methylation is the best-studied epigenetic mark that initiates imprinting, but evidence indicates that other mechanisms exist. Recent studies have revealed that maternal trimethylation of H3 on lysine 27 (H3K27me3) mediates autosomal maternal allele-specific gene silencing and has an important role in imprinted XCI through repression of maternal *Xist*. Furthermore, loss of H3K27me3-mediated imprinting contributes to the developmental defects observed in cloned embryos. This novel maternal H3K27me3-mediated non-canonical imprinting mechanism further emphasizes the important role of parental chromatin in development and could provide the basis for improving the efficiency of embryo cloning.

Pronuclear transfer
A technique that involves moving one or both pronuclei (which are formed from the sperm and oocyte genomes shortly after fertilization) from a fertilized one-cell embryo to a different recipient embryo.

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Most autosomal genes in diploid cells are transcribed at similar levels from both alleles. However, for a small subset of genes, one parental allele is transcriptionally silenced by genomic imprinting, and expression depends on whether the allele is inherited from the oocyte or the sperm[1]. In addition to autosomal imprinting, the paternal X chromosome (Xp) is preferentially silenced in female mouse pre-implantation embryos and placental lineages by a process known as imprinted X-chromosome inactivation (XCI; a process distinct from random XCI, which occurs in post-implantation embryonic lineages in mouse and other mammals)[2] (BOX 1). As these imprints can persist from gametes to the next generation, genomic imprinting and imprinted XCI represent two examples of intergenerational epigenetic inheritance. Together, these two processes are critical for controlling the gene dosage during embryonic development, and their dysregulation can cause developmental defects and diseases. For example, loss of imprinting contributes to childhood disorders such as the Prader–Willi/Angelman and Beckwith–Wiedemann/Silver–Russell syndromes[2,3].

The unequal contributions of parental genomes during development was first demonstrated by elegant pronuclear transfer experiments in the 1980s[4,5]; bi-maternal and bi-paternal mouse embryos generated in these studies were found to be non-viable, indicating that both maternal and paternal genomes are required for normal development. The first imprinted genes were identified in the early 1990s[6–9], and shortly afterwards parental allele-specific DNA methylation was found to be critical for imprinted gene expression[10]. Parental allele-specific DNA methylation originates from differential DNA methylation between oocytes and sperm, and is maintained throughout development[11]. These germline differentially methylated regions (DMRs) are the primary signals for establishing secondary allele-specific epigenetic features such as histone modifications and somatic DMRs that help to achieve imprinted expression[12,13]. Germline DNA methylation-dependent allele-specific expression is the classic form of genomic imprinting, and is therefore referred to here as canonical imprinting.

However, several paternally expressed imprinted genes in mouse placenta do not harbour germline DMRs and their imprinted expression is independent of oocyte DNA methylation[14,15]. Furthermore, germline DNA methylation does not regulate the paternal allele-specific expression of the long non-coding RNA (lncRNA) X-inactive specific transcript (*Xist*) in mouse extra-embryonic cells, which causes the paternal allele-specific silencing of most X-linked genes in this lineage[2,16,17]. Taken together, these observations indicate the existence of a germline DNA methylation-independent imprinting mechanism.

Recently, low-input epigenomic profiling techniques[18] have been used to demonstrate that Polycomb repressive complex 2 (PRC2)-mediated trimethylation of H3 on lysine 27 (H3K27me3) in mouse oocytes is the cause of maternal allele-specific silencing of both the autosomal imprinted genes and the imprinted *Xist*[19,20] in the placenta (BOX 2). Because this new imprinting

Box 1 | **Random and imprinted XCI**

X-chromosome inactivation (XCI) is a mechanism of dosage compensation by which one of the X chromosomes of XX females is transcriptionally silenced so that expression levels of X-linked genes are equalized between XX female and XY male cells[146]. In somatic cells, XCI is random, with either the maternal X chromosome (Xm) or the paternal X chromosome (Xp) being silenced[146]. However, in mouse pre-implantation embryos, XCI is imprinted so that Xp is preferentially repressed[85]. After implantation, Xp remains inactive in the extra-embryonic lineages that contribute to the placenta whereas it is reactivated in the epiblast, which gives rise to the embryo proper and in which random XCI subsequently takes place[89,90,147]. Once random XCI is complete, the inactive X remains stably silenced during cell propagation.

The long non-coding RNA X-inactive specific transcript (*Xist*) is only expressed from the future inactive X chromosome and is required to initiate both imprinted and random XCI in *cis*[16,148,149]. Although the details of *Xist*-induced silencing are not fully understood, it is well established that *Xist* associates with numerous partners to inactivate the entire X chromosome. For example, a recent study revealed that a region of the *Xist* RNA, the repeat A element, recruits the RNA-binding protein SPEN at the onset of XCI to elicit gene silencing. Protein interactome analyses of the SPEN effector domain suggest that SPEN mediates gene silencing by recruiting transcriptional co-repressors to the X chromosome[150]. In addition, the *Xist* RNA repeat B element associates with another RNA-binding protein, hnRNPK, which recruits variant Polycomb repressive complexes 1.3 and 1.5 (vPRC1.3/1.5) to deposit the transcriptional repressive chromatin mark ubiquitination to lysine 119 on histone H2A (H2AK119Ub)[151–153]. Together with other mechanisms, these processes lead to the formation of facultative heterochromatin and stable XCI through depletion of active histone marks (such as trimethylation of histone H3 on lysine 4 (H3K4me3), H3K27ac and H3K9ac) and establishment of the repressive histone marks (such as H2AK119Ub, H3K27me3 and H3K9me2)[2,154]. For more detailed information on how *Xist* induces XCI, readers can refer to recent reviews[155,156].

mechanism uses oocyte-inherited H3K27me3, rather than DNA methylation, to distinguish parental alleles in pre-implantation embryos, it is mechanistically different from classic imprinting and is therefore termed non-canonical imprinting. It should be noted that the non-canonical imprinting referred to here is different from the phenomenon of modest parental expression bias observed in specific brain regions, which has also been referred to as non-canonical imprinting in some contexts[21].

In this Review, we first briefly summarize the mechanisms involved in canonical imprinting. We then describe our current understanding of non-canonical imprinting and compare this with and contrast this to canonical imprinting in terms of its establishment and maintenance. In addition, the role of non-canonical imprinting in imprinted XCI, placental development and animal cloning is discussed. Lastly, we discuss how non-canonical imprinting might be conserved during evolution. Unless otherwise specified, both imprinting mechanisms are discussed in the context of the mouse.

Mechanisms of canonical imprinting

Canonically imprinted genes typically are found in clusters of more than three genes and span genomic regions ranging in size from several kilobases to a few megabases[11]. The allele-specific expression of the transcripts within each cluster is regulated by a *cis*-regulatory element known as the imprinting control region (ICR)[11]. ICRs exhibit germline-derived differential DNA methylation between parental alleles, and genetic manipulation of the ICRs in either in vitro cell culture or in vivo mouse studies can cause loss of imprinting of all genes in an imprinted cluster[11]. Given its essential role, the establishment, maintenance and erasure of allelic DNA methylation at ICRs is controlled by multiple regulators. In addition, ICRs use diverse *cis*-regulatory mechanisms to control imprinted gene expression. However, most mechanisms are not fully understood and, even for well-studied mechanisms such as the insulator model and the lncRNA model described below, it is not clear how applicable they might be to other imprinted loci. For a more comprehensive discussion of canonical imprinting, readers are directed to excellent reviews of the topic[3,11,22,23].

Establishment of canonical imprinting during gametogenesis. Primary imprinting marks need to be established during gametogenesis, a developmental window when the parental genomes are in separate compartments and are subject to different epigenetic modifications (FIG. 1a). At this stage, both global de novo DNA methylation and methylation at individual germline DMRs are deposited by the DNA methyltransferase DNMT3A and its essential non-catalytic cofactor DNMT3L[24,25]. Loss of DNMT3A or DNMT3L in oocytes causes maternal imprinting defects and embryonic lethality, and lack of either protein in the male germ line leads to spermatogenesis defects and de novo methylation failure at two of the three paternally methylated DMRs (that is, the *H19/Igf2* and *Gtl2/Dlk1* ICRs)[24,25]. The other paternally methylated DMR, *Rasgrf1*, depends on the piwi-interacting RNA pathway and the recently identified rodent-specific DNMT3C[26–28].

Whereas paternally methylated DMRs acquire DNA methylation prenatally, maternal DMRs are methylated postnatally during oocyte growth[11]. Despite extensive studies, some aspects of de novo DNA methylation during oogenesis remain elusive. The current working model is that transcription elongation causes an enrichment of dimethylation and trimethylation of histone H3 at lysine 36 (H3K36me2/3) at the transcribed regions, and these histone modifications recruit the DNMT3A/3L complex to establish DNA methylation in oocytes[29–31]. In support of this model, premature termination of transcription at germline DMRs in oocytes leads to reduced H3K36me3 levels and a failure of de novo DNA methylation at these loci[29,31,32]. Furthermore, depletion of the H3K36 methyltransferase SETD2 in oocytes causes genome-wide loss of H3K36me3 and DNA hypomethylation, including at germline DMRs[33]. In addition, removal of histone modifications that antagonize DNA methylation is also important for imprinting establishment, as loss of the H3K4 demethylases KDM1A or KDM1B causes a substantial increase of H3K4me2 in oocytes and results in defective establishment of DNA methylation at maternally methylated DMRs[30,34].

Maintenance of canonical imprinting during development. In canonical imprinting, parental allele-specific DNA methylation at ICRs needs to survive two waves of DNA methylation reprogramming; global demethylation during pre-implantation development; and the subsequent remethylation at implantation[35] (FIG. 1a). Genome-wide DNA methylation profiling has revealed that half of the sperm and oocyte genomes are

DNA methylation
An epigenetic modification in which a methyl group is added to the fifth carbon of a cytosine in a DNA molecule. DNA methylation at gene promoters is generally associated with transcriptional silencing.

Somatic DMRs
Also known as secondary differentially methylated regions (DMRs), somatic DMRs are regions of the genome containing allele-specific DNA methylation that is established after fertilization.

Primordial germ cells
Precursors of the gametes that are specified from the somatic lineage during gastrulation.

differentially methylated; however, most of these DMRs become hypomethylated on both parental alleles before implantation[36]. Germline DMRs that overlap ICRs are protected from this global DNA demethylation by the Krüppel-associated box (KRAB)-containing zinc finger protein 57 (ZFP57) and ZFP445 (REFS[37,38]), as mouse embryos that lack ZFP57 and ZFP445 fail to maintain DNA methylation at most ICRs[37,38]. Mechanistic studies in mouse embryonic stem (ES) cells indicate that ZFP57 and ZFP445 bind to the methylated allele at ICRs and recruit the cofactor KAP1 (also known as TRIM28)[38,39]. The ZFP57/KAP1 complex also associates with other epigenetic modifiers, including the DNA methylation maintenance machinery DNMT1 and UHRF1 and the H3K9 methyltransferase SETDB1, to protect allele-specific DNA methylation at ICRs[39,40]. Recently, *N*-α-acetyltransferase 10 protein (NAA10P) has been shown to facilitate DNMT1 binding to the methylated alleles and loss of NAAP10P causes DNA hypomethylation at ICRs in both mouse embryos and mouse ES cells[41]. How NAA10P recruits DNMT1 and interacts with other imprinting maintenance factors, such as ZFP57 and KAP1, remains to be determined.

In addition to the methylated allele at ICRs escaping global DNA demethylation in pre-implantation development, it is equally important for the unmethylated allele to survive genome-wide remethylation during implantation[42]. Although the underlying mechanism remains unclear, it is believed that the unmethylated allele at ICRs is protected from de novo DNA methylation by the presence of histone marks that can antagonize DNA methylation machinery, such as H3K4me3 and/or other modifications[42,43].

Erasure of canonical imprinting in primordial germ cells. In order to re-establish DNA methylation in the germ line according to the sex of the embryo, the allelic DNA methylation at ICRs must first be erased. The erasure of DNA methylation at ICRs occurs as part of the global DNA demethylation process in the primordial germ cells, which involves passive demethylation of the bulk of the genome by DNA replication followed by active demethylation mainly of imprinted loci and germline-specific genes by the ten–eleven translocation (TET) family enzymes[44–46] (FIG. 1a). TET enzymes can convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) and its derivatives, which are then removed by replication-dependent dilution or by the DNA base excision repair pathway[44]. Genetic studies in mouse indicate that TET1 deficiency causes aberrant DNA hypermethylation at only a subset of ICRs in germ cells and somatic tissues, and results in dysregulated imprinted gene expression[46,47]. For example, DNA demethylation at the *Snrpn* ICR is unaffected even in TET1/TET2 double mutants[47,48], suggesting that demethylation at *Snrpn* ICR occurs through passive dilution but not active demethylation. The mechanism underlying ICR-specific dependency on TET proteins remains unknown, but it has been suggested that the sequence composition of the *Snrpn* ICRs could explain why it does not undergo active demethylation like other imprinted loci[47].

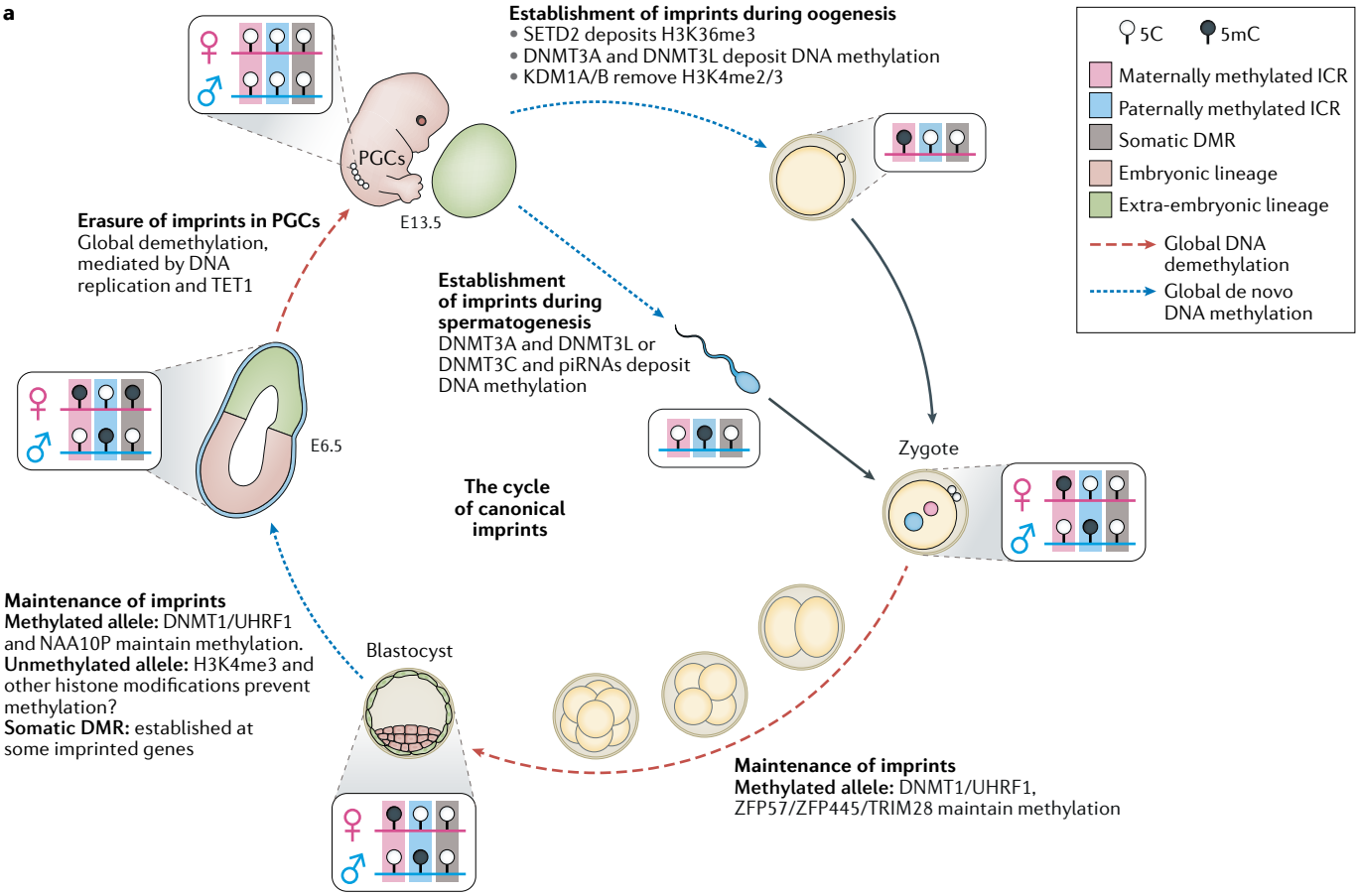
The insulator model of imprinted gene regulation. The insulator model of imprinted gene regulation is best exemplified by the *H19/Igf2* locus, which has been the subject of a series of elegant mouse genetic studies. *H19* is a lncRNA and is maternally expressed, whereas the insulin-like growth factor *Igf2* is paternally expressed[6,8,9]. The *H19/Igf2* ICR is located between the *H19* and *Igf2* genes and is methylated on the paternal allele but unmethylated on the maternal allele[49,50] (FIG. 1b). Deletion of the paternally inherited *H19/Igf2* ICR causes derepression of paternal *H19* and reduced levels of *Igf2*, whereas deletion of the maternally transmitted ICR leads to activation of maternal *Igf2* and repression of *H19* expression[51]. The key to imprinting regulation by the *H19/Igf2* ICR is the DNA methylation-sensitive CCCTC-binding factor (CTCF), which binds only to the unmethylated maternal ICR[52,53]. On the maternal allele, CTCF acts as an insulator and blocks interactions between the *Igf2* promoter and the downstream shared enhancers, preventing *Igf2* expression[52,53]. In addition, CTCF binding facilitates initiation of *H19* expression and prevents ectopic DNA methylation on the unmethylated maternal ICR[54]. Maternal inheritance of an *H19/Igf2* ICR that contains mutated CTCF binding sites abolishes maternal CTCF binding and causes DNA hypermethylation on the maternal allele, preventing *H19* expression[54]. On the paternal allele, however, DNA methylation at the ICR prevents CTCF binding, which allows the enhancers to interact with the *Igf2* promoter to activate *Igf2* expression[52,53]. In addition, DNA

Box 2 | PRC1 and PRC2

Polycomb repressive complex 1 (PRC1) and PRC2 are multisubunit protein complexes that mediate transcriptional repression, mainly by altering chromatin activity[137]. The catalytic core of PRC1 contains one of two E3 ubiquitin ligases, RING1A or RING1B, and one of six PCGF proteins (PCGF1–PCGF6)[157]. PRC1 deposits monoubiquitin to lysine 119 on histone H2A (H2AK119Ub)[158,159]. PRC1 can be subdivided into canonical PRC1 (cPRC1) and variant PRC1 (vPRC1) based on their distinct accessory subunits. The cPRC1 is composed of either PCGF2 or PCGF4 and one of the CBX subunits that can recognize trimethylation of histone H3 on lysine 27 (H3K27me3)[157,160,161]. By contrast, vPRC1 can utilize any of the six PCGF proteins but incorporates either YAF2 or RYBP instead of the CBX subunit[157,162,163]. Therefore, unlike cPRC1, vPRC1 cannot recognize H3K27me3.

The core subunits of PRC2 include one of two histone methyltransferases, EZH1 or EZH2, and the regulatory subunits EED, SUZ12 and either RBAP46 or RBAP48. PRC2 is responsible for monomethylation, dimethylation and trimethylation at lysine 27 on histone H3 (H3K27me1/2/3)[161,164–166]. PRC2 can be further divided into PRC2.1 and PRC2.2. PRC2.1 associates with the PCL1, PCL2 or PCL3 subunits that are known to bind CpG islands[167], whereas PRC2.2 contains AEBP2 and JARID2; JARID2 recognizes H2AK119Ub deposited by PRC1 (REF.[168]).

How PRC1 and PRC2 are recruited to their specific targets and how they exert transcriptional silencing is not fully understood. In the extensively studied mouse embryonic stem (ES) cell model, vPRC1, but not cPRC1 or PRC2, mediates transcriptional silencing of the majority of Polycomb group (PcG) protein targets[169]. However, it should be noted that PRC function is context-dependent as PRC2 and cPRC1 are critical in other processes[170–172]. In mouse ES cells, a compelling model for PRC recruitment is that vPRC1 is first recruited to PcG targets where it deposits H2AK119Ub, which then serves as a docking site for JARID2-mediated recruitment of PRC2.2 (REFS[168,173]). Subsequently, the chromo domain of CBX may bind to PRC2-deposited H3K27me3 to recruit cPRC1 (REF.[161]). Whether this 'vPRC1–PRC2–cPRC1' model applies to other systems remains to be determined. For more detailed information on PRC1 and PRC2 regulation and function, readers may refer to recent reviews[137,174].



methylation at the ICR spreads into the *H19* promoter to silence paternal *H19* expression[51].

The differential access of *H19* and *Igf2* to the shared downstream enhancers indicates distinct three-dimensional conformations between parental alleles at this locus. Using circular chromosome conformation capture with high-throughput sequencing (4C-seq) and DNA fluorescence in situ hybridization (FISH), a recent study indicated that, in addition to allelic CTCF binding at the ICR, biallelic CTCF binding

◀ Fig. 1 | **Germline inherited DNA methylation governs canonical imprinting. a** | During oogenesis, transcription across imprinting control regions (ICRs) recruits the histone methylase SETD2 to deposit trimethylation of histone H3 at lysine 36 (H3K36me3), which then guides the de novo DNA methyltransferases DNMT3A and DNMT3L to establish DNA methylation. Removal of H3K4me2/3 by the demethylases KDM1A/1B is also required for de novo DNA methylation. During spermatogenesis, DNA methylation is dependent on either DNMT3A and DNMT3L (for example, at the *H19/Igf2* and *Gtl2/Dlk1* ICRs) or DNMT3C and the piwi-interacting RNA (piRNA) pathway (for example, at the *Rasgrf1* ICR). Differential DNA methylation at ICRs is protected from global DNA demethylation during pre-implantation development by Krüppel-associated box (KRAB)-containing zinc finger protein 57 (ZFP57), ZFP445 and TRIM28, which bind to the methylated ICRs and recruit the maintenance methyltransferase DNMT1 and its cofactor UHRF1. *N*-α-acetyltransferase 10 protein (NAA10P) is also required to facilitate binding of DNMT1 to the methylated allele for imprinting maintenance. During implantation, the presence of active histone marks, such as H3K4me3 (and possibly others), may prevent unmethylated ICRs from gaining DNA methylation in the wave of global de novo methylation. Secondary allelic DNA methylation (that is, somatic differentially methylated regions (DMRs)) is established at some imprinted genes during this period. To reset imprints for the next generation, allelic DNA methylation at ICRs is erased in primordial germ cells (PGCs). This demethylation process is mainly mediated by passive dilution and ten–eleven translocation 1 (TET1)-mediated oxidation of 5-methylcytosine (5mC) at ICRs. **b** | The insulator model of imprinted gene regulation is illustrated by the *H19/Igf2* cluster. At this locus, the ICR for the long non-coding RNA (lncRNA) *H19* is paternally DNA methylated. In the conceptus, DNA methylation extends to the *H19* promoter to silence its transcription on the paternal allele. DNA methylation at the *H19* ICR also prevents binding of CCCTC-binding factor (CTCF) to the ICR, which results in formation of a topologically associated domain (TAD; blue triangle) that permits transcriptional activation of *Igf2* by the downstream enhancers (long double-headed arrow). On the maternal allele, CTCF binding to the unmethylated *H19* ICR forms two sub-TADs (pink triangles) that prevent the interaction between *Igf2* and the enhancers, and *Igf2* remains transcriptionally repressed. CTCF binding also facilitates the initiation of *H19* transcription by preventing gain of DNA methylation on the maternal allele. **c** | The lncRNA model of imprinted gene regulation is illustrated by the *Kcnq1* cluster. Here, the ICR (also known as KvDMR1) serves as the promoter for the lncRNA *Kcnq1ot1*. On the paternal allele, the unmethylated KvDMR1 allows *Kcnq1ot1* transcription, which recruits Polycomb repressive complexes 1 and 2 (PRC1/2) to deposit trimethylation to lysine 27 on histone H3 (H3K27me3) and monoubiquitin to lysine 119 on histone H2A (H2AK119Ub), respectively, to silence flanking protein coding genes. On the maternal allele, *Kcnq1ot1* is repressed by the methylated ICR, allowing expression of the flanking genes. At this cluster, whereas *Kcnq1ot1* and *Cdkn1c* exhibit ubiquitous imprinting, *Slc22a18* and *Tssc4* are only imprinted in placental lineages. The size and signal of H3K27me3 domains and the allelic gene expression are drawn based on publicly available data sets[20,67,70]. Not all genes in this cluster are shown for simplicity. E, embryonic day. 5C, 5-cytosine.

to sites that flank the *H19/Igf2* locus is also involved in modulating allelic chromatin looping in mouse ES cells[55]. Specifically, the biallelic CTCF binding correlates with a topologically associated domain (TAD) that is common to both alleles. However, on the maternal allele, the additional CTCF binding at the *H19/Igf2* ICR contributes to a sub-TAD, which can override the higher-level TAD and restrict the interaction between *Igf2* and the enhancers (FIG. 1b). It remains to be shown whether this allelic TAD model is universal for other imprinted clusters.

The lncRNA model of imprinted gene regulation. One of the best-characterized imprinted clusters that illustrates the lncRNA model of gene regulation is the *Kcnq1* imprinted cluster. The *Kcnq1* ICR, known as KvDMR1, is unmethylated on the paternal allele and methylated on the maternal allele[56,57] (FIG. 1c). KvDMR1 contains a promoter for the paternally expressed lncRNA *Kcnq1ot1*, which recruits repressive histone modifications H3K27me3 and H3K9me2 to silence ten flanking maternally expressed protein-coding genes, including *Cdkn1c*, *Slc22a18* and *Tssc4* (REFS[58–62]). On the maternal allele, however, DNA methylation of KvDMR1 prevents *Kcnq1ot1* expression, thereby allowing the transcription of flanking genes. Deletion of the *Kcnq1ot1* promoter or premature termination of the lncRNA on the paternal allele causes derepression of the neighbouring protein coding genes in mouse embryos[63,64]. By contrast, maternal transmission of the KvDMR1 deletion has no effect on imprinted regulation at this cluster[63,64].

Although the role of *Kcnq1ot1* in regulating imprinted gene expression is well established, how it recruits epigenetic modifiers and induces chromatin changes remain unknown. It is also unclear how *Kcnq1ot1* mediates gene silencing in a tissue-specific and stage-specific manner. For example, although *Kcnq1ot1* is ubiquitously imprinted, *Slc22a18* and *Tssc4* are imprinted only in the placenta and not in the embryo. Evidence from mouse trophoblast stem cells indicates that the stability and abundance of lncRNAs seem to be critical for determining the level of H3K27me3 enrichment at imprinted loci[65] as overexpression or knockdown of another imprinted lncRNA, *Airn*, in mouse trophoblast stem cells causes enhanced or reduced H3K27me3 levels at the imprinted cluster, respectively[65]. In addition, genomic structures (such as DNA loops and TADs) and DNA sequences (such as CpG islands) also seem to be involved in shaping the H3K27me3 domains[65]. Therefore, the capacity of lncRNAs to induce gene silencing can be influenced by complex factors and their variable activities in different cell lineages may explain how lncRNAs can mediated tissue-specific and stage-specific imprinting.

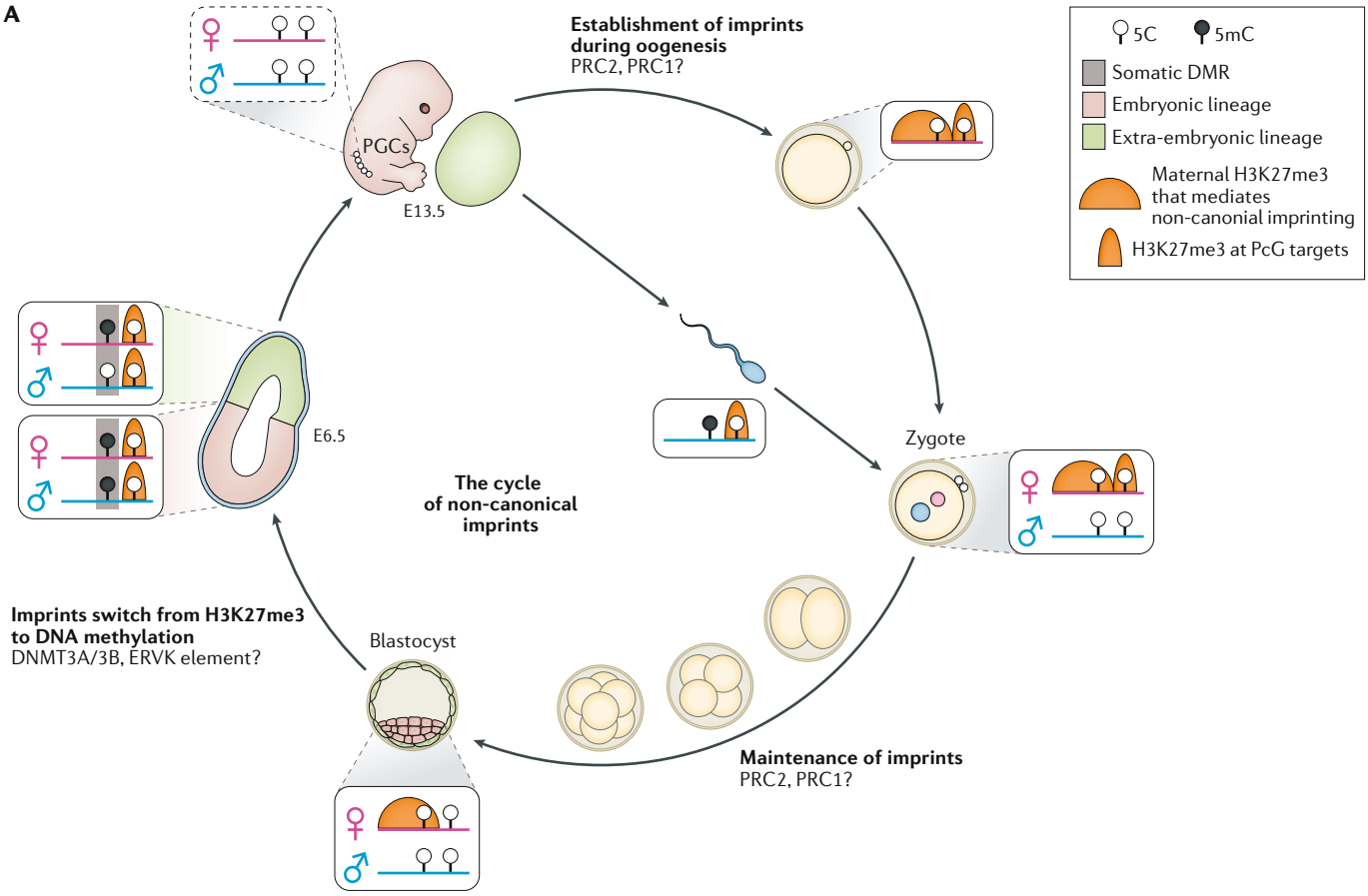
Mechanisms of non-canonical imprinting

Oocyte H3K27me3 and non-canonical imprinting. Although the very different epigenetic landscapes of the sperm and the egg become largely equalized during pre-implantation development, allelic analysis of DNase I hypersensitivity sites (DHSs) in pre-implantation mouse embryos has revealed that known ICRs exhibit differential chromatin accessibility, with the hypomethylated allele showing a higher DHS signal[66]. In addition to these known ICRs, a substantial number of paternal allele-specific DHSs (Ps-DHSs) were detected that are hypomethylated on both alleles, indicating that mechanisms other than DNA methylation determine the allele-specificity of these Ps-DHSs in early embryos[20]. Notably, some of these Ps-DHSs are associated with paternally expressed genes known to be independent of oocyte-derived DNA methylation, such as *Gab1*, *Sfmbt2* and *Slc38a4* (REFS[14,15]). Further analyses indicated that the Ps-DHSs harbour maternal allele-specific H3K27me3 that is inherited from oocytes[67], suggesting that maternal H3K27me3 may reduce chromatin accessibility of the corresponding regions on the maternal allele[20]. Acute depletion of H3K27me3 in mouse pre-implantation embryos by overexpressing the demethylase KDM6B causes biallelic DHSs and gene expression at these loci, demonstrating that maternally inherited H3K27me3 contributes to the Ps-DHSs and paternal allele-specific gene expression[20]. Taken together, these observations suggest that oocyte H3K27me3 can serve as a primary epigenetic mark for imprinted gene expression (FIG. 2).

4C-seq
A sequencing-based method that allows unbiased detection of all genomic regions that interact with a genomic region of interest.

Topologically associated domain
(TAD). A major form of chromatin organization that represents genomic regions with high frequencies of self-interacting events.

CpG islands
Genomic regions with a high density of CpG dinucleotides. In mammalian genomes, CpG islands usually extend from 200 bp to a few kilobase pairs.


A
Establishment of imprints
during oogenesis
PRC2, PRC1?
PGCs
E13.5
5C
5mC
Somatic DMR
Embryonic lineage
Extra-embryonic lineage
Maternal H3K27me3
that mediates
non-canonial imprinting
H3K27me3 at PcG targets
E6.5
The cycle
of non-canonical
imprints
Zygote
Imprints switch from H3K27me3
to DNA methylation
DNMT3A/3B, ERVK element?
Blastocyst
Maintenance of imprints
PRC2, PRC1?

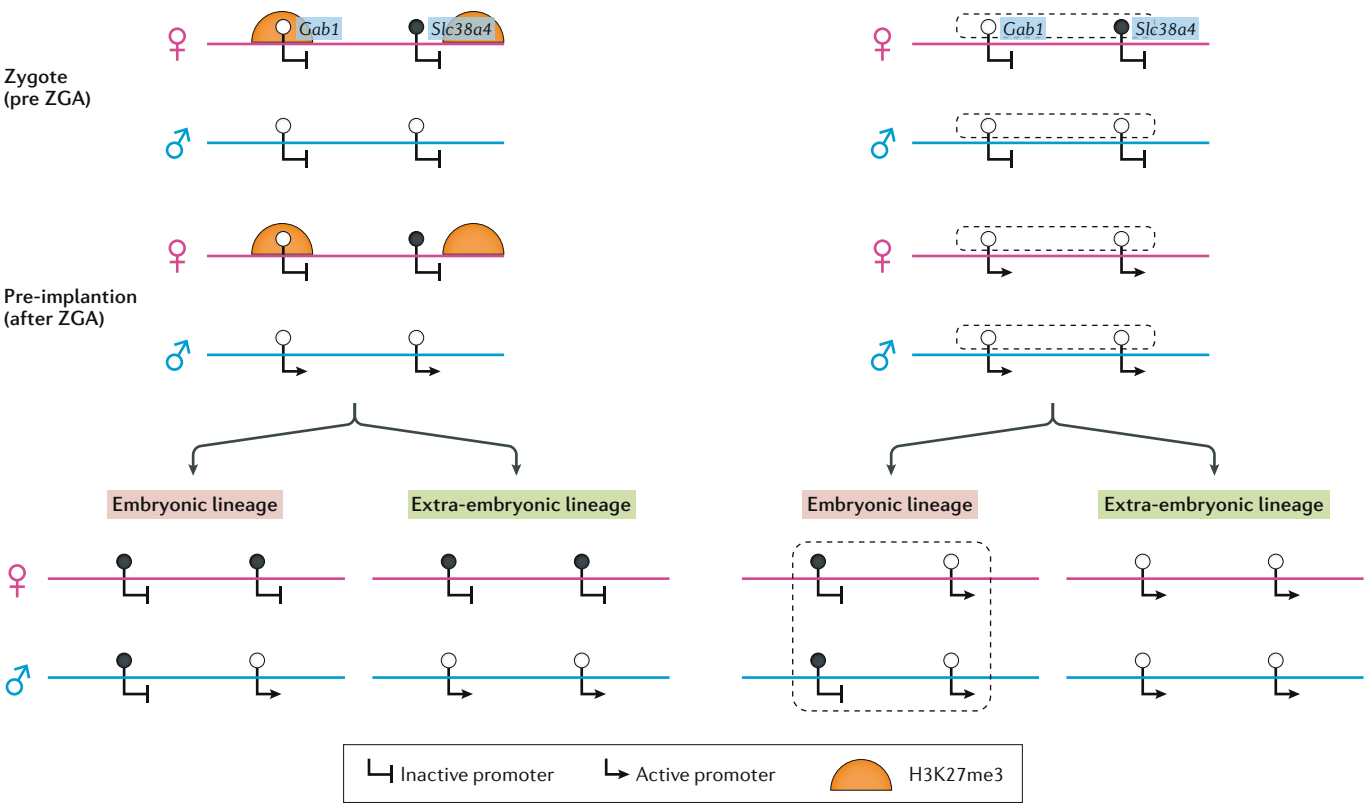

Ba Wild type
Bb Eed maternal knockout
Zygote
(pre ZGA)
Pre-implantion
(after ZGA)
Gab1
Slc38a4
Embryonic lineage
Extra-embryonic lineage
Inactive promoter
Active promoter
H3K27me3

Fig. 2 | **Oocyte inherited H3K27me3 initiates non-canonical imprinting. A** | The dynamics of trimethylation of histone H3 on lysine 27 (H3K27me3) at non-canonical imprinting loci and at genomic targets of Polycomb group (PcG) proteins. Polycomb repressive complex 2 (PRC2) mediates H3K27me3 deposition during oogenesis; whether PRC1-mediated ubiquitination at lysine 119 on histone H2A (H2AK119ub) is involved in PRC2 function in oogenesis remains unknown. After fertilization, H3K27me3 at PcG targets is largely reprogrammed, but maternally inherited H3K27me3 at non-canonically imprinted loci is maintained during pre-implantation development and is responsible for silencing the maternal allele of these genes. After implantation, H3K27me3 is re-established at PcG targets whereas the maternal H3K27me3 that initiates non-canonical imprinting disappears in both the epiblast and the extra-embryonic ectoderm (EXE). The maintenance of some non-canonical imprinting in the EXE depends on the acquisition of somatic differentially methylated regions (DMRs) during implantation via the DNA methyltransferases DNMT3A and DNMT3B. Active endogenous virus K (ERVK) long terminal repeats in the somatic DMRs may have a role in the maintenance of non-canonical imprinting in the placental lineage. In the epiblast, both alleles at non-canonically imprinted loci are repressed by DNA methylation. In primordial germ cells (PGCs), DNA methylation at non-canonical imprinting loci is expected to be erased (dashed line) during the wave of global DNA demethylation mediated by ten–eleven translocation 1 (TET1) and DNA replication. **B** | Typical dynamics of H3K27me3 and DNA methylation at non-canonically imprinted loci (represented by *Gab1*) in wild-type mice (part **Ba**) and *Eed* maternal-knockout mice (part **Bb**). Oocyte-specific depletion of EED, an essential subunit of PRC2, causes loss of H3K27me3 in mature oocytes. Embryos that develop from *Eed*-null oocytes (that is, *Eed* maternal-knockout embryos) lack maternally provided H3K27me3 and lose non-canonical imprinting in both pre-implantation embryos and extra-embryonic cells. Furthermore, somatic DMRs are unmethylated on both alleles in the EXE of these embryos. The *Slc38a4* locus differs from other non-canonically imprinted loci because its DMR is established during oogenesis. However, it becomes hypomethylated in *Eed* maternal-knockout EXE, suggesting that maternal H3K27me3 is essential to maintain differential DNA methylation at this locus. The DNA methylation status of the loci in embryonic lineages of *Eed* maternal-knockout has not been analysed, but predicted patterns are included and indicated by dashed boxes. 5C, 5-cytosine; 5mC, 5-methylcytosine; E, embryonic day; ZGA, zygotic genome activation.

A few differences should be noted between canonical and non-canonical imprinting mechanisms. For canonical imprinting, the imprints (that is, DNA methylation) that govern allele-specific gene expression can be inherited from either oocytes or sperm cells[11] (FIG. 1a). However, the H3K27me3 that mediates non-canonical imprinting is only inherited from oocytes because most sperm DNA is packaged by protamines rather than histones and the minor amount of paternal H3K27me3 is completely reprogrammed at fertilization[67]. The oocyte-inherited H3K27me3 also differs from the allelic H3K27me3 implicated in canonical imprinting, which is secondary to the germline DMRs[68]. Lastly, although canonical imprinting is maintained in both embryonic and extra-embryonic lineages, most non-canonical imprinting is transient, with only some genes important for placental development maintaining their imprinted expression in extra-embryonic cells[20]. As non-canonical imprinting is not maintained in the epiblast lineage that gives rise to germ cells, non-canonical imprints do not need to be erased in primordial germ cells (FIG. 2A); by contrast, canonical imprints must be erased in primordial germ cells to reset imprinting for the next generation (FIG. 1a).

Establishment of non-canonical imprinting during oocyte growth. Analogous to DNA methylation, most H3K27me3 is deposited by PRC2 during oocyte growth[67] (FIG. 2A; BOX 2). However, H3K27me3 is generally anti-correlated with DNA methylation and H3K36me3 in oocytes[33]. In addition, unlike somatic cells, H3K27me3 in oocytes is present not only at the classic genomic targets of Polycomb group (PcG) proteins, such as developmental gene promoters, but also at non-transcribed regions that can be several megabases in size[67,69]. This acquisition of H3K27me3 during oogenesis is independent of DNA methylation as H3K27me3 domains are largely unaffected in *Dnmt3l*-knockout mice[33]. Furthermore, embryos derived from DNA methylation-deficient oocytes maintain intact maternal H3K27me3 domains, explaining why these embryos show normal non-canonical imprinting but abnormal canonical imprinting[70,71]. Conversely, DNA methylation acquisition in oocytes is independent of H3K27me3 as embryos from H3K27me3-deficient oocytes (that is, oocytes from conditional PRC2 null mice) exhibit proper canonical, but not non-canonical, imprinting[72]. Therefore, canonical and non-canonical imprints are independently established during oogenesis.

One intriguing question is how genomic regions are selected for DNA methylation or H3K27me3 during oogenesis, which will determine whether a gene, if imprinted, will be regulated by canonical or non-canonical imprinting. It is likely that H3K27me3 promiscuously marks transcriptionally inactive regions during oogenesis and is antagonized by H3K36me3 at the actively transcribed regions. In support of this notion, H3K27me3 can ectopically occupy regions that are normally marked by H3K36me3 in *Setd2*-null oocytes[33]. However, H3K36me3 cannot be the sole mechanism that defines H3K27me3 boundaries because not all non-transcribed regions in oocytes are marked by H3K27me3 (REF.[33]). Given that disruption of PRC1, a repressive complex that ubiquitinylates lysine 119 of histone H2A (H2AK119ub) (BOX 2), causes more severe defects in oogenesis than disruption of PRC2 (REFS[72–75]), it is likely that PRC1 recruitment may be upstream of H3K27me3 acquisition during oocyte development. Indeed, KDM2B (also known as FBXL10), an H3K36 demethylase[76,77], binds to unmethylated CpG islands and recruits the PRC1 member RING1B to mediate H2AK119ub deposition in mouse ES cells[78–80]. In addition, KDM2B is responsible for protecting genes bound by PRC1 and PRC2 from ectopic de novo DNA methylation in mouse ES cells[81]. However, a role for KDM2B in recruiting PRC1 and antagonizing DNA methylation during oogenesis remains to be demonstrated.

Maintenance of non-canonical imprinting during development. In contrast to DNA methylation at ICRs, which is generally maintained throughout development, the maternally inherited H3K27me3 domains that mediate non-canonical imprinting are only temporarily maintained in pre-implantation embryos[70,71] (FIG. 2A). This maintenance depends on the genomic context. For example, H3K27me3 profiling in mouse early embryos indicates that H3K27me3 at typical PcG targets is erased by the late one-cell stage and then re-established at implantation[67]. Notably, RNA sequencing-based analyses revealed that PcG targets remain inactive even in the absence of H3K27me3 (REF.[67]), suggesting that either transcription factors required for gene activation are

DNase I hypersensitivity sites
(DHSs). Chromatin regions that are less condensed and more sensitive to DNase I enzyme-mediated cleavage than other regions.

Protamines
Basic proteins that replace histones in mature sperm cells and are involved in sperm DNA condensation.

Epiblast
One of the two lineages that are derived from the inner cell mass (ICM) of the blastocyst. The epiblast contributes to all three primary germ layers. The primitive endoderm, the other lineage derived from the ICM, contributes to the yolk sac.

Parthenogenetic activation
A procedure that mimics sperm stimuli to trigger egg activation to initiate embryo development without the contribution of the paternal genome.

Meiotic sex chromosome inactivation
The process of silencing X and Y chromosomes during the meiotic phase of spermatogenesis.

not present or additional repressive epigenetic mechanisms compensate for the loss of H3K27me3 to silence PcG targets in early embryos. Nonetheless, maternally inherited H3K27me3 is essential at this developmental stage to preserve the parental allele-specificity at non-canonically imprinted loci, as acute depletion of H3K27me3 by overexpressing the demethylase KDM6B in mouse pre-implantation embryos results in loss of imprinted expression of these genes[20].

Maternally inherited H3K27me3 diminishes during pre-implantation development and is largely absent after implantation[70,71] (FIG. 2), possibly explaining why most non-canonical imprinting is transient and not maintained beyond implantation[20]. However, the fact that some non-canonically imprinted genes do maintain their imprinted expression in the placental lineage suggests that an additional epigenetic modification takes over from H3K27me3 to repress maternal allele transcription at these loci. Analyses of allelic DNA methylome and H3K27me3 in pre-implantation embryos and post-implantation placental lineages revealed that although these genes lose their maternally inherited H3K27me3, they acquire DNA methylation (that is, somatic DMRs) specifically on the maternal allele to maintain imprinted expression in extra-embryonic cells[70,71] (FIG. 2Ba). Furthermore, the somatic DMR acquisition depends on the zygotic de novo DNA methyltransferases DNMT3A/3B, as DNMT3A/3B double-mutant embryos fail to acquire the somatic DMRs and show derepression of the maternal allele at the non-canonical imprinting loci[70]. It should be noted that this switch from a dependence on maternal H3K27me3 in pre-implantation embryos to allelic DNA methylation after implantation is the opposite of the placenta-specific canonical imprinting that occurs at the *Kcnq1* cluster, in which imprinted expression initially depends on allelic DNA methylation but then switches to H3K27me3 to maintain imprinting[59,61,62] (FIG. 1c).

It remains unclear how these few genes are selected to acquire somatic DMRs and maintain imprinted expression in the placenta. It has been observed that the non-canonically imprinted loci that preferentially acquire somatic DMRs overlap active endogenous retrovirus K (ERVK) long terminal repeats[71]. These ERVK long terminal repeats seem to have a role in maintaining non-canonical imprinting as disruption of the ERVK promoter at the *Gab1* locus caused weakened paternal gene expression bias, although DNA methylation at the promoter was not determined[71]. Furthermore, the somatic DMRs become hypermethylated on both alleles in the epiblast, which explains why non-canonical imprinting is not maintained in this lineage[71]. However, it remains unclear how insertions of ERVK long terminal repeats, but not other repeat types, can maintain imprinting and what placenta-specific transcription factors protect the paternal allele from global de novo DNA methylation at implantation. In addition, it is not known why *Sfmbt2* retains allelic H3K27me3 in early post-implantation development and acquires somatic DMRs later than other non-canonically imprinted loci[70]. Nonetheless, the switch from allelic H3K27me3 to allelic DNA methylation indicates that transient allelic histone modifications in early embryos can have long-term consequences in mouse embryonic development.

Mechanisms of imprinted XCI

Xist imprinting by oocyte H3K27me3. What controls imprinted XCI in mouse pre-implantation embryos has been a long-standing question. In mouse embryos generated by nuclear transfer using either non-growing oocytes or fully grown oocytes, the X chromosome derived from the non-growing oocyte, which resembles a normal Xp, is preferentially silenced[82]. This observation suggests that a maternal imprint is established during oocyte growth to prevent the maternal X chromosome (Xm) from being silenced in early embryos. Consistent with this hypothesis, *Xist* initially remains silenced until the morula stage in diploid bi-maternal mouse embryos generated by parthenogenetic activation[83]. By contrast, it has also been proposed that Xp could inherit a pre-inactive state from the male germ line, in which meiotic sex chromosome inactivation occurs[84]. Although these two possibilities are not mutually exclusive, results from further studies argue against the pre-inactivation of Xp prior to imprinted XCI. An *Xist* transgene on autosomes (which do not undergo meiotic sex chromosome inactivation in the male germ line) can still cause imprinted in *cis* inactivation when paternally inherited[85], indicating that meiotic sex chromosome inactivation is not required for imprinted XCI. In addition, single-cell RNA sequencing of mouse pre-implantation embryos reveals that Xp silencing begins at the four-cell stage instead of being pre-activated[16]. Furthermore, mouse embryos in which the paternal allele of *Xist* has been deleted cannot initiate Xp inactivation[16]. These results suggest that Xp inactivation occurs de novo after zygotic genome activation and is fully dependent on expression of *Xist* from the paternal allele[16]. By contrast, the maternal allele of *Xist* remains repressed in early embryos to keep Xm active (FIG. 3A).

With this in mind, what is the epigenetic imprint that represses maternal *Xist*? Recent studies in early mouse embryos indicate that oocyte-inherited H3K27me3 silences maternal *Xist* expression, whereas paternally expressed *Xist* silences Xp in *cis*[19,72,86] (FIG. 3Ba). This conclusion is supported by several pieces of evidence. Firstly, H3K27me3, but not DNA methylation, is gradually established at the *Xist* locus during oocyte growth and maternally inherited H3K27me3 is maintained until the blastocyst stage[19]. Secondly, acute depletion of H3K27me3 by overexpressing the histone demethylase KDM6B causes loss of maternal H3K27me3 at the *Xist* locus, ectopic maternal *Xist* expression and aberrant maternal XCI in both male and female mouse embryos[19]. Lastly, depletion of EED, a core PRC2 subunit, in oocytes causes loss of maternal H3K27me3, ectopic maternal *Xist* expression and aberrant maternal XCI in embryos of both sexes[72,86] (FIG. 3Bb). Therefore, after fertilization, the oocyte-inherited H3K27me3 silences maternal *Xist* and protects Xm from being inactivated. By contrast, *Xist* on the paternal allele is transcriptionally accessible and is expressed to induce Xp silencing in *cis*[16] (FIG. 3B).

In addition to H3K27me3, maternal H3K9me3 has been proposed to prevent activation of maternal *Xist*

in early embryos because acute depletion of H3K9me3 by overexpressing the H3K9me3 demethylase KDM4B caused a partial derepression of *Xist* in diploid parthogenetic four-cell embryos[87]. However, this result is not reproducible in bi-parental embryos generated by in vitro fertilization[19]. Importantly, neither the *Xist* promoter nor the gene body is enriched for H3K9me3 in fully grown oocytes[88]. Therefore, oocyte H3K9me3 may not be the imprint that suppresses maternal *Xist* in early embryos.

Reactivation of Xp in the inner cell mass. *Xist*-induced paternal XCI is complete by around the 32-cell stage (approximately embryonic day (E) 3.0), and Xp then initiates reactivation in the inner cell mass (ICM) of early blastocysts (approximately E3.5)[89,90]. Single-cell RNA sequencing-based analyses of early and mid ICM revealed that X-linked genes undergo reactivation at different kinetics, with some genes reactivating early at E3.5 and others only fully reactivating at E4.0–4.5 when the epiblast has formed[91]. Notably, the early-reactivated genes in the ICM undergo re-silencing in the ICM-derived primitive endoderm, thus maintaining imprinted XCI in the primitive endoderm that will develop into the yolk sac[91]. Meanwhile, in the ICM-derived epiblast, Xp is fully reactivated and random XCI occurs shortly after.

What controls Xp reactivation remains largely unknown. Initiation of Xp reactivation has been linked to *Xist* repression by pluripotency factors expressed in the ICM[92,93]. In naive female mouse ES cells, in which both Xm and Xp are active, pluripotent factors NANOG, OCT4 and SOX2 bind to *Xist* intron 1 and repress *Xist* transcription[94]. In addition, loss of PRDM14, a guardian of naive pluripotency[95], causes defective Xp reactivation in mouse blastocysts[92]. Mechanistic studies in mouse ES cells suggest that PRDM14 represses *Xist* by directly binding to *Xist* intron 1 and indirectly silencing the *Xist* activator RNF12 (REF.[92]). Therefore, the relationships between the pluripotent factors and *Xist* seem to be complex. To what extent the insights obtained in mouse ES cells are applicable to Xp reactivation in embryos remains to be determined. It is also intriguing that some X-linked genes initiate reactivation before the loss of *Xist* coating and repressive H3K27me3 in the ICM, two cytological hallmarks of Xp reactivation[91,96]. It is unclear how the early reactivation is initiated, although transcription factors such as MYC have been proposed to play a part in driving transcriptional activation of these genes in early blastocysts[91]. On the other hand, erasure of H3K27me3 by histone demethylase KDM6A (also known as UTX) contributes to the transcription of late-reactivated genes[91]. How removal of additional repressive chromatin marks associated with paternal XCI, such as H3K9me2 and H2AK119ub, contributes to Xp reactivation remains to be studied.

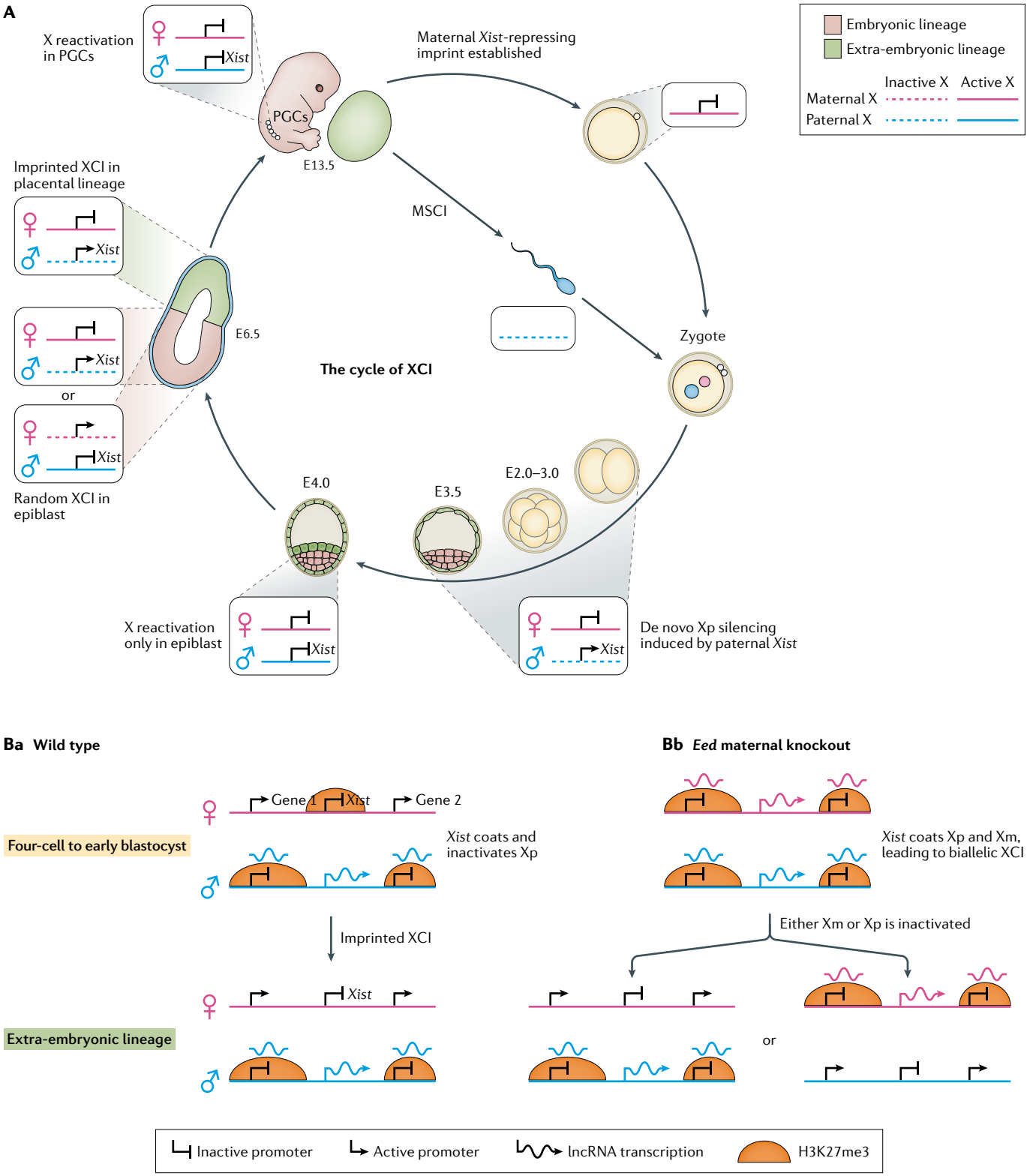
Maintenance of imprinted Xist in the placenta. Following Xp reactivation, random XCI occurs in the embryo proper with *Xist* expressed from either Xm or Xp. By contrast, *Xist* imprinting is maintained in extra-embryonic lineages by the maternally expressed lncRNA *Tsix*, which is transcribed in an antisense direction from the *Xist* locus and represses *Xist* transcription in *cis*[97–99]. When a *Tsix*-knockout allele is maternally inherited, maternal *Xist* is ectopically expressed in extra-embryonic lineages, leading to aberrant maternal XCI and embryonic lethality[98,99]. Given the essential role of *Tsix* in *Xist* imprinting and the absence of H3K27me3 from the *Xist* region after implantation, it is likely that oocyte H3K27me3-mediated maternal repression of *Xist* is replaced by *Tsix*-mediated repression in early post-implantation development. It should be noted that *Tsix* does not initiate maternal *Xist* silencing because it is not expressed until the morula stage[99,100].

As occurs at autosomal non-canonically imprinted loci, the *Xist* promoter becomes differentially methylated in extra-embryonic lineages after implantation[101]. However, disruption of DNMT1 does not affect the imprinted expression of an X-linked reporter gene in extra-embryonic lineages, indicating that this DNA methyltransferase is not responsible for maintaining *Xist* imprinting in this lineage[102]. Furthermore, simultaneous disruption of both DNMT3A and DNMT3B, de novo DNA methyltransferases that potentially compensate for DNMT1 in *Dnmt1* mutants[102], does not affect *Xist* coating and only one X chromosome is inactivated[103]. These observations imply that, unlike autosomal non-canonical imprinting, *Xist* maintains monoallelic expression in the absence of de novo DNA methylation in extra-embryonic lineages.

Another notable difference between autosomal non-canonical imprinting and *Xist* imprinting is the developmental consequences of loss of imprinting in *Eed* maternal-knockout embryos. For non-canonical imprinting on autosomes, loss of maternal EED causes ectopic expression of the maternal allele in both pre-implantation embryos and placental lineages[72] (FIG. 2Bb). By contrast, in *Eed* maternal-knockout embryos, both X chromosomes in females and the sole X chromosome in males are silenced at the morula stage owing to ectopic expression of maternal *Xist*, but aberrant *Xist* imprinting and XCI are resolved at the blastocyst stage[72,86]. Interestingly, *Xist* and XCI are no longer imprinted but either Xm or Xp is inactivated in placental lineages of *Eed* maternal-knockout female embryos[72] (FIG. 3Bb). Analogously, androgenetic XpXp embryos, which do not have oocyte H3K27me3 to repress either *Xist* allele, also show biallelic XCI in early embryos but only one X chromosome is randomly inactivated in later development[104]. The correction of abnormal *Xist* imprinting in *Eed* maternal-knockout and XpXp embryos indicates that an X chromosome counting mechanism exists in early embryos to ensure that a single X chromosome is active regardless of its parental origin. However, aberrant maternal XCI in pre-implantation embryos already causes downregulation of X-linked genes[72] and may contribute to the developmental defects observed in these mouse models.

Non-canonical imprinting in the placenta

As the majority of canonically imprinted genes are expressed prenatally, their functions have been best characterized in fetal development and placental biology[3]. Recently, roles for genomic imprinting in neuronal



processes and adult behaviours have been reported[23], which is consistent with the brain, along with the placenta, being one of the organs with the highest number of imprinted genes[105]. The physiological functions of canonical imprinting have been thoroughly reviewed elsewhere[2,3,23,106], so here we focus on the role of non-canonical imprinting in development.

Most non-canonical imprinting is transient in pre-implantation embryos, with several genes maintaining imprinted expression in the placenta[20]. The function of the transient non-canonical imprinting is unclear and whether it has any long-term effects on development remains to be shown. However, evidence from canonical imprinting indicates that transient imprinting in

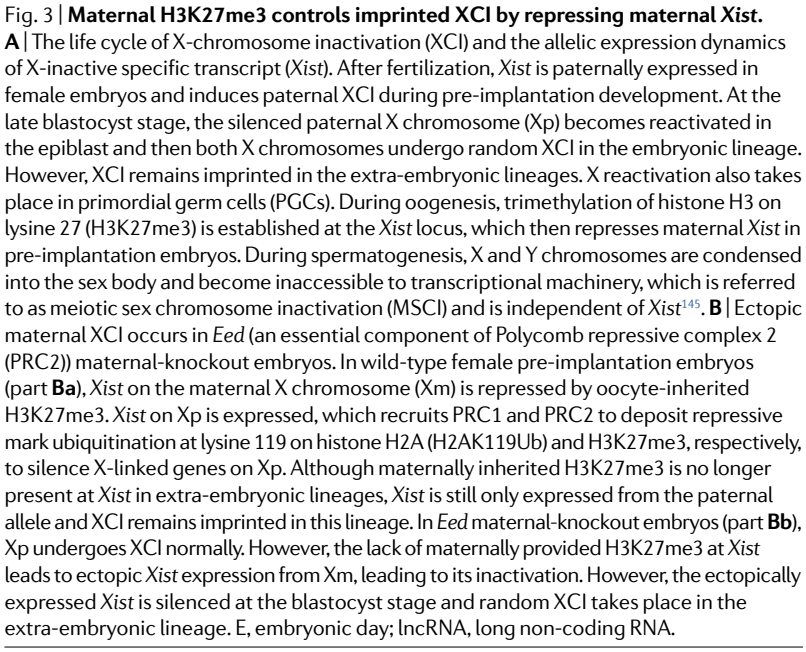


Fig. 3 | **Maternal H3K27me3 controls imprinted XCI by repressing maternal *Xist*.** **A** | The life cycle of X-chromosome inactivation (XCI) and the allelic expression dynamics of X-inactive specific transcript (*Xist*). After fertilization, *Xist* is paternally expressed in female embryos and induces paternal XCI during pre-implantation development. At the late blastocyst stage, the silenced paternal X chromosome (Xp) becomes reactivated in the epiblast and then both X chromosomes undergo random XCI in the embryonic lineage. However, XCI remains imprinted in the extra-embryonic lineages. X reactivation also takes place in primordial germ cells (PGCs). During oogenesis, trimethylation of histone H3 on lysine 27 (H3K27me3) is established at the *Xist* locus, which then represses maternal *Xist* in pre-implantation embryos. During spermatogenesis, X and Y chromosomes are condensed into the sex body and become inaccessible to transcriptional machinery, which is referred to as meiotic sex chromosome inactivation (MSCI) and is independent of *Xist*[145]. **B** | Ectopic maternal XCI occurs in *Eed* (an essential component of Polycomb repressive complex 2 (PRC2)) maternal-knockout embryos. In wild-type female pre-implantation embryos (part **Ba**), *Xist* on the maternal X chromosome (Xm) is repressed by oocyte-inherited H3K27me3. *Xist* on Xp is expressed, which recruits PRC1 and PRC2 to deposit repressive mark ubiquitination at lysine 119 on histone H2A (H2AK119Ub) and H3K27me3, respectively, to silence X-linked genes on Xp. Although maternally inherited H3K27me3 is no longer present at *Xist* in extra-embryonic lineages, *Xist* is still only expressed from the paternal allele and XCI remains imprinted in this lineage. In *Eed* maternal-knockout embryos (part **Bb**), Xp undergoes XCI normally. However, the lack of maternally provided H3K27me3 at *Xist* leads to ectopic *Xist* expression from Xm, leading to its inactivation. However, the ectopically expressed *Xist* is silenced at the blastocyst stage and random XCI takes place in the extra-embryonic lineage. E, embryonic day; lncRNA, long non-coding RNA.

early embryos can regulate somatic DMR acquisition, which affects later physiological processes[107]. Specifically, a transient maternal germline DMR (the *Gpr1/Zdbf2* DMR) causes paternal allele-specific expression of the lncRNA *Liz* in early mouse embryos[108]. *Liz* is required to promote a paternally methylated intergenic somatic DMR (~10 kb upstream of *Zdbf2*), which can antagonize H3K27me3-mediated repression of *Zdbf2* (REF.[107]). Mouse embryos that lack early transient *Liz* expression fail to acquire the somatic DMR and to activate *Zdbf2* in the postnatal brain. These animals also show ~20% body weight reduction through adult life[107]. Therefore, although some transient non-canonical imprinting may be a by-product of transient asymmetric parental H3K27me3 in early embryos, a functional role with lifelong consequences remains possible.

Of the non-canonical imprinted genes whose imprinting state is maintained in the placenta (TABLE 1), *Slc38a4*, *Sfmbt2* and *Gab1* are the best characterized. Knockout mouse models for each of these genes develop placenta hypoplasia and show lethality or sub-lethality[109–111]. For *Slc38a4* and *Sfmbt2*, placental development is defective only when the knockout allele is paternally inherited, consistent with imprinting and silencing of the maternal allele[109,110]. SLC38A4 is an amino acid transporter that is likely involved in transporting amino acids at the maternal–fetal interface[109], which is consistent with the placental hypoplasia, and the subsequent small-body phenotype, observed in mutant mice[110]. SFMBT2 is a mammalian homologue of the *Drosophila* PcG protein Sfmbt but its molecular function remains poorly characterized[112]. Paternal inheritance of a *Sfmbt2*-knockout allele results in embryonic lethality at mid-gestation due to severe placenta defects[110]. Intron 10 of *Sfmbt2* harbours one of the largest microRNA clusters in the mouse, which is imprinted like *Sfmbt2* (REF.[113]). Deletion of this microRNA cluster on the paternal allele severely impairs placental function and approximately one-third of the paternal knockout pups die around mid-gestation[113]. Therefore, *Sfmtb2* regulates placental development through both the SFMBT2 protein and the associated microRNA cluster[113]. Lastly, GAB1 functions as an adaptor protein downstream of tyrosine kinase signalling and *Gab1* homozygous mutant embryos die at late gestation and display developmental defects in the placenta and other organs, such as the heart and skin[111,114].

Although *Gab1*, *Sfmbt2* and *Slc38a4* are required for normal mouse placental development, it has not yet been demonstrated whether these genes need to be imprinted. The maternal alleles at all non-canonically imprinted loci are derepressed in *Eed* maternal-knockout embryos and this mouse model has various developmental defects, including embryonic sub-lethality, growth retardation at gastrulation and postnatal overgrowth[72,74]. However, these defects could be a combined effect of aberrant imprinted XCI, loss of non-canonical imprinting on autosomes and imprinting-independent functions related to maternal H3K27me3 depletion. Given that *Gab1*, *Sfmbt2* and *Slc38a4* mutant mice show placental hypoplasia, it is possible that biallelic expression of these genes may cause an enlarged placenta. Indeed, mouse embryos derived from somatic cell nuclear transfer (SCNT) always express *Gab1*, *Sfmbt2* and *Scl38a4* biallelically and show placenta hyperplasia[115]. However, mouse models with either biallelic expression or paternal duplication of the individual loci are needed to further clarify the role of autosomal non-canonical imprinting in placental development.

Aberrant imprinting and XCI in SCNT

SCNT is a technique by which a differentiated somatic cell nucleus is reprogrammed by an enucleated oocyte to acquire totipotency (FIG. 4a). SCNT makes possible not only reproductive cloning but also derivation of embryonic stem cells from cloned blastocysts[116]. Therefore, SCNT holds great potential for regenerative medicine and the agricultural industry. However, the efficiency of the process has remained low in the past 20 years since it was first used to successfully clone the first mammal[117]. Aberrant genomic imprinting and imprinted XCI are two of the major known barriers impeding post-implantation development of cloned animals[116].

Loss of canonical imprinting in SCNT embryos. The initial assessment of canonical imprinting in cloned mouse embryos revealed that SCNT only alters transcript abundance but not allelic expression of imprinted genes[118]. However, this study was based on the analyses of only a few imprinted genes. Later, a comprehensive RNA sequencing-based study indicated that canonical imprinting is stochastically disrupted in the brain and the placenta of cloned mice and the aberrant imprinting involves both loss of monoallelic gene expression and alterations of transcriptional abundance[15]. It should be noted that some of the imprinting errors in cloned embryos may not be solely caused by SCNT reprogramming as the assisted reproductive techniques (ARTs) used in SCNT, such as superovulation and embryo culture, are also known to induce epimutations[119].

Nonetheless, at least some imprinting errors, such as those seen at the *Gtl2/Dlk1* locus, are likely caused

Table 1 | **List of genes non-canonically imprinted in extra-embryonic cells**

Gene	Molecular function	Mouse knockout phenotype	Antisense imprinted lncRNA?	Germline DMR?	Somatic DMR?	DMR overlapped repeat element
Gab1	A docking protein involved in cell signalling	Embryonic lethality; placenta, heart and skin defects[111,114]	Yes, paternal	No	Yes, E6.5	ERVK:RLTR15
Sfmbt2	A Polycomb group protein	Embryonic lethality due to severe placenta defects[110]	Yes, paternal	No	Yes, E7.5	ERVK:RLTR11B
Slc38a4	An amino acid transporter	Placenta hypoplasia, reduced fetal weight, 20% survival rate[109]	No	Yes	NA[a]	ERVK:MLTR31F
Phf17	A cofactor involved in histone acetylation	NA	No	No	Yes, E6.5	ERVK:RLTR20C and RLTR31B
Smoc1	A matricellular protein involved in cell signalling	Perinatal lethality[144]	Yes, paternal	No	Yes, E6.5	ERVK:RLTR11B
Platr20	A lncRNA with unknown function	NA	Yes, paternal	No	Yes, E6.5	ERVK:RLTR15
Gm32885	A lncRNA with unknown function	NA	No	No	Yes, E6.5	ERVK:RLTR31A

DMR, differentially methylated region; E, embryonic day; ERVK, endogenous retrovirus K; lncRNA, long non-coding RNA; NA, not applicable; RLTR, retrotransposon long terminal repeat. [a]*Slc38a4* germline DMR maintenance requires maternal trimethylation of histone H3 on lysine 27 (H3K27me3) and zygotic de novo DNA methyltransferases DNMT3A/3B.

by SCNT as they are rarely observed in embryos generated with the use of ARTs[120]. It has been previously shown that one-third of SCNT embryos lose imprinting at the *Gtl2/Dlk1* locus. In these embryos, *Dlk1* becomes biallelically expressed and *Gtl2* becomes biallelically repressed[15] (FIG. 4b), and their loss of allele-specific expression is associated with the gain of DNA methylation on the normally unmethylated maternal ICR[15]. It remains unknown how such epimutations are caused by SCNT reprogramming. It is likely that ectopic gain of maternal DNA methylation occurs during the wave of global de novo DNA methylation at implantation as the *Gtl2/Dlk1* maternal ICR is still hypomethylated at the blastocyst stage of SCNT embryos[115]. Loss of imprinting at the *Gtl2/Dlk1* locus is strongly correlated with lethality of SCNT embryos[15], so understanding the mechanisms underlying this epimutation could uncover the means to mitigate its effects and thereby improve cloning efficiency.

Loss of non-canonical imprinting in SCNT embryos. Non-canonically imprinted genes always show biallelic expression in mouse SCNT pre-implantation embryos, placenta and the derived TSCs because somatic cells do not retain the primary imprint, the oocyte-derived H3K27me3 (REFS[15,115,121]) (FIG. 4c). Indeed, maternal-biased H3K27me3 domains that normally exist during pre-implantation development have been shown to be absent in SCNT morula-stage embryos[115]. Intriguingly, the germline DMR on the maternal allele of *Slc38a4* is maintained in SCNT donor cells, but all resulting embryos exhibit biallelic expression and loss of maternal-specific DNA methylation of this locus by the blastocyst stage[15] (FIG. 4c). This observation suggests that the *Slc38a4* germline DMR cannot mediate imprinting in pre-implantation embryos in the absence of maternally inherited H3K27me3. As noted above, *Gab1*-, *Slc38a4*- and *Sfmbt2*-knockout mice show placenta hypoplasia, and biallelic expression of these genes may contribute to the enlarged placenta observed in all cloned mouse embryos[115]. Whether using donor cells that are heterozygous for knockout alleles of all three of these genes can reverse the enlarged placenta phenotype of SCNT embryos remains to be shown.

Similar to non-canonical imprinting on autosomes, maternal H3K27me3 domains at the *Xist* locus also do not persist after implantation. Therefore, all SCNT-derived early embryos ectopically express *Xist* from the maternal allele, resulting in maternal XCI[122] (FIG. 4d). Remarkably, cloning efficiency (in terms of the live pup rate) can be increased by around tenfold by correcting *Xist* expression in SCNT embryos, either by using *Xist*-knockout donor cells or by knocking down *Xist* expression via small interfering RNA (siRNA) injection at the one-cell stage[122,123]. Correcting *Xist* expression in SCNT embryos both reverses the downregulation of X-linked genes owing to aberrant maternal XCI and reduces the number of differentially expressed genes on autosomes[122]. These observations suggest that abnormal XCI in SCNT embryos disturbs the expression of both autosomal and X-linked genes. Similarly, aberrant maternal XCI may also contribute to the embryonic sub-lethality observed in the *Eed* maternal-knockout mouse model[72,74,86].

Conservation of non-canonical imprinting

Non-canonical imprinting is not conserved in humans. In general, genomic imprinting in mice and humans is less conserved in the placenta than in the fetus[124,125]. The oocyte H3K27me3-controlled mouse imprinted genes that have human orthologues, such as *Gab1* and *Sfmbt2*, are also not imprinted in the human placenta[14]. Recently, comprehensive profiling of histone modifications during human early embryonic development revealed that H3K27me3 is globally depleted on both parental alleles at the eight-cell stage[126]. These results indicate that oocyte-derived H3K27me3 in humans is unable to preserve allele-specificity throughout development and is therefore unlikely to serve as an imprinting mark[126]. Although oocyte H3K27me3 does not mediate

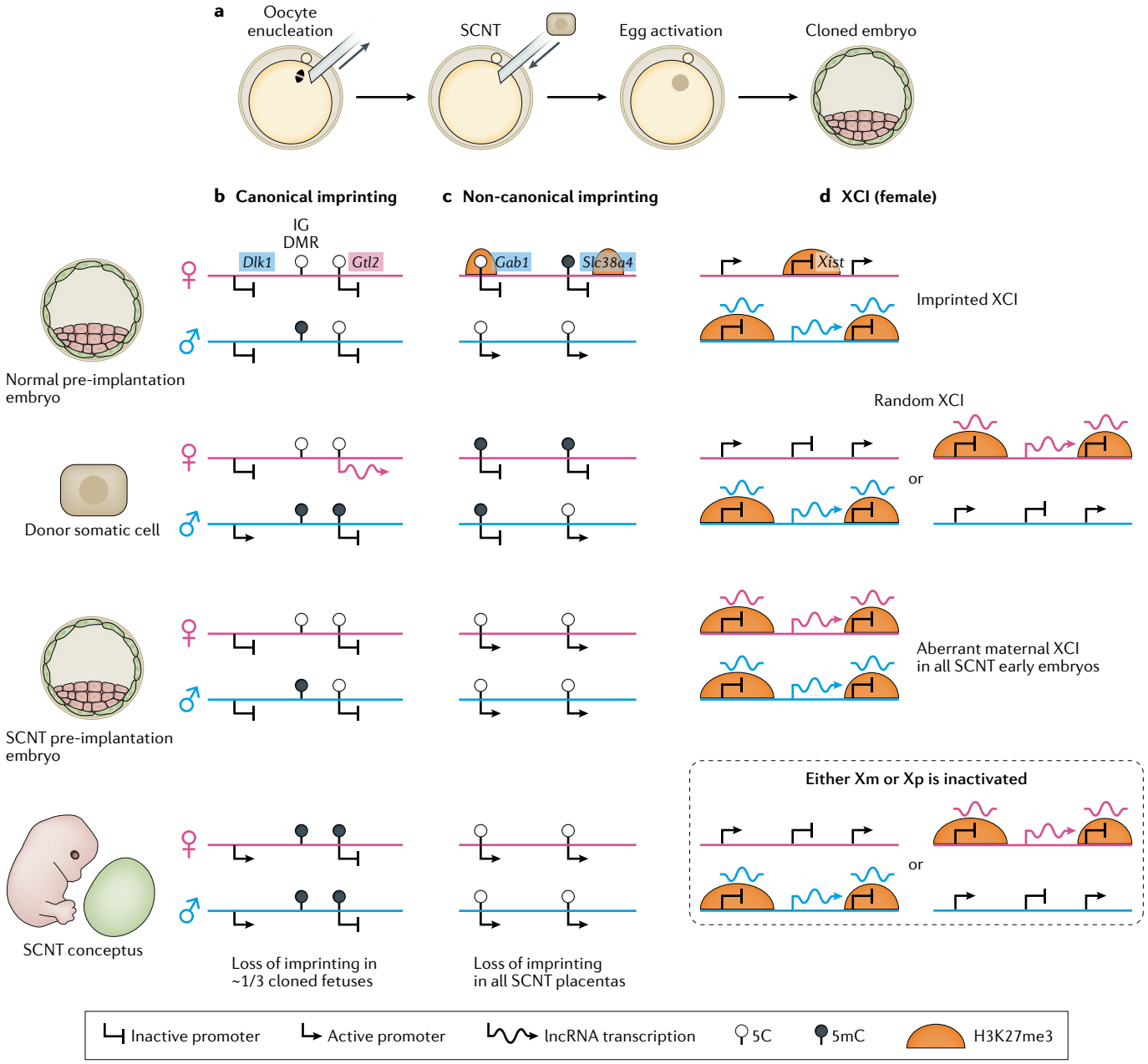


Fig. 4 | **Defects in genomic imprinting and imprinted XCI occur in SCNT reprogramming. a** | The general processes involved in somatic cell nuclear transfer (SCNT). A metaphase II oocyte is first enucleated and a donor cell nucleus from a differentiated somatic cell is transferred to the enucleated oocyte. The oocyte is then artificially activated by applying a chemical or electrical stimulus to initiate the developmental programme to form cloned embryos. **b** | Stochastic loss of canonical imprinting occurs at the *Gtl2/Dlk1* locus in SCNT embryos. At this locus, the imprinting control region is referred to as the intergenic differentially methylated region (IG DMR) and paternal-specific DNA methylation of the donor somatic cell is maintained in pre-implantation embryos derived by SCNT. However, after implantation, around one-third of SCNT embryos exhibit gain of DNA methylation on the normally unmethylated maternal allele. The abnormal gain of DNA methylation is associated with biallelic expression of *Dlk1* and biallelic repression of *Gtl2*, and with fetal lethality. **c** | Imprinting is consistently lost at non-canonically imprinted loci in SCNT embryos. In donor cells, such as cumulus and Sertoli cells, the typical non-canonical imprinted loci (represented by *Gab1*) are marked by neither maternal trimethylation of histone H3 on lysine 27 (H3K27me3) nor somatic DMRs. Therefore, all placenta derived by SNCT show loss of non-canonical imprinting; they are also enlarged, which may be caused by disrupted non-canonical imprinting. Although the DMR is maintained at the non-canonically imprinted *Slc38a4* locus in the donor cell, *Slc38a4* becomes biallelically expressed in all cloned embryos, suggesting that the *Slc38a4* DMR cannot be maintained without maternal H3K27me3 in early embryos. **d** | Aberrant X-chromosome inactivation (XCI) occurs in SCNT embryos. In donor cells, one X chromosome is randomly inactivated and X-inactive specific transcript (*Xist*) is not marked by maternal H3K27me3. Therefore, cloned embryos always express ectopic maternal *Xist* in addition to paternal *Xist*, and both X chromosomes undergo XCI. Analogous to *Eed* maternal-knockout embryos or XpXp androgenetic embryos, the biallelic inactivation of XCI is likely to be resolved at the late blastocyst stage (dashed boxes). However, insufficient expression of X-linked genes in pre-implantation development may still contribute to the post-implantation defects of cloned embryos, and correction of *Xist* expression has been shown to increase cloning efficiency by about tenfold[122]. lncRNA long non-coding RNA; 5C, 5-cytosine; 5mC, 5-methylcytosine; Xm, maternal X chromosome; Xp, paternal X chromosome.

CHG methylation
DNA methylation typically occurs in a CpG context. In CHG methylation, H correspond to A, T or C, but not G.

imprinted gene expression in humans as it does in mice, paternal-specific expression of genes not associated with germline DMRs can occur in human morula embryos, suggesting that a DNA methylation-independent imprinting mechanism may exist[127]. Recently, data from a comprehensive survey of allele-specific gene expression that compared transcriptomes between bi-maternal and bi-paternal human early embryos suggested that around half of maternally or paternally biased gene expression cannot be explained by differential DNA methylation between parental alleles[128]. Whether other epigenetic mechanisms modulate this allele-specific gene expression independently of DNA methylation remains to be investigated.

XCI dynamics in human early development is also distinct from mouse, although the detailed mechanisms remain elusive, partly owing to conflicting data. It was first proposed that one X chromosome is inactive in female human pre-implantation embryos as RNA FISH detected *XIST* coating and some X-linked gene foci on only one of the two X chromosomes[129]. However, another RNA FISH-based study reported that *XIST* coats both X chromosomes in female and the sole X chromosome in male human early embryos[130]. The discrepancies could be due to the different FISH conditions, which may compromise detection of the FISH signal on both alleles[130]. Intriguingly, the *XIST*-coated X chromosome lacks H3K27me3 and a few examined X-linked genes do not undergo silencing at these stages[130]. Recently, data from single-cell RNA sequencing analyses indicated that dosage compensation of X-linked genes is achieved by reducing gene expression levels on both X chromosomes in female embryos[131], although this model was later challenged when the data set was reanalysed using different computational criteria[132]. Despite the conflicting results and analyses, these studies highlight that important differences exist between XCI in human and mouse, and support the view that imprinted XCI is not conserved in human pre-implantation embryos. Interested readers are directed to a detailed review of human XCI dynamics[133].

Germline histone-mediated imprinting occurs in flowering plants. Maternal H3K27me3 has been implicated as a primary imprint in the endosperm of flowering plants[134]. Analogous to the mammalian placenta, the endosperm does not contribute to the next generation but is required for nourishment of the embryos. Similar to non-canonical imprinting in mice, H3K27me3-controlled imprinting in angiosperms is asymmetrically established in gametes and can persist in the endosperm[134]. In addition, maternal H3K27me3 can recruit additional repressive epigenetic marks, including CHG methylation and H3K9me2, which may enforce gene silencing[135,136]. In support of this notion, co-enrichment of H3K27me3, H3K9me2 and CHG methylation was observed on the maternal allele at paternally expressed genes in *Arabidopsis* endosperm[136]. Furthermore, lack of PRC2 causes reduced CHG methylation, suggesting that maternal CHG methylation depends on PRC2 activity[136]. Given that the primary organs for germline H3K27me3-mediated imprinting in both plants and mice are involved in nutrient transfer, it is possible that this imprinting mechanism has evolved to respond to a similar selective pressure.

Conclusions and future perspectives

Recent advances in low-input epigenomic profiling have greatly enhanced our understanding of chromatin dynamics during mammalian parental-to-zygotic transition. Accumulating evidence indicates that histone modifications can be transmitted from gametes to fertilized embryos to exert transcriptional regulation in the next generation. In particular, oocyte-inherited H3K27me3 can govern imprinted XCI and some placenta-specific imprinted genes in mice. These findings expand the known mechanisms by which intergenerational epigenetic inheritance occurs and provide an opportunity to fully understand epigenetic reprogramming and totipotency acquisition in early development.

Although much has been discovered in recent years about the mechanisms underlying non-canonical imprinting, including how it is established and maintained, many details remain to be clarified. Firstly, it remains unknown whether PRC1-mediated H2AK119Ub plays a part in regulating non-canonical imprinting. PRC1-catalysed H2AK119Ub usually overlaps with PRC2-mediated H3K27me3 in mouse ES cells and plays a predominant role in silencing PcG targets and maintaining pluripotency[137]. By contrast, removal of H3K27me3 alone can cause loss of non-canonical imprinting[20,70,72], suggesting a distinct interplay between PRC1 and PRC2 in early embryos, at least at the oocyte H3K27me3-controlled imprinted genes. Secondly, it is not clear why non-canonical imprinting cannot be maintained in the embryonic lineage after implantation. Thirdly, imprinted antisense lncRNAs have been identified upstream of the promoters of *Gab1*, *Sfmbt2* and *Smoc1*, and whether these lncRNAs are involved in imprinting regulation remains to be determined[138]. At least for *Sfmbt2*, the transcription and/or splicing of its antisense RNA contributes to *Sfmbt2* activation, potentially by modulating the chromatin state at the *Sfmbt2* promoter[139]. Fourthly, it remains a point of debate whether expression of the *Slc38a4* gene, which has a germline DMR that maintains paternal allele expression in the epiblast, is controlled by canonical or non-canonical imprinting. The observation that *Slc38a4* imprinting is compromised in *Eed* but not *Dnmt3l* or *Dnmt3a/3b* maternal-knockout embryos indicates that this gene is regulated by the non-canonical mechanism[15,20,70,72]. However, it was reported recently that local oocyte DNA hypomethylation at the *Slc83a4* DMR can cause biallelic expression of *Slc38a4* in the placenta[140]. Whether this discrepancy is caused by alternative promoter usage or lineage-specific imprinting regulation remains to be determined[71,140]. Lastly, it remains challenging to correct canonical or non-canonical imprinting errors to rescue post-implantation defects in cloned embryos. It is unclear whether the modified epigenome of donor cells can persist to the next generation during the dynamic SCNT reprogramming in early embryos. In addition, although targeted DNA methylation or demethylation in oocytes and early embryos has been

achieved[141,142], fixing the imprinting errors in SCNT embryos in an allele-specific manner is still challenging.

Beyond the role of maternal histones in genomic imprinting, the precise mechanisms and the extent to which parental chromatin affects the next generation remain unclear. For example, although oocyte-provided PRC2 in *Drosophila melanogaster* prevents precocious activation of some developmental regulators at zygotic genome activation by restricting enhancer function[143], it remains unknown whether a similar transcriptional repressive mechanism exists in mammals because there is no evidence to support that maternal H3K27me3 in mouse performs an analogous role. Notably, despite considerable achievements in mapping the chromatin landscape in mammalian early development, the dynamic control of this process remains unclear[18]. Thus, the function of, and regulatory mechanisms underlying, parental chromatin dynamics in gametogenesis and early development will remain important areas of research for years to come.

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